CXXI. THE NITROGEN METABOLISM OF AN INSECT (*LUCILIA SERICATA* MG.)

II. AMMONIA AND OTHER METABOLITES

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It was shown [Brown, 1938] that uric acid, or its oxidation product allantoin, is being constantly produced, as a nitrogenous end-product by *Lucilia sericata* throughout its life cycle. It remains to elucidate the role of NH_3 , known to be produced in large quantities by larvae of this species, and to investigate the possible occurrence of any other catabolite. This paper reports studies on the subjects of NH_3 and of its relationship with uric acid and allantoin, of urea, creatine and creatinine, and other nitrogenous substances.

METHODS OF ANALYSIS

Ammonia. Colorimetric method of Folin and Bell, modified for a small aliquot.

Urea. Xanthidrol method of Fosse; after deproteinization with tungstomolybdic acid, centrifuging and filtration through hardened filter paper immediately before the addition of xanthidrol. The urease method of Wakeman and Morrell was also used.

Creatine and creatinine. Alkaline picrate method of Folin; the picric acid was purified by the method of Benedict, the creatine converted by the method of Borsook [1935], and preliminary deproteinization accomplished by trichloroacetic acid (final concentration 2%).

Bicarbonate. Titration method of Van Slyke [1922].

Reducing sugar. Copper titration method of Benedict.

Uric acid and allantoin. The methods of Borsook [1935], as reported in the previous paper.

All the analytical methods enumerated above are taken, unless otherwise indicated, from the compilation of Peters & Van Slyke [1932].

AMMONIA

The excretion of NH_3 by sterile larvae. NH_3 was observed in sterile cultures of *Lucilia* larvae by Hobson [1932], and Michelbacher *et al.* [1932]. It appeared desirable to obtain quantitative figures on the course of excretion of this substance during larval life. Therefore sterile larvae were reared on a casein diet, and the NH_3 produced in a number of cultures estimated on successive days, as previously described [Brown, 1938] for the course of allantoin excretion; fully-fed larvae were removed to sterile sand, and the same technique used. The results together with the body weights of the larvae concerned are plotted in Fig. 1.

The curve for NH_3 excretion will be seen to be in every respect similar to that obtained for non-sterile larvae of *Wohlfahrtia* and *Calliphora* [Brown, 1936]. (Attention is here drawn to a typographical error in that paper, where the units for NH_3 in the text-figures should be cg., and not—as printed—mg.) The assertion of Bogdanow [1906] that this NH_3 production could be attributed to the action of contaminating bacteria is thus conclusively answered.

Fig. 1. The excretion of ammonia during the larval life of *Lucilia*. A, body weight in g. B, daily ammonia excretion in cg. Figures calculated to basis of 100 larvae.

A striking feature is the quantity of NH_3 that is excreted by the larvae—over 15 times the combined amount of uric acid and allantoin. This holds good for the three species of maggot that have been studied, as shown in Table I.

Table I. *Total NH_3 excreted by larvae of some flesh flies*

Figures in mg. per 100 larvae

Species and diet	Total NH_3 excreted mg.	Body-weight mg.	$\frac{\text{NH}_3}{\text{body-weight}}$ %
<i>Lucilia</i> , sterile casein	41	1,440	2.8
<i>Calliphora</i> , casein	216	4,620	4.7
<i>Calliphora</i> , meat	126	5,540	2.3
<i>Wohlfahrtia</i> , meat	534	15,310	3.5

It may be observed that the ratio of NH_3 produced to the body-weight achieved is of the same order in each case. This would suggest that for sarcophagous larvae as a whole, the formation of a given amount of tissue requires the deamination of an identical amount of food protein.

Ammonium bicarbonate. The slightness of the alkalinity of excreta and food residues, not more than pH 7.7 although the NH_3 concentration may be as high as 5 mg. per ml., suggested that this compound might be excreted in a combined form, probably with CO_2 . By a rough quantitative method it was found that although there was no carbonate present in the diets, an appreciable amount appeared after the larvae had grown in them; also that diets made up

with added NH_3 absorbed CO_2 from the air. These preliminary observations suggested the presence of bicarbonate.

Therefore 3 samples of 4-day cultures were analysed accurately for NH_3 and bicarbonate; as also was a check solution of "pure" NH_4HCO_3 (0.4%). The results are shown in Table II.

Table II. *Ammonia and bicarbonate in excreta of sterile larvae*

Culture	pH	NH_3 mg.	HCO_3^- mg.	Ratio $\frac{\text{NH}_3}{\text{HCO}_3^-}$
1	7.6	1.98	9.51	0.208
2	7.4	1.18	5.29	0.223
3	7.5	2.08	9.67	0.215
0.4% NH_4HCO_3		0.72	2.97	0.243 (calc. 0.279)

From the ratio of NH_3 to HCO_3^- , it can be seen that the combination is ammonium bicarbonate, containing even an excess of HCO_3^- over NH_3 . This is probably taken up by the base released by the change HPO_4^- to H_2PO_4^- in the salts of the diet.

Whether the true excretory product is free NH_3 , or combined NH_4HCO_3 , would be difficult to determine. Certainly the proximity of the posterior spiracles to the anus would render respired CO_2 especially liable to combination. In either case, it introduces a complicating factor into respiration experiments; the removal of CO_2 as bicarbonate in the excreta would explain the inordinately low R.Q. figures obtained for *Lucilia* larvae [Rainey, unpublished].

The deaminating enzyme of sterile larvae. An enzyme producing NH_3 from protein and its higher breakdown products has been isolated from *Lucilia* larvae and characterized by Brown & Farber [1936]. Objections might be raised to this work on the ground that, although the active preparations were in the form of acetone powders, the larvae used had been reared on contaminated meat.

Therefore 4 sterile cultures of 4-day larvae on the casein diet were used as material for an acetone powder preparation of 127 mg., made by the method already outlined [Brown, 1938]. This was incubated for 24 hr. at 37.5° with 0.5% casein solution, with a boiled control, and an autolytic control without casein. At the end of the experiment the NH_3 content per digest of 6 ml. was found to be as follows:

NH_3 , mg.	Boiled control	Autolytic control	Experimental	Produced from casein
	0.018	0.036	0.374	0.338

This result gives an activity value of 8.87 mg. NH_3 per g. powder, a figure slightly higher than any that was obtained from larvae on contaminated meat. The experiment shows conclusively that this deaminating enzyme is independent of bacteria, and appears in full strength in larvae reared on a pure protein diet.

NH_3 in the haemolymph. While NH_3 is being produced in such large amounts within the bodies of these larvae, free passage of it into the circulatory fluid would appear to involve a concentration that would almost certainly be toxic. The NH_3 contents of the haemolymph (obtained by thoracic puncture and pressing on to filter paper) and of the remaining tissues of fully-gorged larvae were therefore estimated.

It was found that the haemolymph, comprising in this case 23% of the body-weight, or 32% of the body water, contained only 6% of the total amount of NH_3 in the tissues. That is, the concentration of NH_3 in the haemolymph is more than five times as dilute as it would have been, were the catabolite evenly

distributed throughout the body water. This argues against the free diffusion of NH_3 into the circulatory fluid, and supports the suggestion of Brown & Farber [1936] that in *Lucilia* larvae it is excreted into the mid-gut without ever entering the haemocoel.

The production of NH_3 in egg, pupa and adult. The abundant excretion of NH_3 during the larval feeding period is contrasted with the small amount produced in the other stages. A trace of it may be detected in fully developed eggs, to the extent of 0.009 mg. per g. material (approximately 1200 eggs). There is a small amount present at pupation, gradually increasing till emergence, when it is excreted in the meconium. The ratio of $\text{NH}_3\text{-N}$ to uric acid-N in the meconium of *Lucilia* was found to be 14.5%, somewhat higher than the 9.2% found in *Deilephila* by Heller & Aremowna [1932].

Quantitative determination of NH_3 excretion in adults was effected by allowing samples of the desired age to excrete in sterilized dry culture tubes kept in a desiccator, thus reducing bacterial change to a minimum. The excreta were then analysed for uric acid as well as NH_3 ; the ratio of $\text{NH}_3\text{-N}$ to uric acid-N being fairly constant at 43%, the NH_3 excreted over a given period could be calculated from the known excretion rate of uric acid.

These results are indicated in Fig. 2. It may be noted that the total amount of NH_3 excreted since prepupal life is 2.1 mg. per 100 individuals, being only 5% of the amount excreted during larval feeding.

Fig. 2. The production of ammonia in pupa and adult. A, total produced. B, amount excreted. Figures calculated to basis of 100 larvae.

THE INTERRELATION OF NH_3 , URIC ACID AND ALLANTOIN

Exp. 1. Excretion in starved newly-hatched larvae. For showing experimentally that the NH_3 excretion is dependent on the ingestion of protein food, whereas that of uric acid and allantoin continues independently, newly-hatched larvae were prevented from feeding. A large sample of eggs was inoculated into an autoclaved petri-dish lined with damped filter paper, and sealed with adhesive tape. The larvae hatched and lived under these conditions for 24 hr. at room temperature without mortality. At the end of this period analyses were made of NH_3 , uric acid and allantoin in tissues and excreta, and the amount produced calculated after subtraction of the content in fully-developed eggs. The productions of these substances in starved and feeding larvae may then be compared (Table III).

Table III. *Productions of N catabolites by starved and by feeding larvae. Units mg.*

Catabolite	Eggs	Starved larvae		Produced by starved larvae	Produced by feeding larvae	Starved feeding %
		Tissues	Excreta			
NH_3	0.001	Trace	0.042	0.041	7.800	0.5
Allantoin	0.015	0.192	0.072	0.249	0.390	64.0
Uric acid	0.009	Trace	Negative	Oxidized	Negative	—

This experiment shows that in absence of food the NH_3 production is almost entirely eliminated, whereas the excretion of allantoin is only moderately reduced—a reduction to be expected in view of diminished activity and lack of growth.

However, as repeated experiments showed, sterile starved larvae do excrete a small amount of NH_3 . It may well be that an initial excretion, or secretion, of NH_3 is necessary to produce the alkalinity required by the excreted trypsin, thus enabling the larvae to start the cumulative process of liquefying the medium.

Exp. 2. The effect of adding amino-acid to the diet. In previous work [Brown & Farber, 1936] it has been shown that whereas NH_3 is readily produced from protein and its breakdown products, amino-acids are not deaminated by this enzyme of *Lucilia* larvae. It would therefore seem probable that free amino-acids in the diet would be absorbed intact, and that their N would follow the regular course of metabolism to uric acid and allantoin. As an attempt to demonstrate this assumption sterile larvae were bred on casein diets to which varying amounts of the amino-acid glycine had been added, in the expectation of finding an increased production of uric acid and allantoin.

In making up the diets, water was added only in proportion to the amount of casein, and the slightly acid glycine was neutralized with dilute NaOH. It was found that with 25% and 20% of glycine in the diet, larvae died soon after hatching; with 15%, a few minute larvae survived the experiment; with 10 and 5%, normal growth occurred, being slightly less in the former case. Figures for two of many experiments are given below, a 5-day culture in which only allantoin was excreted, and a 10-day culture in which uric acid was also produced (Table IV).

Table IV. *The production of uric acid and allantoin with free amino-acid added to the diet*

Larvae hatched at 33° and reared at 25°. Units, mg.

% Glycine	Weight of larvae	Allantoin				Uric acid bodies	Total as uric acid	Uric acid per g. larvae
		Excreta	Bodies	Total	As uric acid			
5-day culture	0	696	0.64	0.15	0.79	0.84	Trace	0.84
	0*	361	0.19	0.08	0.27	0.29	Trace	0.29
	5	515	0.24	0.08	0.32	0.35	Trace	0.35
	10	99	0.08	0.03	0.10	0.11	Trace	0.11
10-day culture	0	1610	0.83	0.19	1.02	1.09	0.25	1.34
	5	1465	0.89	0.17	1.06	1.13	0.62	1.75
	10	1289	0.69	0.14	0.83	0.88	0.13	1.01

* Contaminated.

These figures when calculated for a unit weight of larvae, as in the last column of the table, show no correlation of uric acid and allantoin production with the proportion of glycine in the diet. A similar experiment using the amino-acids of a tryptic digest of casein was also unsuccessful. It would seem that the only possible explanation of these results is either a complete utilization without catabolism, or—more probably—the inability of the larvae to absorb such an excess of free amino-acid.

Exp. 3. The effect of adding carbohydrate to the diet. It has been suggested [Brown, 1936] that larvae of *Lucilia* might utilize some dietary constituent other than protein—probably carbohydrate—and that such utilization might be

indicated by the attainment of a given body weight with the excretion of less NH_3 .

Synthetic casein diets were therefore made up with increasing concentrations of glucose (in one experiment) and glycogen (in another). Larvae were found to survive only on diets containing 20 % and under, and on all these, down to a concentration of 5 %, they attained a very small size. The average body-weight at 6 days was about 150 mg. per 100 individuals, the ammonia production only 0.2 mg. per tube, and the pH of the medium only 6.9, scarcely favourable for the extracorporeal action of the excreted trypsin. Contaminated cultures exhibited a slightly better growth, 260 mg. per 100 individuals, due presumably to the increased NH_3 production, 1.2 mg. per tube, and to the more alkaline pH 7.6.

Evidence for hydrolysis of glycogen to glucose by larvae of *Lucilia* was obtained in all cases, one culture containing as much as 98 mg. glucose; considering that the total glycogen in that culture was 250 mg., this probably represents hydrolysis of all the glycogen in the liquefied portion. Hobson [1931] found a weak amylase in the salivary glands of *Lucilia* larvae; and according to Tomita & Kumon [1936], preparations from larvae of *Sarcophaga carnaria* are strongly amylolytic. The corollary of these results is that there was little or no absorption of the glucose so produced.

The effect of the inclusion of carbohydrate in the diet has thus been, if it does not kill outright, to inhibit growth. The cycle of protein digestion, absorption, NH_3 production and digestion again, has been inhibited. It may well be that the mol. concentration of the glucose has produced an osmotic gradient too high for absorption in these organisms, and that concentrations of over 20 % are lethal by reason of their hypertonicity. Such a hypothesis is also suggested by the results of the previous experiment with amino-acid.

Guyénot [1917] found also that the inclusion of carbohydrate in the diet was unfavourable or lethal to larvae of *Drosophila*.

UREA

Distribution in tissues and excreta of all stages. For this study, aqueous extracts were made of the material (1–3 g. being taken) and analysed by the xanthydrol or urease methods, or both. The concentrations of this substance, in mg. per g. material, are given in Table V.

Table V. *Urea and creatine-creatinine in the various stages of Lucilia*

Stage	Urea (xanthydrol) mg./g.	Urea (urease) mg./g.	Creatinine mg./g.	Creatine mg./g.
Eggs (20 hr.)	0.10	0.10	Trace	0.09
Sterile larvae (3½ days)	0.02	—	0.03	0.08
Sterile prepupae (8 days)	0.01	—	0.05	0.09
Pupae (3 days)	0.03	0.04	0.02	0.05
Meconium*	0.03	—	0.05	0.10
Adults†	0.11	0.18	Negative	0.23
Adult excreta	0.07	0.11	0.09	0.15

* For obtaining sterile meconia, mature pupae, surface sterilized with HgCl_2 , were placed in autoclaved culture tubes stoppered with cottonwool and left overnight for emergence.

† For obtaining sterile adults and their excreta, newly-emerged flies were allowed to fly into a sterilized litre flask containing damped cottonwool. One day later, when according to Balzam [1937] they should have lost nearly all pupal bacteria, they were fed with autoclaved sucrose and peptone solutions on filter paper, which were removed 4 hr. later. The next day adults and excreta were analysed.

These results show that although urea is a regular constituent of all stages of *Lucilia*, yet it occurs in such small amounts as to indicate that it is an intermediary product. Only in the egg does its concentration reach any degree of significance. This is similar to the results obtained by Leifert [1935] for *Antherea pernyi*, whose enzyme experiments suggested that here urea was an intermediate in uric acid synthesis.

Occurrence in larval excreta. The excretory residues from 11 cultures of sterile larvae, when tested either by the xanthidrol or the urease method, gave no evidence of the presence of urea. However, Robinson [1937] has reported that urea is present in maggot excretions.

To reconcile these two contradictory results, samples of excreta were taken from larvae on a sterile synthetic diet, a contaminated synthetic diet and on contaminated meat. After deproteinization with tungstomolybdic acid they were stood overnight in the glacial acetic acid, so that filtration gave crystal-clear solutions before the addition of xanthidrol. The results were as follows:

Excreta from sterile synthetic diet: no precipitate (of dioxanthylurea).

Contaminated synthetic diet: doubtful traces of precipitate.

Contaminated meat: definite precipitate.

Thus urea is present in the excreta of larvae raised on meat in the ordinary way, probably from preformed urea in the diet rather than as a product of bacterial hydrolysis of allantoin; since a contaminated culture on a synthetic diet develops only a doubtful trace of this substance.

Absence of urease at any stage. It was possible that the scarcity of urea might be due to its hydrolysis to ammonium carbonate by the enzyme urease. Therefore acetone powders were made of each stage of the insect, egg, larva, prepupa, pupa and adult (500 mg. of each), and incubated with 6% urea at 37.5° for 40 min. In no case was there any NH_3 production. Therefore urease is entirely absent from *Lucilia* throughout its life cycle.

CREATINE AND CREATININE

Distribution in tissues and excreta of all stages. Aqueous extracts of 1-3 g. of the material were analysed by the method of Folin. The creatine and creatinine contents per g. material are given in Table V. From these figures it may be concluded that both substances, but chiefly creatine, are regular constituents of tissues and excreta of *Lucilia sericata*. Leifert [1935] had found creatine, but not creatinine, in significant amounts in many organs of *Antherea*. These two substances continue to be found (in very small amounts) in insects, although there was a tendency to put this evidence in doubt by reason of the invertebrate phosphagen theory [v. Baldwin, 1937].

Metabolism of creatine and creatinine by sterile larvae. In the hope of obtaining some clue as to the role of these substances in the metabolism of *Lucilia*, the fate of added creatine and creatinine in the synthetic diet of sterile larvae was investigated. Approximately 10 mg. of creatine (in one series) and creatinine (in another) were administered per culture tube; after 7 days at 25° the amounts of these substances in larvae and food residues were analysed, as also were control tubes without larvae. The results are tabulated below, being expressed as mg. of creatinine.

It is seen that both series of diets were brought to the same creatinine level by the preliminary autoclaving. However, the results, although approximate, indicate that:

- (1) There is no destruction of total creatine-creatinine by larvae.

Table VI. *Metabolism of creatine and creatinine by sterile larvae*

		Units, mg.					
Culture no.	Weight of larvae	Larvae		Food and excreta		Total creatine and creatinine	
		Creatine	Creatinine	Creatine	Creatinine		
“Creatinine”	1	1506	0.46	0.13	7.13	0.68	8.40
	2	1348	0.29	0.13	7.14	1.04	8.60
	3	1182	0.40	0.10	6.62	0.82	7.94
	Control	—	—	—	1.01	7.14	8.15
“Creatine”	1	735	0.26	0.00	7.69	0.89	9.47
	2	874	0.41	0.13	7.72	0.64	9.00
	3	1054	0.50	0.09	8.11	1.08	10.27
	Control	—	—	—	1.04	9.36	10.40

(2) Creatinine is converted almost entirely into creatine; this is probably due to the alkalinity of the excreta.

(3) A small amount of these two substances is retained within the larvae, possibly entirely accounted for by gut contents.

Thus these experiments indicate that larvae of *Lucilia* treat creatine and creatinine as physiologically inert substances.

MISCELLANEOUS

Allantoic acid and alloxan. Since the enzyme allantoinase has been found in many insects [Rocco, 1936], there was a possibility that in *Lucilia* the allantoin was further catabolized to allantoic acid. Acetone powders (500 mg. in each case) of the various stages were therefore incubated with 0.1 % allantoin solution for 4 hr. at 37.5°, and then digests and boiled controls were analysed for allantoic acid by the method of Borsook. Neither egg, larva, prepupa, pupa nor adult gave any evidence of the presence of allantoinase, and their content of allantoic acid was never more than a trace.

Alloxan, a possible oxidation product of allantoin, was sought in larval excreta by the FeSO₄ test [Plimmer, 1935] with entirely negative results.

Purine oxidases. It was possible that some of the uric acid and allantoin produced by *Lucilia* was the end-product of purine metabolism. Therefore evidence was sought for the production of uric acid or allantoin from adenine by feeding larvae or by their acetone powder preparations; guanine was not used as substrate since Leifert [1935] had found it entirely absent from *Antheraea*.

Incubation of 850 mg. of acetone powder in 0.09 % (saturated) adenine solution at 37.5° for 24 hr., with shaking, gave not a trace of uric acid or allantoin production.

Feeding larvae, reared on the sterile synthetic diet and 5 days old, were transferred to casein diets made up with 0.09 % adenine solution; a control tube was run without the adenine. When the larvae had fed on these for 28 hr. at 33° they and their excreta were analysed for uric acid and allantoin. The results for the two experimental, and the control, tubes are shown in Table VII.

Table VII. *Effect of adenine in the diet on uric acid and allantoin production*

Culture	Number of larvae	Units, mg. Allantoin		Uric acid in bodies	Total as uric acid	Uric acid per 100 larvae
		Excreta	Bodies			
Adenine 1	90	0.006	0.048	0.018	0.075	0.084
Adenine 2	65	0.003	0.042	0.014	0.062	0.095
Water control	70	0.002	0.041	0.026	0.072	0.102

These figures show that larvae feeding on the adenine diet synthesized and excreted no more, even less, uric acid and allantoin than those on the ordinary diet. The conclusion is therefore that *Lucilia* larvae lack the enzyme mechanisms oxidizing purines to uric acid.

DISCUSSION

The results reported in this paper further confirm the thesis that in the N metabolism of *Lucilia sericata* uric acid and allantoin are produced. Therein it resembles all other insects hitherto studied. No other N metabolite could be detected in significant amounts; the very small amount of urea, coupled with the lack of the enzyme urease, suggest that this substance is merely an intermediate in the synthesis of uric acid.

Larvae of this insect represent a special case where ammonia is produced in large quantities as a direct response to protein feeding. The fact that glucose and amino-acids in the diet are not utilized, and even produce lethal effects, suggests that the larval mid-gut is a specialized organ, incapable of dealing with substances of low molecular weight and hence high osmotic pressure; just as the larval deaminating enzyme can act on protein and its higher breakdown products, but not on amino-acids.

SUMMARY

1. In sterile larvae of *Lucilia sericata* the course of NH_3 excretion is similar to that in non-sterile larvae of *Calliphora* and *Wohlfahrtia*.
2. The total amounts of NH_3 excreted in relation to unit body-weight, are approximately equal in these three cases.
3. NH_3 is present in excreta as NH_4HCO_3 .
4. The deaminating enzyme of *Lucilia* is as strong in larvae reared on a sterile casein diet as in those reared on contaminated meat.
5. Whereas the excretion of NH_3 by larvae is closely dependent on protein feeding, the excretion of uric acid and allantoin is independent.
6. Feeding larvae hydrolyse glycogen.
7. Larvae are killed by strong concentrations of amino-acid or glucose in the food, and probably do not absorb moderate concentrations.
8. Urea is present in very small amounts in all stages, but absent from excreta of sterile larvae.
9. Urease and allantoinase are absent at all stages.
10. Small amounts of creatine, and traces of creatinine, are present in all stages; dietary creatinine is not metabolized.
11. Purine oxidases are probably absent.

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