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THE EXCRETION OF AMMONIA AND URIC ACID DURING THE LARVAL LIFE OF CERTAIN MUSCOID FLIES

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(With Five Text-figures)

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IN the early years of this century Weinland (1906) conclusively showed that ammonia constitutes the bulk of the nitrogenous excreta of larvae of the blow-fly, *Calliphora vomitoria*. This was a remarkable observation in a subphylum that was, and still is, considered essentially uricotelic. Pupae and imagines of this species excrete uric acid; Weinland was therefore led to postulate that pupation was basically a physiological change, the change from ammonia to uric acid as the protein catabolite. Twenty-five years later Delaunay (1931), reviewing protein catabolism in invertebrates, quotes Weinland's work and states that "les larves de Calliphora... n'excrètent pas d'acide urique".

During 1933 and 1934 the Sarcophagid fly *Wohlfahrtia vigil* Wlk, an ectoparasite of young mammals, and of occasional clinical occurrence, was being bred for study in the Department of Biology, University of Toronto, by Dr Norma Ford, to whom the author is indebted for material. It being observed that gorged larvae caused a characteristic discoloration of the sawdust into which they were put to pupate, their excreta were Nesslerised, and found to contain large quantities of ammonia. Further analysis of such excreta yielded, surprisingly enough, a significant quantity of uric acid; obviating the possibility of the preformation of this substance in the food, the excreta of larvae reared on a casein diet gave an equally significant quantity of uric acid just before pupation.

Does this uric acid appear only as pupation approaches, or is it a regular constituent of the larval excreted matter? Does it bear a constant ratio to the excreted ammonia, or does it increase as ammonia decreases, in the same relation as that of urinary ammonia to urea in the mammal? To answer such questions the daily excretion was followed, from the newly laid larva (*Wohlfahrtia* is larviparous) continuously up to pupation.

THE PROBLEM OF MICRO-ORGANISMS

Immediately after Weinland's discovery, it was asserted by Bogdanow (1906) that the observed ammonia was due to micrococci present on the blown meat. This objection was met by Weinland. Recently Hobson (1932), and Michelbacher,

Hoskins and Herms (1932) have observed the excretion of ammonia by larvae of *Lucilia sericata* bred under sterile conditions. These workers observed that, on a satisfactory diet (including yeast autolysate vitamins), the only departure from the normal in such sterile cultures was a delay in the inception of larval growth; in their opinion, this may be explained by the lack of ammonifying bacteria to produce, quickly, enough ammonia to give a favourable alkaline reaction for the larval trypsin, which passes out in the excreta and acts extracorporeally (Hobson, 1932).

That in the aggregate the bacterial ammonia production is inconsiderable compared with that produced by the larvae, is suggested by the following observation: Three 16 oz. wide-mouthed bottles, stoppered with cotton-wool, each containing 8 gm. of a casein diet (see below), were exposed under non-sterile conditions at 28° C. for 80 hours. In one were fifty newly hatched larvae of *Calliphora*, which became fully gorged during that period; the other two bottles, without larvae, were used as controls. The ammonia production was as follows:

Bottle with fifty larvae	44·9 mg.
Control A	3·3
Control B	3·0
Average difference	41·8 or 93 per cent.

Accordingly it was not considered necessary to attempt sterile conditions in these preliminary studies, since the main trends would not be obscured in their absence; moreover, the constant use of controls took into account the small bacterial ammonia production.

THE EXCRETION OF AMMONIA AND URIC ACID IN *WOHLFAHRTIA VIGIL* WLK.

Larvae were bred in test-tubes at room temperature, and determinations were made daily on the excreta from the second day after larviposition until pupation. Into each test-tube (5 in. × ¾ in.) was put a piece of lean rabbit muscle about 7 mm. square; the experimental tubes then received five larvae, the remainder, without larvae, being treated as controls. They were closed with firm cotton-wool plugs, thrust down to within 1 in. of the bottom. The larvae immediately burrowed into the meat.

After 24 hours the plug was removed, and 20 c.c. of distilled water were added to each tube. Plug and tube contents were transferred to a 50 c.c. Erlenmeyer flask; during this process the larvae were picked out, dried, weighed, and returned to the next set of tubes similarly prepared. The original tubes were vigorously swilled out with the liquid, which was finally returned to the flask, to be kept at 60° C. for 1 hour; then aliquot amounts were taken for ammonia and uric acid estimation.

For the estimation of ammonia 5 c.c. aliquot samples (2 c.c. or 1 c.c. where necessary) were used. The colorimetric method of Folin and Bell for urinary ammonia (Peters and Van Slyke, 1932), modified for a small aliquot, was employed.

For the estimation of uric acid (10 c.c. aliquot) the colorimetric method of Benedict and Franke (1932) was employed.

The controls were treated in the same way. The average values for the controls were subtracted from the experimental values. The figures, for six groups of five larvae each, are put in the following set of graphs (Fig. 1). All values were multiplied by a factor to a basis "per 100 individuals", a unit introduced into this field of study by Evans (1932). Because of certain irregularities in the intervals between deter-

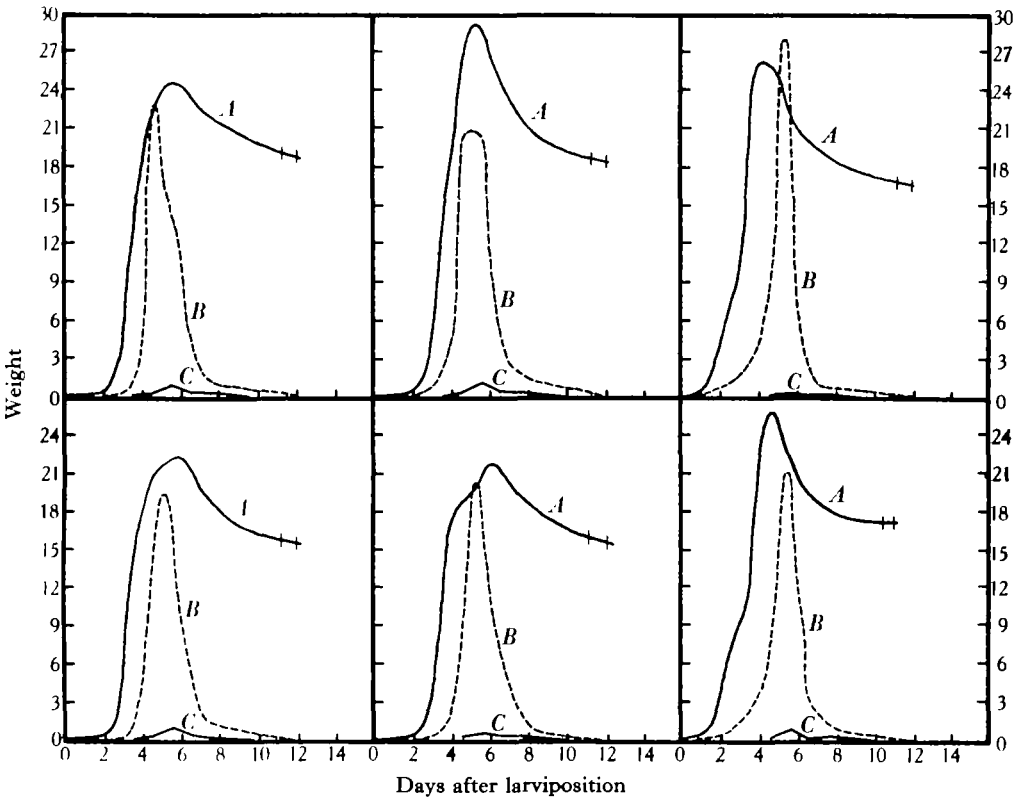

Fig. 1. Excretion of *Wohlfahrtia* larvae on meat diet. Curve *A*. Body weight in gm. (ordinates). Curve *B*. Rate of ammonia excretion in mg. Curve *C*. Rate of uric acid excretion in mg. The two vertical lines cutting the body-weight curve delimit the period of pupation. *Same symbols used throughout all figures.* The explanation of the curves given above is common to al lthe figures.

minations, the figures for ammonia excretion were first plotted on cumulative curves, from which the rate of excretion per 24 hours could be read off by tangents to the curve.

It is seen that all six groups show the same qualitative results, the main trends never being masked by biological variation. They may be summed and averaged to give a single graph for the species *Wohlfahrtia vigil* (Fig. 2), for comparison with the graph for *Calliphora erythrocephala* (Fig. 3).

From these results the following (qualitative) deductions may be drawn:

1. Growth and feeding do not get under way for 1 or 2 days, awaiting probably a sufficient accumulation of ammonia for liquefaction of the meat. This is borne out by the fact that the young larvae normally congregate in an unbroken rank to attack the muscle; if separated they make no headway, and soon die.

2. Ammonia excretion lags about 1 day behind food ingestion.

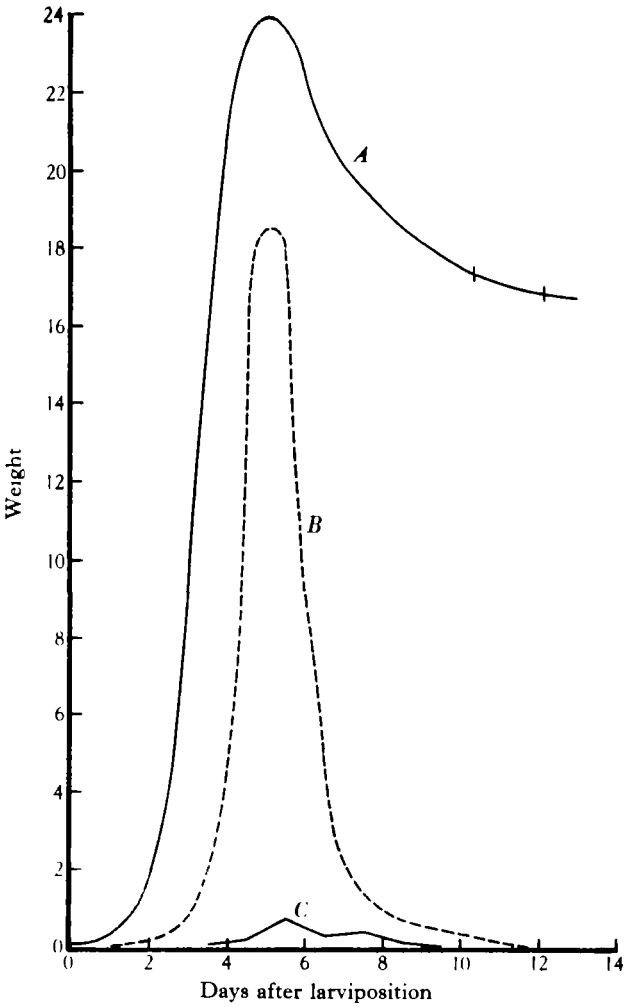

Fig. 2. Average curve; excretion of *Wohlfahrtia* on meat diet.

3. Maximum ammonia excretion occurs approximately at the peak of the body-weight curve, when ingestion and excretion are equal in weight, which is just before the larvae leave the food.

4. Ammonia excretion then falls off suddenly, finally to cease at pupation.

5. Uric acid excretion is proportional to ammonia excretion, and roughly proportional to the total volume of excreta.

The information so obtained is of qualitative value only; the next step was to improve the technique, in order to render quantitative experiments possible.

CRITICISM OF METHODS USED, AND EVOLUTION OF AN IMPROVED METHOD

The original method was examined on the following points:

1. Does loss of ammonia occur by volatilisation from the experimental test-tube? Tubes with gorged and feeding larvae were connected by a U-tube to 0·1 *N* H_2SO_4, air being drawn over slowly at intervals during the 24 hours. By Nesslerisation of the acid (which involves no error) it was shown that only 0·2–0·5 per cent. of the excreted ammonia could be drawn out of the tube, the water of the liquefied food being sufficient to retain it.

2. Solutions of excreta, kept at 60° C., in Erlenmeyer flasks for 1 hour, lost 32 per cent. of the ammonia (in concentrations of approximately 0·1 mg. per c.c.). This, therefore, necessitated a solvent for uric acid in the cold. Lithium carbonate was found to be a satisfactory solvent, when added in the cold to a slightly ammoniated solution and sediment of uric acid.

3. Solutions of excreta to be analysed contained much of the proteins of the food; it was necessary to find a method of protein precipitation which would not (*a*) dilute the original solution too much, nor (*b*) adsorb uric acid when present in very low concentrations. Colloidal iron fulfilled the first requirement, but adsorbed 80–95 per cent. of the uric acid (when in concentrations of approximately 0·2 mg./ml.). Tungstomolybdic acid in acid solution was found to be satisfactory on both counts.

These results led to the adoption of the following improved method. The experimental tube was treated with 19 c.c. of distilled water and 1 c.c. of saturated lithium carbonate solution. The material was transferred to a 50 c.c. Erlenmeyer flask as in the original method, shaking being substituted for heating. Ammonia determination proceeded as before. For uric acid determination, 8 c.c. of the original solution were transferred to a 50 c.c. centrifuge tube, 1 c.c. of the tungstomolybdate solution was added, followed by 1 c.c. of 0·62 *N* H_2SO_4. The precipitate was centrifuged off, leaving a clear solution of almost 10 c.c., to be tested colorimetrically as before.

Evans, in recent work on uric acid in insects (1934), uses sodium carbonate as the solvent, and neutralisation by acetic acid to precipitate the proteins. In employing Evans's method on solutions of excreta, it was found that neutralisation only partially removed the proteins, for they could be precipitated in large quantities from the neutralised filtrate by further acidification. If excess of acid was added to the solution before filtering, the dense precipitate of proteins rendered filtration impossible.

The values given by the tungstomolybdic method and by Evans's method were then compared, using slightly ammoniated aqueous solutions of uric acid of known concentration, containing ovalbumin. The uric acid readings were calculated to a

basis of mg. per c.c. of base-added liquid, and the percentage variation thus calculated:

Actual concentration	Percentage difference by	
mg./c.c.	Evans's method	Tungstomolybdic method
0·0384	−15	+18
0·0304	−15	+ 8
0·0243	−23	+ 8
0·0142	−16	−19
0·0042	−18	−67

In concentrations of 0·02–0·03 mg./c.c., for which Benedict and Franke intended their method to be used, tungstomolybdic precipitation gives a higher figure than Evans's method. The tungstomolybdic method is standard for the protein precipitation of serum, containing 0·020–0·035 mg./c.c. of uric acid. Its other advantages are that it gives a clear protein-free liquid, and it is quicker. Evans's method does not completely remove protein contamination, which according to Benedict and Franke adversely affects the development of the colour. It is therefore suggested that the tungstomolybdic method should be used, avoiding concentrations above 0·030 mg./c.c., where the colour is no longer proportional; and for concentrations below 0·015 mg./c.c. Evans's method may be used.

THE EXCRETION OF AMMONIA AND URIC ACID IN *CALLIPHORA ERYTHROCEPHALA* MG.

For these quantitative experiments the larvae of *Calliphora erythrocephala*, the common blow-fly, were used; this species is oviparous. Experiments on them could be begun within 4 hours after hatching, and were continued up to pupation. Diets of meat, casein and ovalbumin were used. The meat was fresh lean beef; the protein diets consisted of 96 per cent. of the particular protein and 4 per cent. of the McCollum-Simmonds (1918) salt quota, made into a thick paste with distilled water, with the addition of a little autoclaved yeast suspension.

The method of rearing was as in *Wohlfahrtia*, the artificial diets being added in lumps with a little filter paper and a drop of water. Towards the end of larval life, when the larvae had ceased feeding but continued to excrete, a little dampened cotton-wool with sawdust replaced the food. Seven larvae per experimental tube, and control tubes without larvae, were used. Analyses and weighings were made at the same hour throughout the larval period.

The experimental results are summarised in Figs. 3, 4 and 5. Consideration of the growth and excretion on the meat diet yields the same conclusions as for *Wohlfahrtia*, with these modifications:

1. The feeding and growth of young larvae are well under way by the first day after hatching.

2. There is no lag of ammonia excretion behind food ingestion. This may be due to the shorter alimentary tract of *Calliphora*, in comparison with the larger *Wohlfahrtia*.

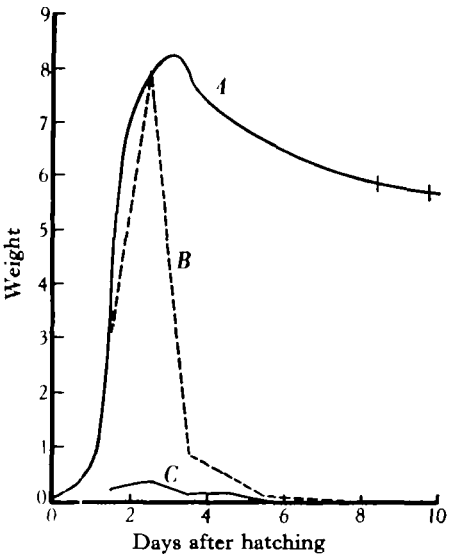

Fig. 3. Excretion of *Calliphora* on meat diet.

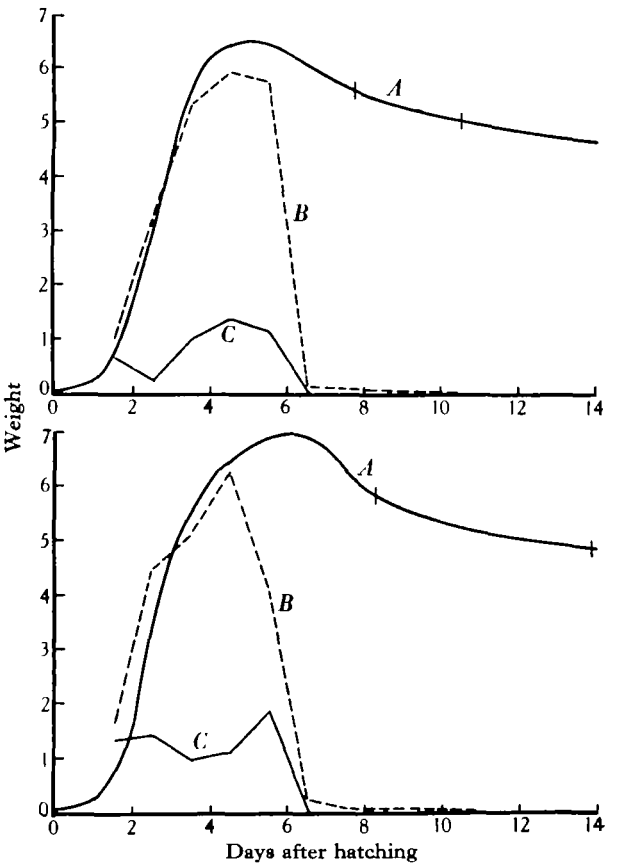

Fig. 4. Excretion of *Calliphora* on casein diet.

These two points, it will be seen, are interdependent. Two additional points, good for both species, also become evident:

1. There is no correlation of the peaks of uric acid excretion to the larval moults. These are at the 10th hour and 3rd day of larval life in *Wohlfahrtia*, and correspondingly a little earlier in *Calliphora* (Ford and Walker, unpublished).

2. The ratio of the body weight attained to the amount of protein metabolised—as measured by ammonia excretion—is approximately the same for both species (allowing for the loss in ammonia analyses in *Wohlfahrtia*).

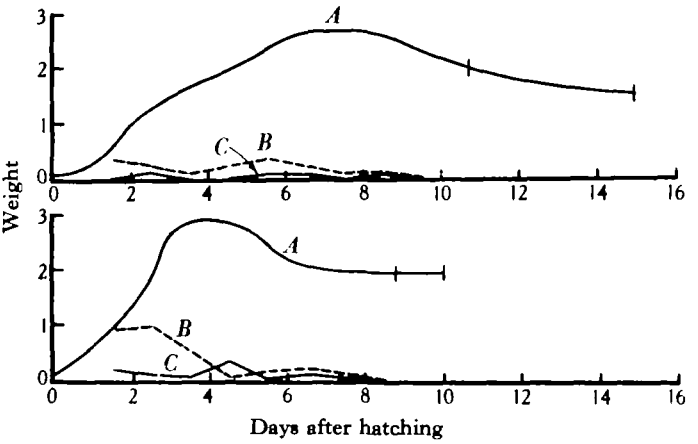

Fig. 5. Excretion of *Calliphora* on ovalbumin diet.

Comparison of the body-weight curves on diets of meat, casein and ovalbumin show that this is the order of their biological value. Diminished larval weight, protraction of the pupating period, and failure of the adult to emerge are the signs of decreased value of the food. The casein diet appears to be adequate, since 87 per cent. of the normal weight on a meat diet was attained, and most of the adults emerged. The ovalbumin diet is definitely inferior, with only a few successful emergents; it is a frequent observation that uncooked ovalbumin is resistant to mammalian enzymes, only a small percentage of it being utilised. Also Hobson (1933) reports the presence in aqueous extracts of egg-white of substances repugnant to growth of blowfly larvae; these might have been adsorbed on the ovalbumin in its technical preparation.

The utilisation by the larvae of a substance, other than protein in the meat, is suggested upon two counts by a comparison of the results on a casein diet with those on meat:

1. The relation between the body weight attained and the amount of protein metabolised to attain it may be studied by using the ratio of the total ammonia excreted during larval life (in mg.) to the body weight at pupation (in gm.), as follows:

$$\text{For meat } \frac{8{\cdot}80}{0{\cdot}39} = 22{\cdot}7; \qquad \text{for casein } \frac{15{\cdot}29}{0{\cdot}34} = 45{\cdot}9; \qquad \frac{15{\cdot}13}{0{\cdot}32} = 46{\cdot}7.$$

Thus, for achievement of a given body weight, twice as much protein is metabolised on a casein diet as on a meat diet; this suggests that meat offers some metabolic fuel other than protein.

2. A striking point is the unusually high excretion of uric acid by larvae on the casein diet. If it can be assumed that uric acid is the product of endogenous protein metabolism, any excess production of that substance from a given diet might be due to the specific dynamic action of that diet. Therefore it is indicated that larvae on a meat diet supplement protein with some substance less S.D.A.-promoting than protein.

In this connection, it may be noted that there is present in meat varying amounts of carbohydrate, and up to 20 per cent. of fat. Wollmann (1922) has found an amylolytic enzyme in larvae of *Calliphora vomitoria*, and there is a weak amylase present in the salivary glands of *Lucilia* larvae (Hobson, 1931). Future experiments will therefore investigate the effects of adding hexose carbohydrate, unsaturated fat, and saturated fat to a casein diet, and the effect of substituting "myosin" for casein.

With regard to the retention of uric acid in the body of the larvae, figures obtained indicate that the amount is independent of the amount excreted, and is of the order of 0·04 mg. per gm. of body weight.

SUMMARY

1. Uric acid is present in small amounts, as a true catabolite, in tissues and excreta of larvae of *Calliphora erythrocephala* Mg. and *Wohlfahrtia vigil* Wlk.

2. The excretion of ammonia increases commensurate with the rise in body weight of the larvae, lagging a day behind it in *Wohlfahrtia*, this lag being almost absent in *Calliphora*; it falls off rapidly a few days before, finally disappearing at pupation.

3. The excretion of uric acid is similar in its course, but in much smaller amount.

4. The adequacy of casein as the sole caloric source, and the low biological value of ovalbumin, is demonstrated in *Calliphora*.

5. The pure protein (casein) diet involves an excretion of ammonia and uric acid about twice as great as the normal meat diet.

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