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

I. URIC ACID, ALLANTOIN AND URICASE

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THE purpose of the following two papers of this series is to demonstrate the synthesis and excretion of nitrogenous substances by *Lucilia sericata* throughout its life-cycle, and to elucidate their origin and the role they play in the metabolism of the insect. This first paper deals with the main catabolite uric acid, with its oxidation product allantoin, and with the oxidative enzyme uricase.

MATERIAL AND METHODS

The insect used for this study was the common flesh-fly or sheep blowfly, *Lucilia sericata* Mg.; it was obtained from a stock bred continuously in the laboratory for several years.

Methods of analysis

Uric acid was determined colorimetrically by the Zn precipitation method of Borsook [1935]; proteins were first precipitated by tungstomolybdic acid [Peters & Van Slyke, 1932].

Allantoin was assayed by the colorimetric method of Borsook [1935].

Extracts were made by grinding the material in 1 part of a saturated solution of Li_2CO_3, and making up to volume with 24 parts of distilled water.

Synthetic diet for sterile larvae

The diet was essentially that developed by Michelbacher *et al.* [1932], consisting of:

Casein (acetic-precipitated B.D.H.)	90·0 parts
Brewers' yeast powder	9·0
Salt quota [McCollum & Simmonds, 1918]	0·9
Agar	3·0

For each culture tube (5 in. × 1 in.), 2·5 g. of this powder mixture were made up with 5 ml. of an emulsion of lanolin, produced by boiling adeps lanae B.D.H. with 200 parts of water and shaking until cool. Glass beads may be added to the diet to prevent drowning of larvae. The tubes were stoppered with cottonwool and autoclaved for 20 min. at 17 lb. pressure.

Eggs were surface-sterilized by the method of Hobson [1932], and about 100 eggs were inoculated into each tube. Cultures were raised at 33°; in some cases they were hatched at this temperature and later kept at 25°. At 33° they achieved 60% and at the lower temperature 90% of the size of normal larvae on contaminated meat; adult flies emerged in due course. Cultures with butter as source of sterol, or with the lanolin enriched by cholesteryl acetate, showed no difference in amount of growth.

Tests for sterility were performed on serum broth and by subculture into deep gelatin incubated at 33°. About 90% of the cultures so tested were sterile by these methods.

Uric acid and allantoin

In this study all cultures were kept at a constant temperature of 33° and results were calculated to the standard basis of mg. per 100 individuals. All graphs represent cumulative figures.

Eggs. During embryonic development both uric acid and allantoin accumulate. Eggs ready to hatch were found to contain 0·178 mg. uric acid and 0·106 mg. allantoin per g. fresh material, or respectively 0·0015 and 0·0009 mg. per 100 individuals.

Sterile larvae and prepupae. The factors of bacterial change and of catabolites preformed in the food were eliminated under these circumscribed conditions of rearing. Sterilized eggs were inoculated into 8 tubes containing the sterile casein diet. Five of these cultures were analysed, for content of uric acid and allantoin of larval tissues and of excreta, on 5 successive days, until the larvae had stopped feeding. The prepupae in the remaining 3 tubes were transferred with sterile forceps to similar tubes containing damp quartz sand (acid-washed) and cotton-wool, all sterilized. At the end of the 8th day several individuals had become white pupae. The results are shown graphically in Fig. 1.

It may be seen that both allantoin and uric acid are produced by sterile larvae. The production of allantoin is steady, increasing slightly with age, throughout larval and prepupal life. Uric acid does not appear until the 3rd day of larval life and then its amount in the tissues increases; it is not excreted.

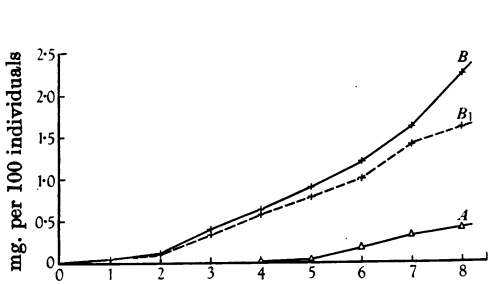

Fig. 1.

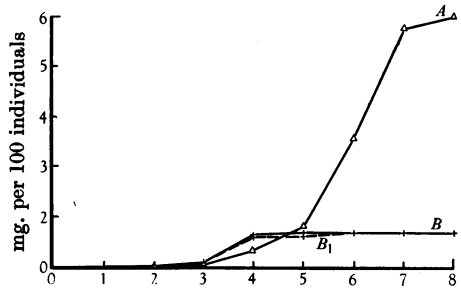

Fig. 2.

Fig. 1. Uric acid and allantoin in sterile larvae on a casein diet. *A*, Uric acid in bodies. *B*, Total allantoin produced. B_1, Allantion excreted.

Fig. 2. Uric acid and allantoin in normal larvae on a meat diet. *A*, Uric acid in bodies. *B*, Total allantoin produced. B_1, Allantoin excreted.

Normal larvae. A culture of larvae was raised on non-sterile lean beef, being allowed to migrate into sand when fully gorged. Every day a sample was taken and washed; of this a portion was analysed for uric acid and allantoin content, the remainder being placed on silver sand in a test tube and the excreta analysed 24 hr. later. The results, calculated similarly, are given in Fig. 2.

In contrast to sterile larvae, allantoin excretion in normal larvae is confined to the first 4 days, and occurs mainly on the 4th; thereafter there is a pronounced accumulation of uric acid. The uric acid, again, is never excreted but is carried over into the pupa.

Pupae. Samples were obtained from an even-aged batch of stock pupae kept in damp sand, 25 being taken daily for analysis. For obtaining emergence and the resulting meconia on the 5th day, a sample of pupae was transferred on the 4th day to sterile dry test tubes plugged with cottonwool. The temperature of the experiment (33°) was high for pupal development, but mortality occurred only after the adult had fully formed within the puparium. The results are plotted in Fig. 3.

Thus during metamorphosis uric acid is being produced in increased amount; some 90 % of it is excreted in the meconium. Allantoin is almost entirely absent throughout pupal life, but appears in the meconium to the extent of 11 % of the uric acid.

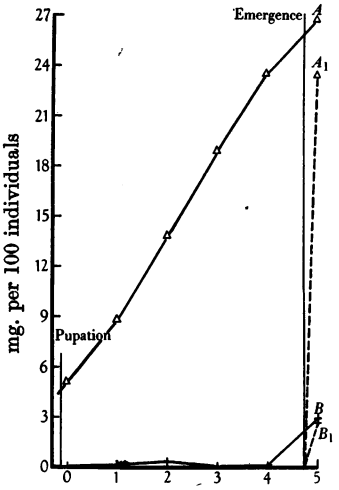

Fig. 3.

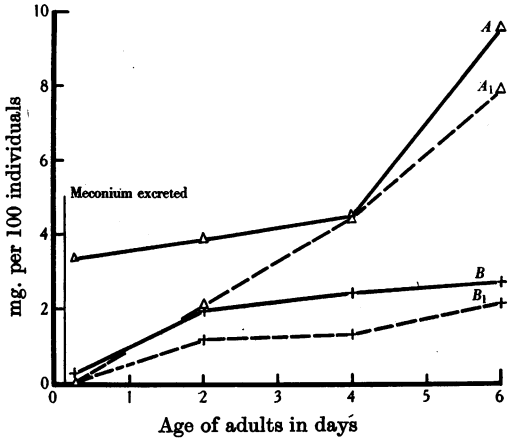

Fig. 4.

Fig. 3. Uric acid and allantoin in pupae. *A*, Uric acid in pupae. A_1, Uric acid excreted in meconium. *B*, Allantoin in pupae. B_1, Allantoin excreted in meconium.

Fig. 4. Uric acid and allantoin in adults. *A*, Total uric acid produced. A_1, Uric acid excreted. *B*, Total allantoin produced. B_1, Allantoin excreted.

Adults. For this study precautions were taken to reduce bacterial contamination. Mature pupae were surface-sterilized by $HgCl_2$ and put in autoclaved culture tubes. Then 4 wide-mouthed glass jars (250 ml.) containing lump sugar, serum broth on filter paper and a little water, were stoppered with cottonwool and sterilized by autoclaving. About 20 of the newly emerged flies were transferred from the tubes to each jar by phototropism. Sterile water was added from time to time; death from desiccation occurred, but was allowed for by a correction factor. Uric acid and allantoin in bodies and excreta of the adults were analysed in jars left for 2, 4 and 6 days respectively. Adult excreta appear as small black spots, contrasted with the larger creamy granulations of the meconia; they contain the same yellow-brown pigment however. The results, calculated to the standard unit, are shown in Fig. 4.

Uric acid is being produced in the adult at a rate about half of that in the pupa. The process of excretion almost completely eliminates this and the carry-over from the pupa, though uric acid accumulates again in later life. The produc-

tion of allantoin is quite high—about half that of uric acid, in early adult life, though later it falls off markedly.

Interfering substances in uric acid analysis. The following substances were found to give faint colours with the arsenophosphotungstic acid reagent: K_2S, resorcinol, cysteine and tyrosine; incubation at 37° for 24 hr. reduced the colour-producing power of K_2S to a trace [cf. Truszkowski & Chajkinowna, 1935].

The advantage of Borsook's method is that it first precipitates uric acid free from interfering substances, which it was found to do quantitatively for concentrations as low as 0·002 %. The original direct method and Borsook's method were compared for tissues and excreta of *Lucilia* larvae on a casein diet, after preliminary tungstomolybdic acid precipitation; the uric acid values in mg. per 100 individuals were:

	By direct method	By Borsook's method
For body tissues	0·66	0·40
For excreta	0·42	0·00

Thus interfering substances are present in both tissues and excreta, being most significant in the latter. It is unlikely that sulphides or thio-compounds are concerned, since standing at 37° for 24 hr. has no effect; nor does allantoin produce any colour.

The fact that *Lucilia* excreta, though actually containing no uric acid, yet appear to contain a small amount when analysed by the direct method of Benedict & Franke [1922], led to a re-examination of the claim of uric acid excretion in larvae of *Calliphora erythrocephala* [Brown, 1936]. Larvae were raised on a non-sterile casein diet as described in that paper and the excreta and food residues were analysed daily by the direct method and the method of Borsook. No uric acid was found by the latter, though analysis by the former method gave figures ranging up to 0·29 mg. per 100 individuals, daily production. Thus it can be seen that larvae of *Calliphora*, like those of *Lucilia*, do not excrete uric acid; the interfering substances are probably free tyrosine and other compounds produced in the liquefaction of casein.

Uricase

The variation of uricase throughout the life cycle. The results reported above show that allantoin appears as a catabolite in eggs, larvae and adults, whereas uric acid accumulates as the only end-product in prepupae and pupae, to be excreted in the meconium. These facts could be explained by the presence of the enzyme uricase—oxidizing uric acid to allantoin—in eggs, larvae and adults, and its absence from pupae. The amount of uricase was therefore determined quantitatively for the various stages in the life cycle of *Lucilia*.

Material for this study was taken from stock reared at 25°; the sterile larvae were raised on the casein diet at 33°. The enzyme preparations were obtained in a concentrated and fat-free form by precipitating the ground tissue in 20 volumes of acetone, washing by repeated centrifuging and decantation with acetone and drying in a vacuum desiccator. The resulting fine powders were kept in the desiccator until used. About 725 mg. fresh material gave 100 mg. "acetone powder". The technique for assay of uricase content was as follows.

Enzyme solution: 200 to 500 mg. powder ground in water and made up to 10 ml.

Substrate (0·1 % uric acid) solution: 100 mg. uric acid dissolved in 50 ml. boiling saturated Li_2CO_3 solution, made up to 100 ml., cooled and filtered; used immediately.

Digests: 3 ml. enzyme incubated with 2 ml. substrate for 24 hr. at 37·5°, with continuous shaking under toluene in L-shaped rocker tubes. Control digest boiled for 3 min. before incubation, experimental digests (two) for 3 min. at end of incubation.

Uric acid and allantoin were then determined in the two experimental and the control digests. The amount of uric acid destroyed by the enzyme was obtained by subtracting the experimental value from the control, and the amount of allantoin produced by the reverse process. Thus, allowing for the fact that 168 mg. of uric acid would give 158 mg. of allantoin, the percentage of oxidized uric acid appearing as allantoin could be calculated. The uricase contents of the different stages were compared by multiplying the results so as to give the uricolytic activity of 1 g. of tissue powder. In many cases the powders of two or more samples of material of the same stage were assayed, so that Table I represents a summary of results.

Table I. *The uricolytic activities of the various stages of* Lucilia

Stage	Exact age	Uric acid destroyed mg.	Allantoin produced mg.	$\frac{\text{Allantoin}}{\text{Uric acid}}$ %	Uricase per g. powder mg. uric acid
Eggs	20 hr. after oviposition	4·1	2·8	74	9·6
Larvae	3½ days after hatching	3·7	3·2	91	14·2
Prepupae	7 days after hatching	0·0	0·0	—	0·0
Pupae	3 days after hatching	0·0	0·0	—	0·0
Adults	1 to 5 days after emergence	4·2	3·5	90	21·2
Sterile larvae	3 days	2·9	1·3	50	17·3
	5½ days	5·8	3·5	64	10·4
	8 days (prepupae)	3·6	2·7	78	15·4

These results explain quite adequately the excretion figures of the first half of the paper. They show that:

1. As uric acid is oxidized by the enzyme preparations from *Lucilia*, allantoin is produced; this is characteristic of uricase.

2. The enzyme uricase is present in eggs, larvae and adults, and absent from pupae.

An interesting finding is that whereas the enzyme is definitely absent from normal prepupae, in sterile larvae it persists until pupation. Thus the difference between the results of Fig. 1 and Fig. 2 is explained.

The occurrence of uricase in adults of *Lucilia sericata* is paralleled by that reported for *Musca carnaria* by Truszkowski & Chajkinowna [1935].

The formation of allantoin in alkaline solution. Robinson [1935], who discovered allantoin in larval excreta, suggested that it arose from the oxidation of uric acid, which "is said to occur in alkaline solution". In previous work [Brown, 1937], it had been found that extracts of excreta of *Melanoplus bivittatus* in 0·2 % Li_2CO_3 solution showed a gradual destruction of their uric acid content; suggesting that the alkalinity of such a solution was responsible for the change.

Therefore uric acid was dissolved in half-saturated Li_2CO_3 solution, to the extent of 100 mg. per 100 ml. The *p*H of this solution was 9·6 (colorimetric). This was kept at room temperature, and the increase in its content of allantoin was followed by the method of Borsook, viz.:

Days after uric acid dissolved	0	3	9	18
Allantoin, mg. per 100 ml.	0·15	3·35	24·5	61·5

At 18 days the content of uric acid had dropped to only 12 % of the initial amount. Thus the formation of allantoin from uric acid at this alkalinity is definite.

It therefore remained to discover whether the alkalinity of the larval excreta was sufficient to promote such a change. 1 mg. of crystalline uric acid was added to the excretory residues of 8 cultures of sterile larvae, and incubated at 37·5° for 24 hr. It was found that from it 0·11 mg. of allantoin was produced, while the amount of uric acid in solution had fallen from 0·09 mg. to 0·06 mg. The *p*H of the medium was 7·7. Thus, though the alkalinity of larval excreta allows a certain amount of oxidation to allantoin, it may be considered insufficient to mask the excretion of uric acid were such to occur.

The disappearance of uricase from prepupae. Previous experiments having shown that uricolytic activity, although strong in feeding larvae, is totally absent from prepupae 3 days after leaving the meat, an attempt was made to determine the exact time at which it disappeared, and to correlate it with the course of synthesis and excretion of uric acid and allantoin.

A culture of larvae was raised on non-sterile meat at 25°; it was arranged so that when they left the meat, which occurred at $3\frac{1}{2}$ days after hatching, they should fall into the lower chamber of a clean glass desiccator. Every day from the 2nd to the 5th inclusive, a large sample was taken for the preparation of acetone powders, the uricase content of which was assayed by the methods given above. The daily production of uric acid and allantoin in tissues and excreta was also followed.

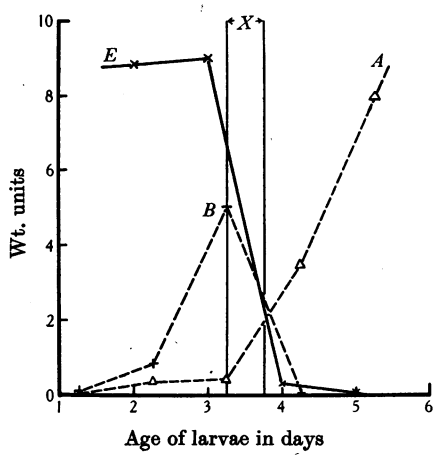

Fig. 5. The disappearance of uricase from prepupae and the change from allantoin to uric acid as the catabolite. *E*, uricase content of larvae, in mg. uric acid destroyed per g. powder. *A*, uric acid accumulated in bodies. *B*, allantoin excreted. In mg. × 10 per 100 individuals. *X*, larvae leaving meat during period indicated by two vertical lines.

The uricase content of these larvae day by day is plotted in Fig. 5, together with the production of uric acid and allantoin for comparison. It is seen that the enzyme uricase disappears quite suddenly between the 3rd and 4th days. At this time the excretion of allantoin ceases, and the accumulation of uric acid in the tissues is correspondingly increased. This is precisely the period when the larvae are leaving the meat and become prepupae. Thus the disappearance of uricase is a clear-cut symptom of the transition from larva to prepupa.

To follow up these results, and in view of the retention of uricase and the persistence of allantoin excretion in larvae kept on sterile synthetic diets, an investigation was made of the effect on uricolytic activity of (*a*) removing the

larvae from the meat prematurely, and of (*b*) keeping them on it forcibly for an extra day. The following findings were obtained, using the same technique:

(*a*) Larvae removed from meat at $2\frac{1}{4}$ days, and kept in silver sand till $3\frac{1}{4}$ days, contained a uricolytic activity of 14·2 mg. uric acid per g. powder.

(*b*) Larvae kept on meat in cottonwool-plugged culture tubes for $4\frac{1}{2}$ days retained a uricolytic activity of 13·2 mg. uric acid per g. powder.

Thus it can be seen that the mere removal of larvae from the meat is not sufficient to cause their uricase to disappear and that there is some other metabolic factor in operation. Retention on the meat appeared to postpone the disappearance of uricase, though in this case the larvae were under unusual conditions, being engulfed and rendered immobile by the meat.

The appearance of uricase on emergence. Just as the loss of uricolytic activity in normal larvae could be successfully localized within 1 day, so it seemed feasible to determine the time at which the adult gained its uricase.

A sample of pupae was kept at 25°. When emergence had just started, a number were dissected and divided into two classes according to their degree of development, viz.:

1. Eyes red, body not yet pigmented, taken as $1\frac{1}{4}$ days before emergence.
2. Eyes and body pigmented, taken as $\frac{1}{4}$ day before emergence. Acetone powders were made of each class. The remaining pupae were allowed to emerge. A portion of these was taken soon after emergence (0 to 8 hr.) for an acetone powder of newly-emerged flies. The remainder were kept in a large flask with damped cottonwool for $1\frac{1}{2}$ days, and then made into acetone powder.

These four age-classes were then tested for uricolytic activity by the methods outlined above. The results are represented graphically in Fig. 6.

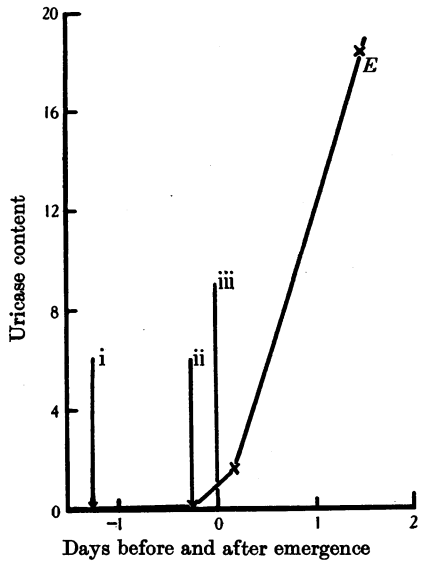

Fig. 6. The appearance of uricase on emergence of adult. *E*, uricase content of insect, in mg. uric acid destroyed per g. powder. i, eyes only pigmented. ii, body pigmented. iii, emergence.

It may be seen that uricase does not appear until the fly has actually emerged, and the first day of adult life witnesses a great increase in its activity. During this period too the adult is disinfecting itself of bacteria carried over from pupal life [Balzam, 1937].

Discussion

The results described in this paper show that in *Lucilia sericata* N is metabolized to uric acid; at various stages in the life cycle—the egg, the adult, and notably the larva—it is further oxidized to allantoin. It was this last fact, coupled with the high excretion of NH_3, which caused *Lucilia* larvae to be considered as exceptions to the uricotelic rule. However, the presence of allantoin, confirming the work of Robinson, and of the enzyme uricase, has shown the path of N metabolism in this insect to be through uric acid.

It would be difficult to speculate on the reasons for *Lucilia* larvae excreting allantoin instead of uric acid; firstly, owing to the fortuitous distribution of uricolytic powers in mammals and, secondly, because the work of Leifert [1935] indicates that uricase and allantoin are perhaps more widely distributed in insects than has been hitherto supposed. However, the semi-aquatic existence of these larvae would eliminate the need of an excretory product adapted to dry conditions of life, thereby rendering available the energy yielded by oxidizing uric acid to allantoin.

Summary

1. Sterile larvae of *Lucilia sericata* have been reared on a synthetic diet, with lanolin as the source of sterol.

2. Larvae excrete allantoin but not uric acid; the latter accumulates in the tissues.

3. Pupae produce large quantities of uric acid but no allantoin.

4. Adults and eggs produce both allantoin and uric acid.

5. Uricase is present in eggs, larvae, and adults, but absent from prepupae and pupae.

6. Uricase disappears suddenly and entirely when the larvae leave the meat and become prepupae.

7. The reappearance of uricase is equally abrupt, occurring immediately after emergence.

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REFERENCES

Balzam (1937). *Ann. Inst. Pasteur*, **58**, 181.
Benedict & Franke (1922). *J. biol. Chem.* **52**, 387.
Borsook (1935). *J. biol. Chem.* **110**, 481.
Brown (1936). *J. exp. Biol.* **13**, 131.
—— (1937). *J. exp. Biol.* **14**, 87.
Hobson (1932). *J. exp. Biol.* **9**, 128.
Leifert (1935). *Zool. Jahrb. Abt. allg. Zool. Physiol. Tiere*, **55**, 131.
McCollum & Simmonds (1918). *J. biol. Chem.* **33**, 303.
Michelbacher, Hoskins & Herms (1932). *J. exp. Zool.* **64**, 109.
Peters & Van Slyke (1932). Quantitative Clinical Chemistry, **2**.
Robinson (1935). *J. Bone Jt. Surg.* **17**, 267.
Truszkowski & Chajkinowna (1935). *Biochem. J.* **29**, 2361.