PEPTIDASE ACTIVITIES OF EXTRACTS OF SALIVARY GLANDS OF DROSOPHILA MELANOGASTER*‡

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Analysis of cell growth in chemical terms is essentially an analysis of the formation and activity of enzymes. The enzymes must first be characterized as a basis for the measurement of their activities during the growth of suitable biological material. The work reported here served this function preparatory to a subsequent study of changes in enzyme content during growth of the larval salivary gland of Drosophila melanogaster. The peptidases were selected for studies of growth because their specificities and their distribution in tissues have suggested a correlation with protein synthesis (Linderstrøm-Lang and Holter, 1932; Fruton, 1941; Bergmann, 1942). Discussion of the problems raised by this possibility and of the choice of biological material will be deferred until the data on growth are presented (Patterson, Dackerman, and Schultz, 1949).

Since the peptidases of Drosophila had not previously been studied, it was first necessary to ascertain whether the larval salivary gland contained a sufficient quantity of recognizable enzymes to make its use feasible in experimental work. The titrimetric methods of Linderstrøm-Lang and Holter (1940) proved suitable for determination of peptidase activity on individual salivary glands or extracts from them.

Proteolytic enzymes are classified into exo- and endopeptidases according to the type of simple peptide substrate attacked (Bergmann, 1942). Five substrates for determination of exopeptidase activity and one suitable for identification of a type of endopeptidase activity were available. Crude glycerine extracts of salivary glands were analyzed for peptidase activity toward these different substrates under various conditions of activation and inhibition. The data were then compared with those obtained from partially purified enzyme preparations by other workers, and were interpreted in terms of specific peptidases in the extracts. This rough survey proved sufficient to demonstrate the presence of one enzyme with a high enough activity to make practicable its use for the study of changes during growth. This enzyme, a peptidase splitting alanylglycine, was further studied in the extracts not only to assure its identity

607

with the usual alanylglycine peptidase, but also to find the best conditions for its measurement.   Accordingly, the behavior of the enzyme towards activators and inhibitors was investigated in greater detail.   In addition, its pH activity, time, and concentration curves were determined.

Altogether, the results have shown that both in the variety of enzymes and in the ease with which their activity can be measured by micro methods, the *Drosophila* salivary glands are favorable material.

EXPERIMENTAL PROCEDURES

Biological Material

Of the three stocks of *Drosophila melanogaster* used in these experiments two were wild type: Tuscaloosa (Tusc) and the highly inbred Oregon R (Ore R).   The third, a giant mutant, was chosen to provide individuals with large salivary glands.   This stock (gt w^a Ore R) was isogenic with Ore R, except for the giant mutant and an apricot allel at the white locus.

Salivary glands were taken from individuals at three stages of development: (*a*) late third instar larvae (LL) that had crawled up the sides of the bottle and were no longer feeding; (*b*) prepupae (5), time at 5 hours (25°C.) after the larva with the pupal horns everted was immobile; and (*c*) everted pupae (ev); *i.e.*, approximately 12 hour pupae in which eversion of the imaginal discs (pupation proper) has just occurred. In the first two stages, the larval glands were still growing; at the later stage they were beginning to disintegrate, although still grossly whole in appearance.

Dissection

In view of the instability of the peptidases, precautions were taken to minimize inactivation during dissection of the glands out of the larva.   The larvae and pupae were rinsed in fly-Ringer's solution (Ephrussi and Beadle, 1936) and any adherent food was removed before they were ice-cooled.   They were then dissected in this Ringer's solution on a cold stage which consisted of a specially designed insulated ice chamber fitted into the stage of a binocular dissecting microscope.   The glands were removed by the usual method of decapitation of the larva, or by cutting off the tip of the pupa.

The next step, the dissection of the fat body from the glands, was the most time-consuming and painstaking part of the experiment.   The fat body is closely adherent to the late larval glands, is easily removed from the 5 hour pupal glands, and is rarely present on the everted pupal glands.   Extracts of the fat body taken out of the same larvae from which the glands had been dissected, showed considerable peptidase activity (see Table IV).   It was therefore necessary to dissect the fat away with care.   This operation, the removal of the ducts and the ring of imaginal cells at the junction of duct and gland, and the transfer of the glands to a fresh drop of Ringer's, were all accomplished with steel needles.   Smooth tipped glass needles were used for the rapid transfer of the glands, one by one, from the Ringer's through a rinsing drop of glycerine buffer into the final medium.

Preparation of Extracts

The extraction medium used for the exopeptidase experiments was 30 per cent glycerine buffered to pH 7.4 with $M/60$ phosphate. Without glycerine to stabilize the enzyme, the activity of extracts in splitting alanylglycine is rapidly lost (Linderstrøm-Lang, 1929, 1930). A standard extract[1] consisted of 36 glands added successively to 234 (extracts 1 to 15) or 305 μl. (extracts 16 to 26 and 29) of glycerine buffer in a 1 ml. centrifuge tube with ground glass stopper. All volumes under 1 ml. were pipetted into the tubes by the Levy (1936) type constriction pipettes.

For the endopeptidase experiments, the extraction medium was 30 per cent glycerine buffered with $M/10$ citrate to pH 5. Two concentrated extracts were prepared, each containing 108 glands in 234 μl. of buffer.

Great care was taken to ensure both clean and sterile conditions in the extracts and the reaction tubes. Dust contamination reduced the enzymatic activity and bacterial contamination added enzymes other than those measured. Therefore, after the earliest experiments, all work was carried out in an air-conditioned room, rendered dust-proof as far as possible. Finally, as the extracts were prepared, they were sterilized by freezing and thawing seven times. Dry ice–acetone mixtures were used for the quick freezing. Routine cultures of extracts, buffers, and substrate solutions were made. The solutions rarely showed contamination and the data from experiments using contaminated solutions are omitted.

During extraction the glands underwent no chemical or physical treatment other than freezing and thawing. The use of whole cells was advocated by Linderstrøm-Lang (1933) in order to preserve the enzyme more nearly in its native state than it would be in cells exposed to mechanical handling or autolysis. Rapid freezing and thawing neither changed the enzymatic activity nor cytolyzed the cells of the Drosophila salivary gland. When other usual cytolytic procedures were tried a lowered enzymatic activity resulted, hence no bound enzyme could be freed in this way. With this material, therefore, the use of whole glands in which the cells were not cytolyzed seemed advisable, especially since no plans had been made at this time to study fragments of the glands or cells.

During the dissection period the extract tubes remained at room temperature (23–24°C.); thereafter extraction was allowed to proceed at 4°C. Under these conditions the peptidase activity of the extracts was found to increase for about 13 days, remaining constant after that time. Extracts were generally used in the period between 13 and 23 days' extraction, but comparisons were made only between extracts of comparable extraction periods. A check on the completeness of extraction of the enzyme was carried out by reextracting the glands used for the extracts. No enzyme activity was detected in these second extracts.

The Measurement of Peptide Hydrolysis

The substrates used for the exopeptidase experiments included dl-alanylglycine (AG), dl-leucylglycine (LG), l-leucylglycylglycine (LGG), glycylglycylglycine (GGG) and glycylglycine (GG). The racemic mixtures were made up to be 0.18 M and the

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[1] We wish to thank Miss Dorothy Newmeyer for help in preparing extracts 1 to 19.

l-forms 0.09 M. They were adjusted to a given pH at 25°C. with 0.1 N NaOH using a Beckman pH meter. Since the experiments were carried out at 40°C., the pH readings at 25°C. had to be converted to the correct values for the higher temperature, making allowance for the change in pK of the peptide with temperature (Cohn and Edsall, 1943). Actual readings at 40°C. with the pH meter showed that the corrections were accurate for AG. In all but the pH activity experiments, a pH of 7.60 ± 0.05 at 40°C. was used. This corresponds to a pH of 7.95 ± 0.05 at 25°C., in the case of AG. This pH was close to the optimum found for the splitting of AG and was considered near enough to the published optima for enzymes splitting the other substrates to be used for these survey experiments. The buffering capacity of the substrate was sufficient so that during the course of an experiment no measurable change in pH occurred, even at maximal observed splitting (8 μl. N/20 HCl).

α-*N*-Benzoyl-*l*-arginineamide[2] (BAA) served as substrate for the endopeptidase work (Fruton, Irving, and Bergmann, 1941). The BAA was made up to give a final concentration of 0.05 M. Here 30 per cent glycerine buffered to pH 5.00 with citrate (0.04 M final concentration) was used as the extraction medium. Cysteine (0.01 M) was employed for activation.

The hydrolysis was carried out according to the methods of Linderstrøm-Lang and Holter (1940); (see also Linderstrøm-Lang, 1938). Small reaction tubes were used to which were added a 7 μl. drop of extract or buffer and a 7μl. drop of substrate solution. All experiments were run in a water bath at 40.0°C. and the temperature was maintained constant to ±0.03°C. When AG or LG served as substrate, a reaction time of 4 hours sufficed. The other substrates required a longer period of hydrolysis (20 to 24 hours).

The reaction tubes were set up at 6 minute intervals, and after having been capped, remained in the bath for the allotted time. Reactions were stopped, maintaining the requisite 6 minute intervals, by the addition of 30 μl. of titration fluid (N/20 HCl in 97 per cent alcohol) from an automatic pipette. About 150 μl. of indicator (*ca.* 0.001 per cent napthyl red in 90 per cent acetone) were added and the titration of the amino groups was carried out immediately with the aid of a contact type burette,[3] which holds 100 μl. and can be read to 0.02 μl. This procedure allowed no time for evaporation of the titration fluid with which the reaction was stopped, thus eliminating a possible source of error.

Three sets of tubes were used in each experiment: "determinations," "enzyme blanks," and "reagent blanks." All were titrated to a standard pH at the end of a given reaction time. A "determination" consisted of a 7 μl. aliquot of enzyme extract to which was added a 7 μl. drop of substrate solution, the two drops being mixed with the aid of a stirring bead (flea) and magnet. The "enzyme blanks" consisted likewise of a drop of enzyme extract, but in this case the substrate drop was placed on the side

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of the tube. These tubes were suspended in the water bath in a horizontal position so that there would be no contact between enzyme and substrate. The "reagent blanks" consisted of a drop of glycerine buffer mixed with a drop of substrate.

The difference between the titration values of determination and enzyme blank was used as a measure of the hydrolysis of the substrate caused by the enzyme. For simplicity, all enzyme contents are recorded in terms of this titration value. The values recorded in the tables and graphs represent the mean of three determinations minus the mean of two or three enzyme blanks. When the average deviation is given followed by a +, this means that one of the enzyme blanks was lost. Average deviation is used here rather than standard deviation since the numbers are very small. The difference between the titration values of the enzyme blank and the reagent blank gave the "enzyme blank value," *i.e.* the value due to any free amino acid or other titrable acid or alkali originally present in the glands or extract, or due to autolysis. This value was small, averaging 0.20 μl. in the extracts buffered at pH 7.4. In the extracts prepared for determination of endopeptidase activity, however, it was very high, rising with the time of extraction. These extracts were buffered to pH 5.0.

When experiments were carried out to test the effect of added substances, a small drop (usually 1 μl.) of the solution in question was added to the 7 μl. drop of enzyme extract or buffer. The concentration of added substance given in the tables is that of the final mixture after substrate addition. The drops were thoroughly mixed and the tube incubated in the water bath for one-half hour before addition of the substrate drop. Control experiments were carried out in which the extract and buffer drops were diluted with a similar volume of water.

RESULTS

Exopeptidase Activity of Extracts

The activity of gland extracts in hydrolyzing various peptide substrates is given in Table I. The hydrolysis of AG is at least twice as great as that of LG whether or not the latter is activated by $MgSO_4$. Glycylglycine shows very little splitting even with a reaction time of 20 hours. The hydrolysis of the tripeptides LGG and GGG by gland extracts is low, but it is measurable in the case of LGG in 20 hours. The splitting of GGG is always very low.

It will be evident from an inspection of the data that the activities in the extracts from male glands are consistently lower than those from female glands. For the AG hydrolysis, this will be discussed when data from single gland experiments are considered (Patterson, Dackerman and Schultz, 1949). Further data are required to determine how the activities toward the other substrates vary in the extracts of glands from the two sexes. The present experiments indicate that differences of this kind may exist.

As seen from the average deviations, the variability in the case of some of the determinations is great. Since the average deviation in the reagent blanks containing glycerine buffer and alanylglycine was only about ±0.06 μl. N/20 HCl the variability was probably caused by difficulty in pipetting aliquots of

TABLE I

Hydrolysis of Peptide Substrates by Extracts of Salivary Glands

(pH 7.60 ± 0.05, 40°C.)

Extract No.	Stock	Stage	Sex	Time extracted	Substrate	Time reaction	MnSO₄ concentration	Hydrolysis*
				days		*hrs.*	*M*	*μl.N/20 HCl*
14	Tusc	ev	♀	22	LG	4	0.003	2.21 ± 0.16
				27	LGG	4	0.003	0.38 ± 0.10
				27	GGG	4	—	0.38 ± 0.04
				28	LG	4	0.003	1.83 ± 0.01 +
15	Tusc	ev	♂	25	LG	4	0.003	1.48 ± 0.35
				28	LG	4	0.003	1.69 ± 0.06
				28	LGG	20	0.003	1.96 ± 0.13
				28	GGG	20	—	0.33 ± 0.23
16	Tusc	ev	♀	16	LG	4	0.003	2.02 ± 0.27
				22	LGG	20	0.003	3.25 ± 0.13
				22	GGG	20	—	0.37 ± 0.17
				26	AG	4	—	4.39 ± 0.22
17	Tusc	ev	♂	16	LG	4	0.003	1.27 ± 0.27
				20	LGG	20	0.003	1.66 ± 0.26
				20	GGG	20	—	0.43 ± 0.14
				26	AG	4	—	2.72 ± 0.08
18	Tusc	LL	♀	17	LG	4	0.003	1.64 ± 0.04
				21	LGG	20	0.003	1.97 ± 0.30
				21	GGG	20	—	0.47 ± 0.08 +
				24	AG	4	—	3.84 ± 0.21
19	Tusc	LL	♂	18	LG	4	0.003	1.16 ± 0.06
				20	LGG	20	0.003	1.16 ± 0.36
				20	GGG	20	—	0.94 ± 0.10
				24	AG	4	—	3.35 ± 0.04
20	Ore R	5	♀	13	AG	4	—	4.57 ± 0.39
				24‡	LG	4	0.001	1.94 ± 0.40 +
22	Ore R	5	♀	21‡	AG	4		3.37 ± 0.04 +
				21‡	GG	4		0.24 ± 0.26
				21‡	GG	20		0.87 ± 0.32
24§	Ore R	5	♀	16	AG	4		6.21 ± 0.36
				20	LG	4		2.60 ± 0.12
26‖	gt wᵃ	5	♀	15	AG	4		5.28 ± 0.43
	Ore R			15	LG	4		2.36 ± 0.31

* Titration value given by a 7 μl. aliquot of gland extract. These values may be converted to splitting per gland by multiplying by the factor 0.89 in extracts 1 to 15; 1.16 in extracts 16 to 23; 1.10 in extracts 24, 25, and 29, and 2.32 in extract 26.

‡ Frozen at −70°C. after 14 days.

§ 38 glands added.

‖ Extract diluted 1:1 with glycerine buffer.

the highly viscous gland extracts. This viscosity, together with the ultraviolet absorption spectrum of the extracts (Fig. 1) indicated that they contained considerable amounts of nucleic acid. Calculations based on the phosphorus content and the absorption at 2600 Å of a solution of yeast nucleic acid (pH 6.8),
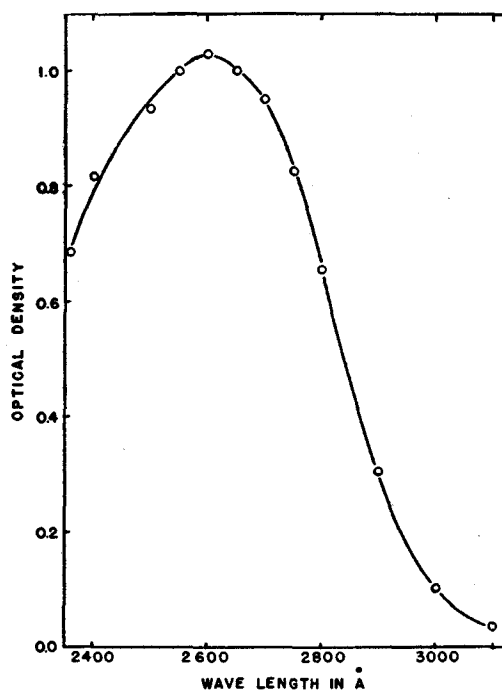

FIG. 1. Ultraviolet absorption spectrum of a buffered (M/60 phosphate, pH 7.4) 30 per cent glycerine extract of salivary glands from gt w^a Ore R 5 hour female pupae. After 14 days at 4°C. the extract (36 glands in 305 μl. buffer) was diluted 1:1 with glycerine buffer and the absorption measured against a glycerine buffer blank in a Beckman spectrophotometer. Micro cells (Lowry and Bessey, 1946) containing 60 μl. drops were used.

purchased from Schwarz Laboratories, gave a concentration of 0.05 mg. per ml. for five of the standard gland extracts.[4]

Endopeptidase Activity: Benzoyl-l-Arginineamide Hydrolysis

The endopeptidase experiments with BAA as substrate serve to indicate the presence of cathepsin II (Fruton, Irving, and Bergmann, 1941) in pH 5.0 glycerine buffer extracts of both males and females (ev). Without cysteine activa-

[4] *Note Added in Proof.*—Analysis by the orcinol reaction has given values of the same order of magnitude, showing the nucleic acid to be of the pentose type. Tests with the diphenylamine reaction have proved negative.

tion, there was no activity towards BAA; but with the activator, a low hydrolysis was observed, for example, 0.98 ± 0.14 μl. N/20 HCl in 24 hours at 40°C. This value should perhaps be considered a minimal measure of enzyme content, since its high enzyme blank (4.37 ± 0.14) shows the presence of split products. This high autolysis, after a 21 day extraction period at pH 5.0, 4°C, obviously indicates the presence of other enzymes, possibly other peptidases. Further experiments are necessary at diverse pH using short extraction periods and a variety of substrates.

Activity of Extracts in the Presence of Added Substances

The differences in the activity of the extracts toward diverse substrates indicated that there were probably several enzymes present. Since AG, LG, and LGG were the substrates showing the greatest and therefore the most accurately measurable splitting, only the enzymes concerned in their hydrolysis were considered. These enzymes might not be present in fully activated form in the extracts and it was therefore necessary to carry out a series of experiments to test the effects of addition of various substances known as activators of peptidases.

It has become increasingly clear recently that the dipeptidases and aminopeptidases are metal enzymes (Maschmann, 1943; Smith, 1946, 1948 b). Manganese or magnesium, zinc, and cobalt are the metals whose action as coenzymes has been demonstrated. The peptidase for which the greatest volume of data exists is l-leucineaminopeptidase (Berger and Johnson, 1939, 1940; Smith and Bergmann, 1944). This enzyme splits LG and LGG at equal rates and is activated primarily by manganese and to a lesser degree by magnesium. When the gland extracts were incubated with $MnSO_4$ (0.003 M) for one half-hour before substrate was added, it was found (Table II) that the splitting of LG was not activated whereas the much slower hydrolysis of LGG was enhanced by this metal. Since Smith (1946) has shown the metal-protein combination to be a slow reaction, a 4 hour preincubation of the extract with Mn^{++} was tried in an attempt to activate the LG-splitting enzyme. No appreciable increase in hydrolysis was observed. These results are discussed in a later section as evidence for the presence of two distinct enzymes whose activity is measured here by the splitting of LG and LGG.

Since activation experiments did not succeed in bringing the value for the hydrolysis of LG up to those shown by the gland extracts when AG was used as a substrate, AG was obviously the most convenient substrate to use. The next step was to find out whether the splitting of AG could be activated. As seen from Table III when Mn^{++}, Zn^{++}, or Co^{++} were added in the concentrations commonly found to give activation, no clear-cut enhancement or inhibition of splitting was observed. From these experiments it follows that whatever the relation between enzyme and metal, no gain in activity results from the addition of these metals to the extracts when the substrate AG is used. If

sufficient metal is present in the extracts, addition of more might produce only an inhibition.

TABLE II

Effect of Manganese on the Hydrolysis of Leucylglycine and Leucylglycylglycine by Extracts of Salivary Glands of Drosophila melanogaster

(pH 7.60 ± 0.05, 40°C.)

Extract No.	Stock	Stage	Sex	Time ex- tracted	Substrate	Time reaction	MnSO4 concen- tration	Time act	Hydrolysis*
				days		*hrs.*	*M*	*hrs.*	*µl.N/20 HCl*
14	Tusc	ev	♀	22	LG	4	0.003	0.5	2.21 ± 0.16
				22	LG	4	—	0.5	2.01 ± 0.10
				27	LGG	4	0.003	0.5	0.38 ± 0.04
				27	LGG	4	—	0.5	0.00
15	Tusc	ev	♂	25	LG	4	0.003	0.5	1.48 ± 0.35
				25	LG	4	—	0.5	0.72 ± 0.30
				28	LGG	20	0.003	0.5	1.96 ± 0.13
				28	LGG	20	—	0.5	0.90 ± 0.37
16	Tusc	ev	♀	16	LG	4	0.003	0.5	2.02 ± 0.27
				16	LG	4	—	0.5	1.97 ± 0.17
				22	LGG	20	0.003	0.5	3.25 ± 0.13
				22	LGG	20	—	0.5	1.07 ± 0.28
17	Tusc	ev	♂	16	LG	4	0.003	0.5	1.29 ± 0.27
				16	LG	4	—	0.5	1.52 ± 0.11
				20	LGG	20	0.003	0.5	1.66 ± 0.26
				20	LGG	20	—	0.5	0.64 ± 0.07
18	Tusc	LL	♀	19	LG	4	0.003	0.5	1.64 ± 0.04 +
				19	LG	4	—	0.5	1.86 ± 0.06 +
				21	LGG	20	0.003	0.5	1.97 ± 0.30
				21	LGG	20	—	0.5	1.47 ± 0.35
19	Tusc	LL	♂	18	LG	4	0.003	0.5	1.16 ± 0.06
				18	LG	4	—	0.5	1.90 ± 0.16 +
				20	LGG	20	0.003	0.5	1.16 ± 0.36
				20	LGG	20	—	0.5	0.67 ± 0.08 +
20	Ore R	5	♀	24‡	LG	4	0.001	4	1.94 ± 0.40 +
				24‡	LG	4	—	4	1.45 ± 0.04 +

Notes as under Table I.

It seemed desirable to determine whether the enzymes of these extracts showed a conventional behavior toward known inhibitors of peptidases (Grassmann and Dyckerhoff, 1928; Gailey and Johnson, 1941). In Table IV experiments with cysteine (SH inhibition) are given and the resulting inhibition com-

TABLE III

Effect of Metals on the Hydrolysis of Alanylglycine by Extracts of Salivary Glands of Drosophila melanogaster

(pH 7.60 ± 0.05, 40°C., 4 hours)

Extract No.	Stock	Stage	Sex	Time extracted	Metal salt	Concentration	Hydrolysis*
				days		M	$\mu l.N/20$ HCl
18	Tusc	LL	♀	24	MnSO₄	0.003	4.04 ± 0.47
					—		3.84 ± 0.21
19	Tusc	LL	♂	24	MnSO₄	0.003	2.42 ± 0.19
					—		3.35 ± 0.04 +
23	Ore R	5	♂	23‡	ZnSO₄	0.0004	1.53 ± 0.65
					—		0.82 ± 0.34
25§	Ore R	5	♂	17	ZnSO₄	0.0004	3.01 ± 0.52
					—		4.51 ± 0.19
29§	Ore R	5	♀	8	CoCl₂	0.001	1.40 ± 0.34
					—		2.37 ± 0.32

Notes as under Table I.

TABLE IV

Effect of Inhibitors on the Hydrolysis of Peptides by Extracts of Salivary Glands and Fat Body of Drosophila melanogaster

(pH 7.60 ± 0.05, 40°C., 4 hours)

Extract No.	Stock	Material	Sex	Time extracted	Substrate	Inhibitor	Concentration	Hydrolysis*	Inhibition
				days			M	$\mu l.N/20$ HCl	*per cent*
7	Tusc	Fat body	♀	28	AG	Cysteine	0.01	0.84 ± 0.40	90
						—		8.07 ± 0.15 +	
6	Tusc	Fat body	♂	27	AG	Cysteine	0.01	0.51 ± 0.11 +	93
						—		7.13 ± 0.15	
23	Ore R	5	♂	20‡	AG	Cysteine	0.01	0.00	
						—		1.90 ± 0.21	100
25	Ore R	5	♂	23	AG	Cysteine	0.001	2.08 ± 0.12	40
						—		3.57 ± 0.06	
24§	Ore R	5	♀	20	LG	Cysteine	0.01	1.07 ± 0.47	(59)¶
						Cysteine	0.001	2.38 ± 0.44	(15)
						—		2.60 ± 0.12	
25§	Ore R	5	♂	17	AG	Alanine	0.01	4.27 ± 0.13	(5)
						—		4.51 ± 0.19	

Notes as under Table I.

¶ Values in parentheses only qualitatively significant.

616

pared with that by another amino acid, alanine. AG splitting was inhibited 90 to 100 per cent by cysteine at 0.01 M and about 40 per cent at 0.001 M. The hydrolysis of LG was also inhibited but to a lesser degree, again giving evidence for the possible existence of separate enzymes of different specificity. When AG was used as substrate, alanine at 0.01 M gave only the expected slight inhibitory effect of a split product.

The experiments in this section show that the extracts of salivary glands on the whole behave in a manner similar to preparations of peptidases from other sources. They also provide some justification for using the splitting of AG
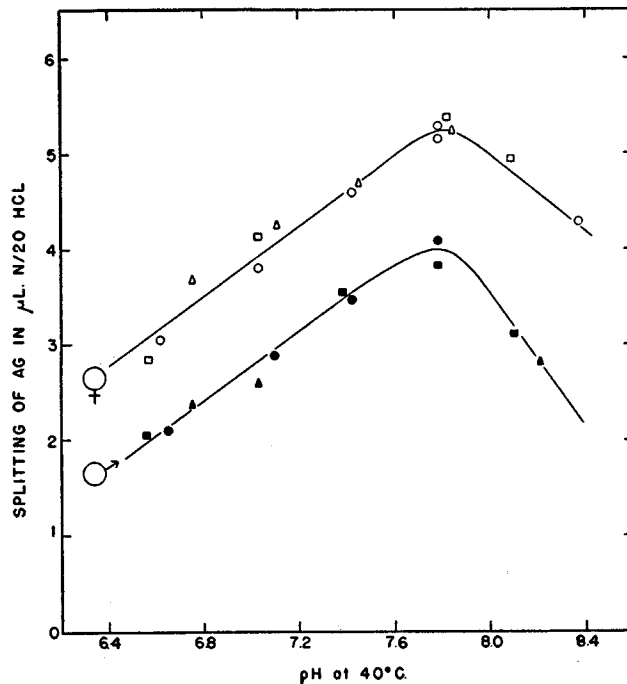

FIG. 2. pH activity curve of the enzyme-splitting AG in extracts of salivary glands from the Tusc stock. The values were taken from different extracts. The solid symbols represent extracts from males prepared simultaneously with those from females (open symbols); O, △ extracts from late larvae, and □ from pupae.

without addition of metal activators as an index of the peptidase content of the salivary glands.

Determination of Optimal Conditions for Measurement of AG Peptidase

In the preceding brief survey of the effects of activators, etc., some of the conditions for the use of measurement of AG hydrolysis as an index of enzyme content have been established. In the present section, other necessary data are presented: measurement of the activity of the enzyme extracts at a series of different pH, concentrations, and reaction times.

The pH activity curves (Fig. 2) were constructed from determinations made on a series of comparable extracts, the substrate adjusted to the required pH being added to the respective aliquots. The optimal pH range was found to be broad, extending from 7.55 to 7.95 (40°C.) This was true for extracts from both males and females, the quantitative difference between the two remaining approximately constant at all pH. The peak, pH 7.8, falls within the range (pH 7.4–7.8) of values occurring in typical AG-peptidases (for example, Linder-strøm-Lang and Sato, 1929; Duspiva, 1936; Holter and Doyle, 1938; Palmer
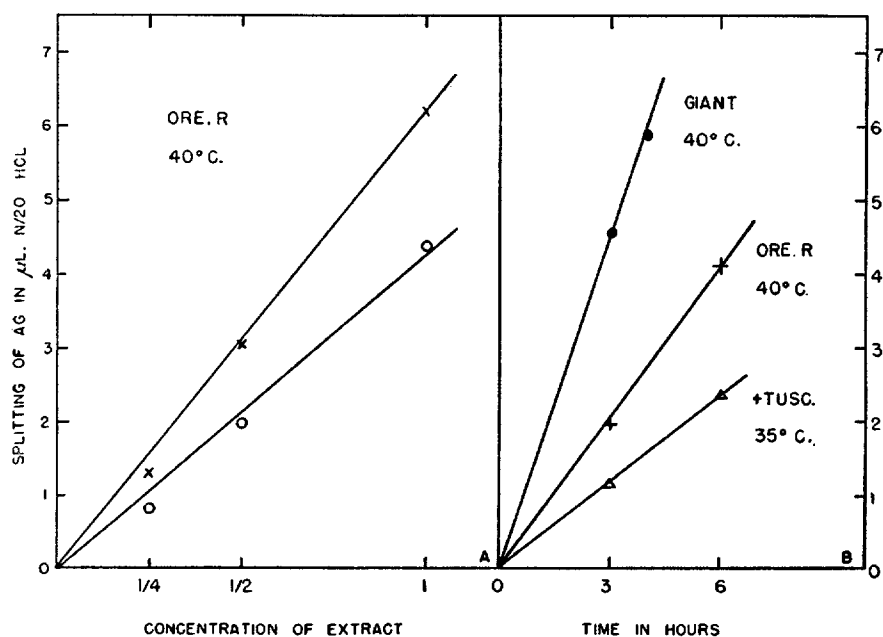

FIG. 3. Variation of AG-peptidase activity (A) with concentration of the enzyme extract, and (B) with reaction time. In A two extracts of salivary glands from Ore R 5 hour pupae (X), and larvae (O) were diluted to ½ and ¼ concentration with glycerine buffer and the hydrolysis carried out at 40°C. for 4 hours. In B three extracts of standard concentration were used and the reaction carried out for varying times ●, gt w^a Ore R 5 hour pupae; +, Ore R 5 hour pupae, and △, Tusc, late larvae.

and Levy, 1940). For most of the experiments a pH of 7.6 which falls well within the range of maximal activity was chosen in order to avoid spontaneous splitting of the substrate which even at 4°C. occurs perceptibly at higher pH after prolonged storage.

In the experiments in which concentrations of extract or reaction time was varied, the amount of substrate added was the same as that used in other experiments. At all times the substrate was present in excess. Over a fourfold range of extract dilutions, the relation between concentration and activity was linear (Fig. 3 A). If a complex of enzyme and some inhibitor present in these

extracts had been dissociated on dilution, a deviation from linearity would have been found at the higher concentrations. With the linear relation established, support is provided for the use of activity as a measure of enzyme content in extracts of comparable concentration. Within the experimental range, splitting was found to be proportional to reaction time in extracts from three different stocks (Fig. 3 B). Thus, with the concentration of substrate employed, the degree of hydrolysis is proportional to concentration of extract and time of reaction up to a titration value of 6 μl. $N/20$ HCl.

The usual temperature at which peptidase experiments are carried out is 40°C. The upper range for the normal development of *Drosophila melanogaster* occurs between 25 and 30°C. It was conceivable, although unlikely, that the enzymatic activity might increase at a lower temperature, one more physiological for *Drosophila*. In the time experiments just discussed, the Tusc extract was allowed to act at 35°C. It had previously been determined (*cf.* Table I, extracts 16 and 20) that Tusc and Ore R gave comparable values at 40°C. Consequently comparison of the curves from the two stocks, at the two temperatures (Fig. 3 B), shows an increase of activity at the higher temperature.

DISCUSSION

The attempt to analyze the enzymatic constitution of these crude extracts was necessarily based on the information available about the behavior of partially purified enzymes. Activators and/or inhibitors intrinsic to crude extracts may, however, distort the activity relationships which serve as a basis for identification of the enzymes. For this reason, substances which are present in the extracts and which might influence the enzyme activities deserve consideration.

Under the experimental conditions given, the substances most influential in determining the activity of a peptidase will be first the products of the catalyzed reaction, and second the substances (metals as coenzymes, inhibitors, etc.) affecting the enzyme directly either by way of influence on the active groups, or by non-specific reactions with the enzyme protein. The reaction products here—the free amino acids—are not present in the salivary glands or the extracts to any appreciable extent: the low titration value of the enzyme blanks agrees with the chromatographic analysis of LaCour and Drew (1947) on this point. For the metals, there is no analysis of the extracts available at present; and, of course, the absence of activation with certain of the metals may be due to their presence in sufficient quantities in the extract. The reducing systems have not been investigated in detail; there is no good evidence for the presence of much free —SH. Finally, as has been mentioned earlier, appreciable quantities of nucleic acid, about 0.05 mg. per ml., appear to be present in the phosphate buffer extracts. The nucleic acids are known to inhibit the action of a different type of proteolytic enzyme, a carboxypeptidase

at pH 5 (Mims, Swendseid, and Bird, 1947). Hence, they merit attention when present in the enzyme extract. It may well be that the low values found for the endopeptidase characterized by cysteine-activated BAA splitting (pH 5.0) are due to the inhibitory effect of the nucleic acid in the extracts—a point that requires further investigation.

The activities exhibited by gland extracts toward the different exopeptidase substrates are evidence for the existence of a minimum of three enzymes: an AG-dipeptidase, a LG-dipeptidase, a leucineaminopeptidase; there may also be an aminopolypeptidase. It will be recalled that the rates at which the different substrates are split differ greatly. To facilitate comparison of the rates, the values of peptide hydrolysis by the different extracts can be averaged. If an arbitrary value of 1 is given to the rate for GGG, the approximate rates of the rest follow: GG, 2.5; LGG-Mn, 5; LG, 20; AG, 40. Clearly the high rate at which AG is hydrolyzed is distinctive and suggests that the enzyme is similar to the peptidase I of Linderstrøm-Lang (Linderstrøm-Lang and Sato, 1929; Linderstrøm-Lang, 1930). The other properties of the *Drosophila* enzyme support this conclusion: the rapid loss of activity in phosphate buffer without glycerine, the pH optimum at 7.8, and (Gailey and Johnson, 1941; Maschmann, 1941) the strong inhibition by cysteine. The data on activity of the enzyme when temperature, concentration of extract, or time are varied, are also consistent with the known behavior of AG-peptidases.

Since the hydrolysis of GG proceeded at a very low rate, it would appear possible that the AG-peptidase is responsible for this activity. A more probable alternative, however, is that a specific GG-peptidase (Smith, 1948 *b*) is involved. This cobalt enzyme is very labile and, if present, could have been largely inactivated during the prolonged extraction period. In the absence of experiments with Co++ activation, this possibility cannot be evaluated.

The data from the LG and LGG experiments require the assumption of two separate enzymes for their explanation. Were the typical leucineaminopeptidase (originally peptidase II of Linderstrøm-Lang, 1929; Smith and Bergmann, 1944; Smith, 1946; Berger and Johnson, 1939, 1940) responsible for both hydrolyses, the two substrates should have been split at the same rate, and activation by Mn++ should have occurred in both cases. However, with the *Drosophila* extracts, the rate of hydrolysis of LG-Mn is four times that of LGG-Mn. Moreover, LG hydrolysis is not activated by Mn while that of LGG is doubled. The conditions required by the assumption of a single leucineaminopeptidase are therefore not fulfilled. It might be assumed alternatively that the AG-dipeptidase is responsible for the LG splitting. But, aside from the objection to such an argument from the point of view of enzyme specificity, the different percentage inhibition with 0.01 M cysteine (90 per cent, AG; 60 per cent, LG) indicates the presence of two enzymes. A simpler interpretation would regard the LGG splitting as due to the usual leucineaminopep-

tidase and not to the non-Mn-activated lymphopeptidase (Fruton, Smith, and Driscoll, 1948); the LG splitting would then be attributed to a dipeptidase hydrolyzing LG, but not activated by Mn (Smith, 1948 a).

Finally, the presence of a still different enzyme may be indicated by the hydrolysis of GGG at a slower rate than LGG. A leucineaminopeptidase seems unlikely as the enzyme involved since the one studied by Smith (1948 b) does not act on GGG. This enzyme splitting GGG may possibly be similar to the aminopolypeptidase purified by Ågren (1945) and/or the lymphopeptidase of Fruton, Smith, and Driscoll (1948).

SUMMARY

1. Peptide-splitting enzymes have been studied in buffered glycerine extracts of larval salivary glands of three stocks of *Drosophila melanogaster*.

2. The ultraviolet absorption spectrum of the glycerine extracts indicates the presence of a considerable amount of nucleic acid.

3. Alanylglycine (AG), leucylglycine (LG), leucylglycylglycine (LGG), glycylglycine (GG), and diglycylglycine (GGG) are split by the gland extracts in descending order of activity.

4. Of the various metals added, manganese was the only one found to give clear cut activation and that only with LGG as substrate. Cysteine inhibited the splitting of both AG and LG.

5. Comparison of the data with those published indicates the presence in the extracts in descending order of activity (at pH 7.6, 40°C.) of at least four enzymes: an AG-dipeptidase, an LG-dipeptidase, a leucineaminopeptidase, and possibly an aminopolypeptidase.

6. Optimum conditions for the measurement of the enzyme splitting AG were determined. The pH activity and kinetic data are typical for an AG-dipeptidase.

7. An enzyme (probably cathepsin II) splitting benzoyl-*l*-arginineamide (pH 5.0) with cysteine activation was observed to occur with very low activity in gland extracts.

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