CLIX. ON THE DEAMINATING ENZYME OF FLESH-FLY LARVAE.

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INTRODUCTION.

THE production of ammonia as their main nitrogenous end-product by flesh-fly larvae is now well known. The mechanism of its production however is still obscure [Messer and McLellan, 1935]. Hobson [1931] for instance was unable to find any evidence of deamination of glycine at p_H 6.8 by aqueous extracts of *Lucilia sericata* larvae. One of us [Brown, 1935] carried out the same experiment using larvae of *Wohlfahrtia vigil* on both glycine and tyrosine and varying the p_H from 4.0 to 8.0 with entirely negative results.

Some hope for the elucidation of this problem lay in an observation of Weinland [1908], who found that in his peptone brei of *Calliphora* tissue the reaction became definitely alkaline to litmus presumably owing to the production of ammonia. In the present communication the presence of a deaminating enzyme has been established and some of its properties and conditions of action have been determined.

ANALYTICAL METHODS.

Ammonia was determined by permittit adsorption and nesslerisation [Peters and Van Slyke, 1932]. In analysing solutions containing protein or its breakdown products the colour on nesslerisation was unsatisfactory owing to a straw-yellow tinge which developed; this could be obviated by repeated washings of the permittit after ammonia adsorption or by substitution of permittit adsorption by aeration into 0.1 N HCl.

Amino-nitrogen was estimated by alcoholic titration [Willstätter and Waldschmidt-Leitz, 1921] using *N*/50 alcoholic NaOH with cresolphthalein as indicator.

Protein-nitrogen was measured by precipitating the protein with trichloroacetic acid and determining the nitrogen in the washed precipitate using the technique described by Farber and Wynne [1935].

Uric acid was determined by the method of Benedict and Franke [*v. Peters and Van Slyke*, 1932].

The organisms used in this investigation were *Lucilia sericata* Mg. and *Calliphora erythrocephala* Mg., both flesh-flies of the same ecological habit. Notes on their laboratory breeding are published elsewhere [Brown, 1936, 2].

Preliminary investigations on brei.

Brei was prepared in the following manner. Large numbers of washed larvae were ground in a mortar for 20 min. Of the resultant sticky mass portions were removed in a horn spoon, weighed and transferred to another mortar. They were then each ground with one-half their weight of the appropriate solution of the substrate and enough water (measured) was added to make the resultant brei thin enough to pour into a weighed centrifuge-tube which was then weighed

with its contents. These mixtures were incubated under toluene for 24 hours at 30°. They behaved exactly as described by Weinland [1908]. At the end of the digestion period water was added to 40 ml., the contents were well stirred and then centrifuged and aliquot amounts of the supernatant liquid were analysed for ammonia. Controls without substrate were also set up and the small value of their autolytic ammonia production, calculated on the same unit weight of original tissue, was subtracted from the experimental values.

It was found that ammonia was produced in large quantities from Witte peptone. Mere alkalinity (p_H 7.5–9.5) did not give rise to any production of ammonia from peptone under these conditions.

It was necessary to establish the variations in the deaminating activity throughout the life-cycle of the organism. Using brei of *Lucilia* on Witte peptone the results in Table I were obtained.

Table I. *Variation in deaminating activity of tissue brei during the life-cycle of Lucilia.*

	Stage of life-cycle	NH ₃ per g. tissue mg.	Weight per 100 larvae g.	NH ₃ per 100 larvae mg.
Larvae	Half-gorged	8.66	5.26	45.0
		8.47	—	—
	Fully gorged	11.11	4.80	53.3
	25% with gut evacuated	10.74	4.49	46.1
		9.69	—	—
	2% pupated	7.63	3.53	26.9
Pupae	1–3 days after pupation	1.32	4.74*	6.5
Adults	1–3 days after emergence	0.00	1.60	0.0

* Batch of exceptionally large-sized pupae.

The deamination therefore is at a maximum during the period of the most intensive digestion of protein by the larvae. It drops rapidly, being low in pupae and disappearing finally in the adult. It seems best to take larvae just before

Table II. *Deamination of various nitrogenous compounds by tissue brei of Lucilia.*

Substrate	NH ₃ in mg. per g. of tissue				
	Total production		Autolysis control II	In substrate III	Actual production I minus II plus III
	Values	Average I			
Witte peptone	13.23	13.14	4.50	0.07	8.57
	13.04				
Caseinogen, B.D.H.	16.07	14.94	4.50	0.07	10.27
	13.82				
Ovalbumin, Merck	2.75	2.89	2.28	0.30	0.31
	3.03				
Bactopeptone, Difco	3.68	3.84	2.28	0.15	1.41
	4.00				
Glycine	3.84	3.84	3.85	0.00	–0.01
Also no NH ₃ production from these other amino-acids:					
	α -Alanine	Aspartic acid	Phenylalanine		
	β -Alanine	Glutamic acid	Tyrosine		
	Leucine	Arginine	Histidine		
	isoLeucine				

pupation, when (a) their gut is empty of contents which might interfere with the experiments, and (b) they can be easily obtained in a clean dry condition from the sawdust into which the larvae crawl on leaving the meat; although their deaminating power is not at its height, it is quite enough to work with.

The deamination of various nitrogenous compounds by larval brei was next investigated; the results are summarised in Table II.

The proteins and their more complex derivatives furnish the best substrates. Bactopeptone is composed of lower breakdown products than Witte peptone. Ovalbumin is exceptional, presumably owing to its resistance to proteolysis [Farber and Wynne, 1935]; moreover, it is a poor substrate for growth and ammonia production for these organisms *in vivo* [Brown, 1936, 1]. All the amino-acids studied are resistant to deamination.

Active preparations in powder form.

Powders were prepared in large quantities in the following manner. Up to 100 g. of larvae were sifted out from sawdust, washed under water for 1 hour, dried on filter-paper and well macerated in a large mortar. The tissue was then poured into acetone (ether, light petroleum or alcohol partially inactivated the enzyme) and the first extract was decanted through cheese-cloth. By kneading the material in the cloth with a stirring rod in frequent changes of acetone all the tissue except the skins came out in a coarse suspension which was poured through a filter. It dried on the filter-paper within an hour and could be readily pulverised; after passing through a 60-mesh wire screen it gave a powder of the texture of Witte peptone.

The activity of such powders was tested and found to equal that of the dried tissue; moreover, preparations of *Calliphora* and *Lucilia* showed the same order of activity. These experiments were allowed to continue for 4 days by decanting off the supernatant liquid (for ammonia determination) and leaving the centrifugate; they were stopped when the boiled controls began to show some ammonia production from bacterial contamination. At this point nearly 40% of the peptone-nitrogen had been converted into ammonia. Fig. 1 illustrates the results. These powders could be kept in the refrigerator without perceptible loss of activity over a period of weeks.

The substitution of extract for brei.

The solubility in water and in 50% glycerol of the enzyme system was next investigated. Larval powders were triturated in the solvent and centrifuged and the supernatant and centrifugate were tested. Both proteinolytic and deaminating activities were investigated; the results are shown in Table III.

Three types of active preparations were used:

- (1) The dry larval powder triturated in water.
- (2) A 50% glycerol extract of the larval powder.
- (3) A 50% glycerol extract of freshly ground larvae.

Fig. 1. Deaminating activity of dry preparations from brei. A, dried tissue, *Lucilia*; B, acetone powder, *Calliphora*; C, acetone powder, *Lucilia*.

Table III. *Solubilities of deaminase and proteinase of larval powders in water and 50% glycerol.*

	Water				50% glycerol			
	Supernatant		Centrifugate		Supernatant		Centrifugate	
	Protein-N	NH ₃ -N	Protein-N	NH ₃ -N	Protein-N	NH ₃ -N	Protein-N	NH ₃ -N
Initial, mg.	20.1	0.77	25.3	0.29	27.0	0.79	24.6	0.30
24 hours' digestion, mg.	6.5	2.50	3.9	3.47	9.5	4.27	7.8	3.20
Change in mg.	13.6	1.73	21.4	3.18	17.5	3.48	16.8	2.90
% of total activity	39	35	61	65	51	56	49	44

The digestion of caseinogen by the larval enzyme system.

Duplicate digests were set up of the larval powder with 1% caseinogen (B.D.H., later Kahlbaum's preparation according to Hammarsten was used) solution of an initial p_H of 8.6 in Erlenmeyer flasks at 37.5° with shaking under toluene. The p_H dropped to 7.0 during digestion. Residual protein-N, free carboxyl groups and ammonia were determined at appropriate intervals. The progress of two different experiments (with different enzyme preparations) is shown in Fig. 2.

Fig. 2. The breakdown of caseinogen by larval preparations. A, acetone powder 2 (*Calliphora*); B, acetone powder 4 (*Calliphora*).

A, protein-N, decrease; B, amino-N; C, ammonia-N, scale magnified $\times 5$.

It is evident that the protein decreases and free amino-groups increase in a fashion characteristic of "tryptic" digestion [Farber and Wynne, 1935]. The production of ammonia however occurring in considerable amounts after a short lag period is quite different from that in a tryptic digest.

In order to show the difference between digestion by trypsin and by the larval preparations a "switch" experiment was devised. A tryptic digest of caseinogen was set up; at three different points in the digestion a portion was removed, boiled and submitted to the larval enzyme. The addition of the larval enzyme immediately accelerated the production of ammonia. Data are presented in Fig. 3.

Three conclusions may be drawn:

- (1) The larval enzyme produces ammonia much more quickly and in far greater amounts than does trypsin.
- (2) The stimulating effect of the addition of the larval enzyme is also shown in the accelerated production of free amino-groups.

(3) The best substrates for the larval deaminase are those products which occur in the middle of the tryptic digestion, when the fragments of protein breakdown are still large.

Fig. 3. "Switch" experiment. A, protein-; B, amino-; C, ammonia-nitrogen. Continuous lines from origin represent tryptic digestion. Samples removed at 23, 55 and 114 hours, boiled and submitted to larval enzyme.

The substrate for the larval deaminase.

The last experiment was repeated in greater detail: frequent samples of a tryptic digest were taken, boiled, made up to their original volumes and submitted to the action of the larval enzyme (in the form of a 50 % glycerol extract of fresh tissue) for 24 hours at 37.5° under toluene; the p_H was adjusted to 7.6, the optimum of the deaminase (see below). Typical results are given in Table IV. The first column represents the length of time for which the sample had been submitted to tryptic digestion and the second column the amount of ammonia that was produced from it by the larval enzyme.

Table IV. *Variation in deamination of tryptic digest of caseinogen by a glycerol extract of Calliphora.*

Time hours	NH ₃ -N mg.	Time hours	NH ₃ -N mg.	Time hours	NH ₃ -N mg.
0.0	0.77	13.2	0.86	152.0	0.58
1.2	0.93	22.5	0.90	Erepsin added	
3.6	0.89	25.8	1.03	152.5	0.46
4.7	0.95	29.0	0.91	157.5	0.53
6.7	0.99	35.0	0.73	166.5	0.66
8.5	1.00	37.8	0.91	193.0	0.30
10.2	0.99	48.0	0.59	286.0	0.36
12.0	0.95	77.2	0.41	359.0	0.27

Erepsin, in the form of a 50 % glycerol extract of fresh hog intestinal mucosa, was added to complete the digestion. Although the individual values are erratic, the following conclusions may be drawn tentatively:

(1) The original protein is not as good a substrate as its higher breakdown products.

(2) The most effective substrate is present over a broad range of tryptic digestion.

(3) Deamination falls off as the digest approaches the free amino-acid stage.

(4) Addition of the intestinal extract has a short stimulating effect on deamination, presumably due to deamination of the breakdown products of the protein contained in it.

The question of the best substrate was then examined from another angle. Although amino-acids were resistant to deamination, it seemed possible that mixtures of them would be susceptible or that there might be some uninvestigated susceptible amino-acids. The mono-amino-acid fractions therefore from a tryptic digest of caseinogen, from an acid digest of caseinogen and from an acid digest of gelatin were submitted to the larval enzyme and found in no case to be deaminated.

Samples of two dipeptides (glycylglycine, leucylglycine) and of a tripeptide¹ (leucylglycylglycine) were next tested as substrates. In each case there was considerable hydrolytic activity but no deamination: see Table V.

Table V. *Behaviour of larval enzymes towards simple peptides.*

Substrate	M/10 glycylglycine		M/40 leucylglycine		M/50 leucylglycylglycine	
	Amino-N	NH ₃ -N	Amino-N	NH ₃ -N	Amino-N	NH ₃ -N
Initial, mg.	10.56	0.256	7.35	0.139	5.86	0.139
24 hours' digestion, mg.	17.76	0.264	14.70	0.132	11.72	0.126
Produced, mg.	7.20	0.008	7.35	-0.007	5.86	-0.013

Since these organisms are strongly uricolytic [Truszkowski and Chajkinówna, 1935] it was thought possible that the ammonia might be produced from the breakdown of uric acid. Digests were therefore made up of 0.1% uric acid in a Li₂CO₃ solution buffered at *p*_H 7.6 and submitted to the action of the larval powder and of a 50% glycerol extract of fresh larvae, respectively. Samples were taken after 24 and 48 hours, controls with the enzyme preparations alone being run concurrently. The results are presented in Table VI.

Table VI. *Relation of uricolysis to production of ammonia by larval preparations.*

Hours	Powder			Glycerol extract		
	Uric acid Digest	NH ₃ -N Digest	NH ₃ -N Control	Uric acid Digest	NH ₃ -N Digest	NH ₃ -N Control
	mg. per 90 ml.			mg. per 90 ml.		
0	40.0	—	0.35	37.8	—	0.67
24	28.0	1.25	1.18	29.2	1.09	1.04
48	17.9	1.68	1.32	18.4	1.09	1.08

The results indicate that the enzyme preparations are strongly uricolytic, but that an inconsiderable amount of ammonia is produced as a result of this process; this is in keeping with the suggestion of Robinson [1935] that the allantoin present in the excreta of these organisms is the product of uricolysis.

The possible presence of a "deamidase" in the enzyme preparations was next

¹ The authors wish to express their indebtedness and thanks to Dr Max Bergmann of the Rockefeller Institute for his very great generosity and courtesy in supplying samples of the peptides.

tested using 0.05 % asparagine as substrate at p_H 7.6. There was a slight production of ammonia; its small significance is indicated by the following data for digests of 10 ml. substrate-buffer and 2 ml. 50 % glycerol preparation.

	mg.
NH ₃ -N produced by action of larval enzyme	0.172
Total amide-N in substrate	5.300
NH ₃ -N produced by same enzyme preparation on tryptic digest of caseinogen	1.028

It had been hoped that the deaminase could be activated, *e.g.* by protein breakdown products. A long-term tryptic digest of caseinogen and a similar larval digest of caseinogen were dialysed for 2 days; the dialysate and non-dialysed residue in both cases were added in small quantities to a digest of 0.1 % glycine with a 50 % glycerol extract of fresh larvae. Controls without the addition of "activator" and also without glycine but with "activator" were run concurrently. The results are presented in Table VII.

Table VII. *The effect of addition of various fractions on the production of ammonia from glycine by larval preparations.*

All values: mg. NH₃-N per 12 ml. digest.

"Activator"	Experimental with glycine I	Control without glycine II	"Activator" as substrate I minus III	"Activation" I minus II
Caseinogen digest dialysate	0.262	0.254	0.038	0.008
Caseinogen digest residue	0.282	0.282	0.066	0.000
Larval digest dialysate	0.231	0.230	0.045	0.001
Larval digest residue	0.242	0.242	0.026	0.000
Water, III	0.186	—	—	—

The data clearly show that there is no indication of activation. Incidentally, it may be seen that the non-dialysed residues may act slightly as substrates and the dialysates to a lesser degree.

Fig. 4. Effect of temperature on deaminating activity.

Conditions regulating the activity of the enzyme.

The effect of temperature.

The activities of two different powder preparations of the deaminase at various temperatures are shown in Fig. 4.

The relationship between temperature and deaminase activity is linear over the temperature range investigated; temperature coefficients are as follows:

	Powder No. 2	Powder No. 4
10-20°	1.50	—
20-30°	1.94	2.04
30-40°	1.46	1.49

The effect of hydrogen ion concentration.

An incomplete study of the effect of p_H on the deaminase activity indicates that the optimum is about p_H 7.6. Fig. 5 shows the results obtained for two different enzyme preparations.

Fig. 5. Effect of p_H on deaminase activity.

Influence of oxygen.

For a long time it had been suspected that this deamination was an anaerobic process as suggested by Weinland's observations on brei. The first experiment in which digestion was carried out in presence of different gases is summarised in Table VIII a. The substitution of oxygen for air did not affect the deaminase activity whereas the presence of nitrogen accelerated the ammonia production. The low value under CO_2 is explicable by the depressing effect of the gas on the p_H .

In the second experiment digests in air were compared with those *in vacuo* using Thunberg tubes; the results are given in Table VIII b. Both sets of data show that the deaminase activity is greater under anaerobic conditions.

Table VIII. *Effect of gases on ammonia production from caseinogen by larval preparations.*

<i>a</i>			
Gaseous medium	Total production of NH ₃ -N per g. powder	Actual production of NH ₃ -N per g. powder	Final <i>p</i> _H
CO ₂	6.08	4.32	5.9
O ₂	8.09	6.33	6.3
N ₂	9.48	7.72	6.5
Air	8.34	6.58	6.6
Control	1.76	—	—

<i>b</i>			
Conditions	Total production of NH ₃ -N per 5 ml. digest	Actual production of NH ₃ -N per 5 ml. digest	Average
Aerobic	0.638	0.395	0.391
	0.630	0.387	
Anaerobic	0.862	0.619	0.613
	0.850	0.607	
Control	0.243	—	—

Is this deamination oxidative or hydrolytic?

The fact that deamination proceeds better under anaerobic conditions is striking. It may be argued that this is an oxidative process in which oxygen is replaced as a hydrogen acceptor by other substances. Yet it seems unlikely that glycerol extracts of larval powders would be rich in such substances and moreover the process operates better in absence of oxygen; nor is the oxidative deamination in the mammalian kidney stimulated by addition of hydrogen acceptors [Krebs, 1933]. The enzyme itself cannot be destroyed by oxygen since the activity of preparations kept for long periods in air is unimpaired.

Moreover, α -hydroxy-acids have been detected in the deaminated residues. Difficulties were encountered in attempting to estimate hydroxyl groups by benzoyl chloride precipitation or by acetylation since these reactions are also given by amino-groups. Recourse was therefore had to methods of determination of lactic acid, which estimate also in lesser degree other α -hydroxy-acids. Residues in digests of the larval enzyme on caseinogen contained appreciable quantities of α -hydroxy-acids as indicated by the phenol-ferric chloride (Uffelman's) test and by quantitative estimation with the method of Friedemann *et al.* [1927]. A digest was set up with gelatin, which has a relatively high alanine content, and the production of ammonia and "lactic acid" was followed. α -Hydroxy-acids were found to be produced at a rate roughly proportional to the ammonia production; however, in this substrate, the amount of digestion was too small to be of any great quantitative significance.

It is generally assumed that a deamination which is unimpaired by the absence of oxygen and which leaves α -hydroxy-acids in the deaminated residue is hydrolytic. But further work on the mechanism of deamination is indicated.

Separation of proteolytic and deaminating activities.

The enzyme preparations used are both proteolytic and deaminating; experiments hitherto conducted (*v.*, *e.g.*, Fig. 2 and Table III) gave no indication of their separate identities. In order to show that this ammonia production

involves something more than a special modification of proteolysis the two enzymes must be separated. Three types of experiment were devised with this end in view.

In the first a glycerol extract was precipitated with alcohol, but the results were not conclusive.

Experiments were then carried out in an attempt to differentiate between the two types of enzyme by varying the p_H . The optimum p_H for ammonia production is 7.6 (see above); however, the proteolytic activity at this p_H is not at its optimum. The p_H curves of proteolysis show two peaks, one in the neighbourhood of p_H 4-5 and the other at p_H 8.3. This suggests the existence of a tissue proteinase of the cathepsin type and of a proteinase of the trypsin type.

The third experiment was suggested by two observations of other workers; the one made by Hobson [1932] that excreta of *Lucilia* are proteolytic and the other made by Weinland [1907] that the excreta of *Calliphora* do not produce ammonia. A large number of *Lucilia* larvae were taken at the point of their maximum ammonia excretion [v. Brown, 1936, 1] and dropped into 100 ml. of distilled water; in this way a "washing" containing some of the excretory principles was obtained. The washed larvae were then placed on a sintered glass filter overnight, water being slowly added; in this way a solution of excreta was collected. The two solutions, "washings" and "excreta", were incubated at p_H 7.6 with caseinogen and with a tryptic digest of caseinogen, each digest being finally tested both for proteolysis and ammonia production. The results are shown in Table IX.

Table IX. *Proteolytic and deaminating activities of excreta of Lucilia.*

Digest	Substrate	"Enzyme"	NH ₃ -N		Protein-N	
			Initial	Final	Initial	Final
1	Caseinogen	Washings	0.04	0.04	14.1	1.7
1 A			—	0.04	13.8	1.6
2		Excreta	0.05	0.07	12.0	0.0
2 A			—	0.07	12.1	0.0
3	Tryptic digest of caseinogen	Washings	0.10	0.17	—	—
3 A			—	0.18	—	—
4		Excreta	0.13	0.17	—	—
4 A			—	0.17	—	—

The excreta are strongly proteolytic, but during this proteolysis virtually no ammonia is produced. This experiment thus demonstrates the separate identity of the proteinase and the deaminase and suggests that the latter, since it does not pass out from the digestive tract, is an intracellular enzyme. This is in accordance with the findings of Weinland [1908].

DISCUSSION.

The evidence now obtained agrees with the results of other workers and it seems permissible to attempt a tentative sketch of the protein metabolism in these organisms which are purely protein feeders. Ingested protein is broken down initially by proteinase thereby becoming a substrate for deamination which may take place in the cells of the posterior segment of the mid-intestine. The ammonia so produced may be excreted into the digestive tract without entering into the circulatory fluid where it would be injurious in such high concentrations; the deaminated products, probably α -hydroxy-fatty acids, diffuse into

the haemolymph and are used chiefly for fuel or may be synthesised into fat *in situ* [Weinland, 1908]. The products of proteolysis that escape deamination by proceeding too far towards the amino-acid stage may be absorbed into the blood and be used in the endogenous metabolism; they are finally excreted as allantoin [Robinson, 1935] which may be derived from uric acid [Brown, 1936, 1] by the action of uricase [Truszkowski and Chajkinówna, 1935].

The deaminase also possesses peculiarities of exceptional interest. In the first place, it is water-soluble; the only other water-soluble deaminase, as far as the authors are aware, is a bacterial aspartase [Virtanen and Tarnanen, 1931]. Secondly, it is entirely without action upon amino-acids and simple peptides, but is active on higher protein breakdown products; this appears to be unique. The only other instance in any way similar is that of an enzyme obtained from rose petals [Grassmann and Bayerle, 1934] which deaminates di- and tri-peptides more readily than amino-acids. This deaminase, however, required oxygen for its operation.

The third peculiarity of the deaminase of flesh-fly larvae is that its optimum activity is under anaerobic conditions. Examples of anaerobic deaminases are rare; kidney histidase, aspartase and the so-called "spontaneous" deamination of excised organs and tumours may be mentioned, but their function appears to be quite specialised; perhaps the same may be said of the interesting enzyme present in kidney, dehydropeptidase [Bergmann and Schleich, 1931], whose mode of operation is apparently hydrolytic. The only really parallel instance, *i.e.* involving the deamination of protein breakdown products anaerobically, with which we are familiar, is the production of α -hydroxy-isocaproic acid from leucine by *Clostridium acetobutylicum* [Schmidt *et al.*, 1924].

SUMMARY.

The deaminase of flesh-fly larvae has been extracted and studied.

1. The enzyme operates better under anaerobic conditions.
2. The optimum p_H for its operation is 7.6.
3. The relation of its activity with temperature is linear over the range 10–37.5°.
4. The enzyme is water-soluble.
5. It is an intracellular enzyme.
6. The substrates are not simple amino-acids, dipeptides or tripeptides but the higher breakdown products of proteins.

Preparations from these organisms were found to contain also proteinase, polypeptidase, dipeptidase, uricase and very slight deamidase activities.

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