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THE PHYSIOLOGY OF EXCRETION IN THE COTTON STAINER, *DYSDERCUS FASCIATUS* SIGNORET

III. NITROGEN EXCRETION AND EXCRETORY METABOLISM

By M. J. BERRIDGE
*Department of Zoology, University of Cambridge**

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INTRODUCTION

The presence of uric acid in the excreta of insects has been reported by numerous authors. Quantitative information has substantiated that it represents the main nitrogenous excretory substance of most terrestrial insects (Wigglesworth, 1953; Terzian, Irreverre & Stahler, 1957; Irreverre & Terzian, 1959; Hudson, Bartel & Craig, 1959; Razet, 1961).

Uric acid has solubility properties which well suit it to the role of an excretory product in a system where water retention is important. Uric acid enters the excretory system as the soluble sodium or potassium salt, which is then precipitated as free acid by reabsorption of base and water. In most insects this precipitation occurs in the rectum, although in *Rhodnius* it takes place in the proximal portion of the Malpighian tubule (Wigglesworth, 1931*b*). The continuous recycling of base and water through the excretory system gradually results in a large accumulation of uric acid in the rectum. The osmotic pressure of the latter does not increase appreciably because of the low solubility of free uric acid. Such a system of continuous filtration and reabsorption of salts and water can only occur if intake of inorganic ions is low, or if these ions are osmotically inactivated by precipitating them as insoluble salts.

Purines, other than uric acid, are rarely found in insect excreta. The hypoxanthine and xanthine present in the excreta of *Melophagus ovinus* (Nelson, 1958), *Drosophila melanogaster* (Kursteiner, 1961) and *Galleria mellonella* (Nation, 1963) probably reflect peculiarities of purine metabolism, which may have a genetic basis. For example, only the mutants of *D. melanogaster* which lack the enzyme xanthine oxidase (Forrest, Glassmann & Mitchell, 1956) are found to excrete xanthine and hypoxanthine. The absence of the enzyme guanase may account for the presence of guanine in the excreta of the boll weevil, *Anthonomus grandis* (Mitlin, Vickers & Hedin, 1964).

The degradation products of uric acid, however, are commonly present in the excreta of insects. Razet (1954, 1956, 1961) has shown that the excretion of allantoin, and to a lesser extent, allantoic acid, may be more general than has been thought previously. The enzymes responsible for the stepwise breakdown of uric acid to allantoin and allantoic acid, uricase and allantoinase, respectively, have a correspondingly wide distribution in insects (Leifert, 1935; Brown, 1938*a*; Duchâteau, Florkin & Frappez, 1940; Razet, 1953, 1961; Desai & Kilby, 1958; Ross, 1959; Lisa & Ludwig, 1959).

* Present address: Department of Biology, University of Virginia, Charlottesville, Virginia, U.S.A.

Studies on the rate of urine excretion in *Dysdercus* clearly indicated the existence of two distinct phases of excretion (Berridge, 1965*a*). The excretory phase is characterized by a rapid excretion of inorganic ions, which is probably related to the copious flow of urine during this phase (Berridge, 1965*b*). Since, unlike most terrestrial insects, *Dysdercus* does not appear to conserve water in its excretory system, it was of considerable interest to determine how nitrogen is excreted in this insect.

MATERIAL AND METHODS

Female fifth-instar larvae have been used throughout these studies; the methods for culturing these larvae have been described in a previous paper (Berridge, 1965*a*).

The various nitrogenous substances in urine, haemolymph or tissue extracts were identified by chromatography. Samples of urine were applied directly to chromatography paper. Haemolymph samples were deproteinized by the addition of absolute alcohol to a final concentration of 80%, the protein precipitate being removed by centrifugation and the supernatant applied to the chromatography paper. Uric acid in tissues was extracted by homogenizing the tissue in a mixture of equal volumes of 0·1% lithium carbonate and chloroform. This homogenate was centrifuged and the supernatant was applied directly to the chromatography paper. Chloroform-soluble substances, which seriously interfere with chromatography, remained in the chloroform layer at the bottom.

Unknown substances were identified by comparison of R_f values in different solvent systems, and by running standard substances together with the unknown.

Uric acid, allantoin and urea could be identified on a single chromatogram run in 70% *n*-propanol; identification was facilitated by the observation that all these substances react positively when chromatograms are taken through the procedure of Rydon & Smith (1952). Amino acid identification was carried out by two-dimensional chromatography using *n*-butanol/acetic acid/water (4:1:5) followed by aqueous phenol. The dried chromatograms were sprayed with a 0·1% solution of ninhydrin in acetone; spots were produced by heating the paper to 100° C. for 3 min. Creatine and creatinine were identified on a single unidimensional chromatogram run in *n*-butanol/ethanol/water (4:1:1). These two substances appeared as orange spots against a yellow background when chromatograms were treated with an alkaline picric acid reagent (Block, Durrum & Zweig, 1958).

The general procedures for collecting and treating urine and haemolymph for quantitative analysis have already been described (Berridge, 1965*b*).

Total nitrogen and mucopolysaccharide nitrogen in urine were measured by the micro-Kjeldahl procedure of Shaw & Beadle (1949); mucopolysaccharide was precipitated with absolute alcohol before being analysed for its nitrogen content. Amino acid nitrogen was determined colorimetrically by the method of Rosen (1957). Allantoin nitrogen was assayed by the colorimetric method of Christman, Foster & Esterer (1944). The colorimetric method used to estimate urea was non-specific; urea was therefore separated from contaminants by two-dimensional chromatography using *n*-butanol/acetic acid/water (4:1:5), followed by methanol/1% ammonia (7:3) as solvents. The position of kynurenine, which could be located by viewing the chromatogram under ultraviolet light, was used as a marker to locate the urea spot;

the latter consistently travelled slightly farther than kynurenine in both solvents. The urea was eluted off the small area of chromatogram and estimated by a colorimetric method (Beale & Croft, 1961). When urine samples, which had previously been treated with urease, were taken through the above procedure, they gave readings which were indistinguishable from ordinary blanks.

The problem of measuring uric acid in the body was extremely difficult because of the presence of large amounts of pteridines which interfere with uric-acid analysis. It was necessary to separate uric acid from these pteridines by chromatography. The insect, previously dried to constant weight, was homogenized in a mixture of 0·4 ml. of 0·4% lithium carbonate and 0·2 ml. of chloroform and the homogenate was centrifuged. Aliquots of the clear supernatant were applied to chromatograms for two-dimensional development in iso-propanol/1% ammonia (7:3) followed by methanol/1% ammonia (7:3). This was the only solvent pair tried which would adequately separate uric acid from the pteridines in the body. Uric acid was then eluted off the paper with a phosphate buffer (pH 8·0), the eluate was made up to 5 ml. and its absorption was determined at the wavelength of maximum absorption 290 mμ, and at a point of weak absorption 320 mμ; the difference was taken and the uric acid value was recorded from a standard curve. When known amounts of uric acid were chromatographed, eluted and estimated, the recovery averaged 92%.

A manometric technique was used to measure uricase activity; oxygen uptake in the presence of uric acid was measured by the conventional Warburg method at 37° C. The various tissues, dissected out under ice-cold Ringer, were homogenized in a Potter–Elvehjem vessel with 1·0 ml. of 0·2 M borate buffer (pH 9·6) and transferred to the Warburg flask with 1·5 ml. of buffer; the sidearm was charged with 0·4 ml. substrate (300 mg. uric acid/100 ml. 0·4% lithium carbonate). The centre well contained 0·2 ml. 10% potassium hydroxide. After 30 min. temperature equilibration, enzyme and substrate were mixed and oxygen uptake was measured for 1 hr. It was difficult to weigh the very small tissues used in these determinations; for this reason enzyme activity of whole organs was determined and expressed as mm.^{3} O_2 consumed/hr./tissue.

The method used for preparing isolated Malpighian tubules is described elsewhere (Berridge, 1965*c*).

The vertical lines on the graphs represent ± twice the standard error of each mean.

RESULTS

Identification of nitrogenous excretory products

Allantoin and urea were identified on chromatograms of urine run in 70% *n*-propanol. Confirmation was provided by a comparison of their R_f values with those of standard allantoin and urea in a large number of solvent systems. Both substances also gave a characteristic yellow colour when chromatograms were sprayed with a dimethylaminobenzaldehyde reagent (Block *et al.* 1958). Uric acid was completely absent from samples of urine taken from both phases of excretion; a positive reaction was obtained with small amounts of standard uric acid. Overloading the chromatogram with 50 μl. of urine still failed to reveal the presence of uric acid by any of the following detection methods: the reagent of Rydon & Smith (1952), the mercury-

diphenyl-carbazone reagent (Dickstein, Bergmann & Chaimovitz, 1956), or viewing chromatograms under ultraviolet light. A large number of ninhydrin-positive substances were present in the urine; the individual amino acids have not been identified.

When a chromatogram of urine, which had been run unidimensionally in 70% *n*-propanol, was viewed under ultraviolet light, a blue-green fluorescent spot was visible at R_f 0·55. This spot gave a mauve colour with ninhydrin and an orange colour with the dimethylaminobenzaldehyde reagent. These reactions, together with the nature of its fluorescence under ultraviolet light, suggested that the substance

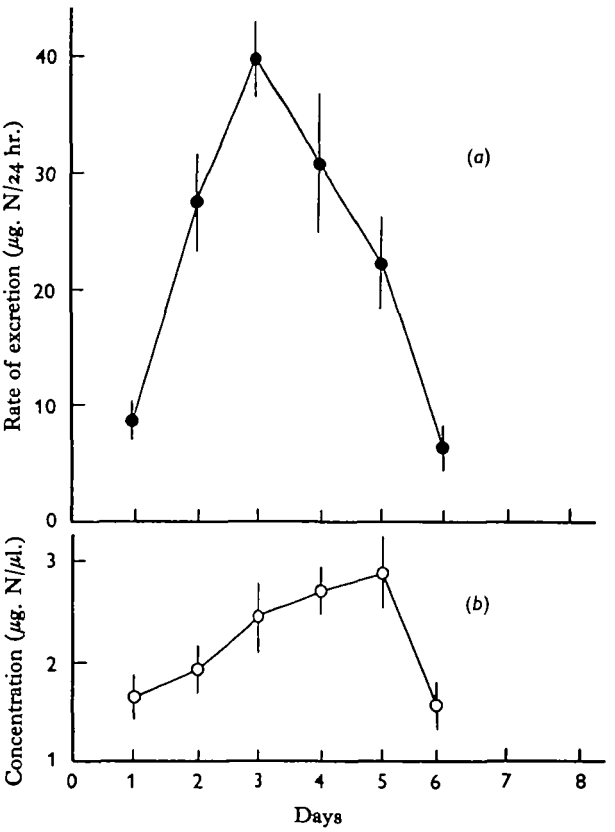


Fig. 1. Total nitrogen excretion. (*a*) Rate of nitrogen excretion. (*b*) Concentration of total nitrogen in the urine.

might be kynurenine. This view has been confirmed by spectral analysis. The unknown substance has an absorption spectrum identical with that of standard kynurenine.

The urine also contains a substance which has the properties of a mucopolysaccharide. A precipitate was formed in the urine on addition of alcohol or acetone, but not with trichloroacetic acid. Hydrolysis of this precipitate with 6 N-HCl at 100° C. for 12 hr. resulted in the appearance of amino acids and simple sugars. The precipitate also gave a positive periodic acid-Schiff reaction which confirmed that it is most probably a mucopolysaccharide.

Creatine, creatinine and pteridines are completely absent from the urine.

Apart from the protein–polysaccharide complex all nitrogenous substances reported in the urine were detected in the haemolymph. In addition uric acid was identified, although, as described above, it was not present in the urine. Since other purines are liable to behave in a similar way to uric acid, more positive identification was obtained by studying the absorption spectrum of the unknown spot. When a chromatogram of deproteinized haemolymph was run two-dimensionally in methanol/1% ammonia (7:3), followed by *n*-butanol/acetic acid/water (4:1:5), a single absorbing spot was apparent with R_f values of 0·4 and 0·34 respectively. This spot, when eluted off the paper with 0·2 M phosphate buffer (pH 8·0), gave an absorption spectrum identical with that of standard uric acid ($E_{max.}$ 290 mμ; $E_{min.}$ 262 mμ).

Table 1. *Contribution of nitrogenous excretory substances to the total nitrogen excreted by* Dysdercus *on the third day of the fifth instar*

Excretory substance	% of total N excreted on third day
Allantoin	61·0
Amino acids	13·2
Urea	12·4
Mucopolysaccharide	5·6
Non-identified N	7·8

Quantitative analyses of nitrogen excretion throughout the fifth instar

Total nitrogen excretion

The rate of nitrogen excretion throughout the fifth instar closely parallels the rate of urine production (Berridge, 1965*a*). There is a sharp peak of nitrogen excretion on the third day, which is followed by an equally sharp decline as the second phase of excretion is approached (Fig. 1). The concentration of nitrogen in the urine increases regularly until the fifth day, but then decreases considerably on the sixth day. Furthermore, the constant nitrogen content of the urine retained in the rectum during the post-excretory phase suggests that there is no further nitrogen excretion after the fifth day (Berridge, 1965*b*). The contribution of the various nitrogenous substances to the total nitrogen excreted is shown in Table 1. Allantoin clearly represents the major nitrogenous excretory product; the other components are of relatively minor importance. The amount of kynurenine excreted has not been measured, but it may make up a considerable part of the residual nitrogen which has not been partitioned.

Excretion of allantoin

Since allantoin represents the main nitrogenous excretory product, the total excreted and the concentration in the urine (Fig. 2) show similarities to total nitrogen excretion (Fig. 1). The allantoin concentration in the haemolymph is also shown in Fig. 2. Except on the fourth day the concentration of allantoin in the urine is significantly less than that in the haemolymph. It seems unlikely that allantoin is reabsorbed by the rectum, and so the above observation probably resulted from a low permeability of the Malpighian tubules to this substance. This possibility has been tested by studying the clearance of allantoin by isolated Malpighian tubules. The

preparation and behaviour of these isolated tubules will be described in considerable detail in a later paper (Berridge, 1965*c*). Tubules were set up in serum containing different concentrations of allantoin. The concentration of allantoin in the urine produced by these tubules over a period of 6 hr. was compared with that of the serum which bathed them (Fig. 3). The allantoin concentration of the urine was always less than that of the serum; the relationship appeared to be consistent throughout the range of serum allantoin concentration employed. The average U/P ratio of all the observations is $0{\cdot}45 \pm 0{\cdot}1$.

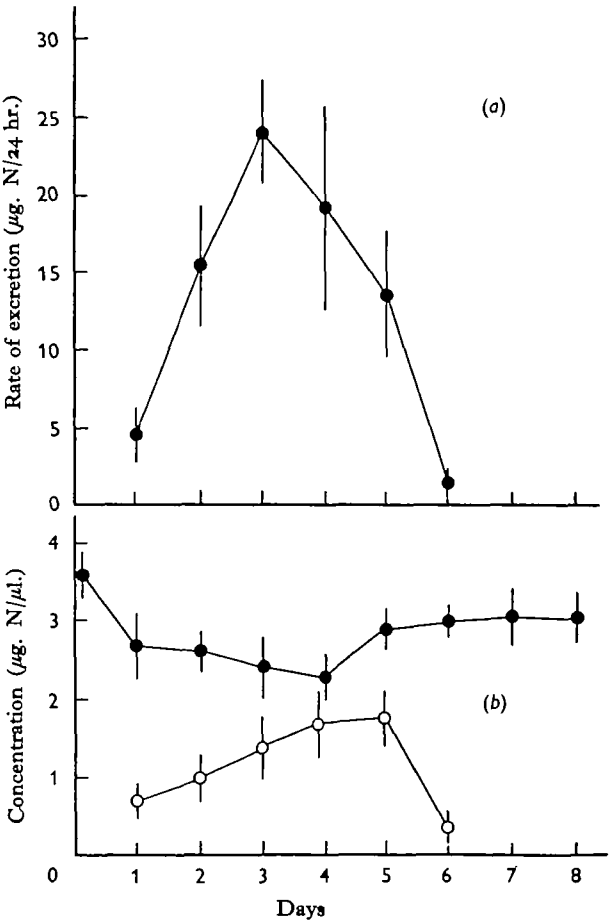


Fig. 2. Allantoin excretion. (*a*) Rate of allantoin excretion. (*b*) Concentration of allantoin in urine and haemolymph. ○, Urine; ●, haemolymph.

Considerable caution must be exercised in attempting to integrate such data from isolated tubules with observations obtained from the intact animal. It would appear however, that the low concentration of allantoin in the urine excreted relative to the haemolymph can be explained by the low permeability of the Malpighian tubules to this substance.

The low concentration of allantoin in the urine which is finally excreted suggests that there is relatively little reabsorption of water from the liquid leaving the Mal-

pighian tubules. A rough estimate of the amount of water reabsorbed by the rectum can be obtained from the following calculation. The amount of allantoin nitrogen excreted on the third day of the instar is 24·5 μg. N and the concentration of allantoin in the haemolymph is 2·46 μg. N/μl. (Fig. 2). If it is assumed that the U/P ratio for the passage of allantoin through the Malpighian tubule is 0·45 (this value was obtained from studies, just described, on isolated tubules), then the initial concentration of allantoin in the urine will be $2{\cdot}46 \times 0{\cdot}45 = 1{\cdot}11$ μg. N/μl. Therefore the amount of

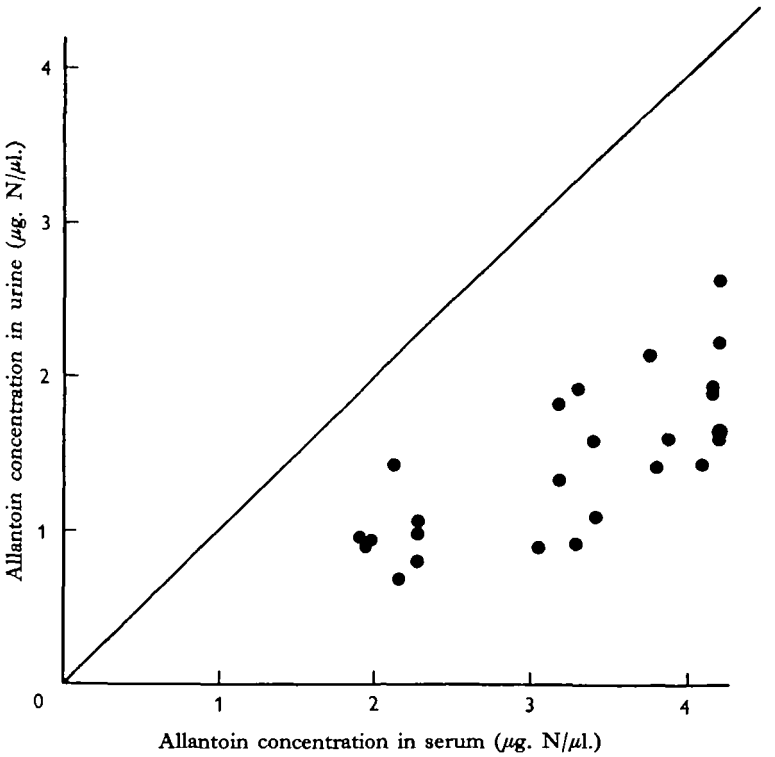


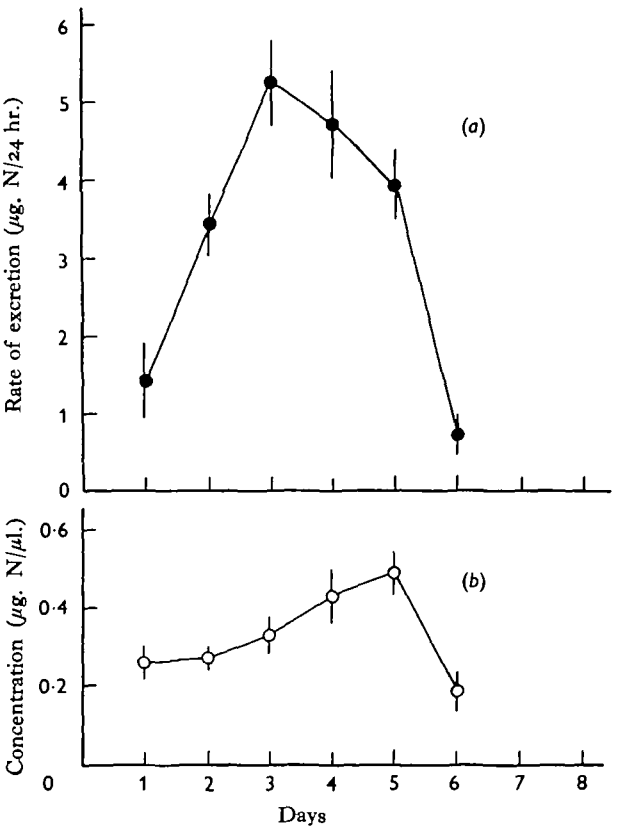
Fig. 3. Relationship between concentration of allantoin in the urine secreted by isolated Malpighian tubules and its concentration in the serum. The straight line from the origin indicates equal concentrations of allantoin in urine and serum.

urine which must be formed by the Malpighian tubules in order to excrete 24·5 μg. N will be $24{\cdot}5/1{\cdot}11 = 22{\cdot}0$ μl. The actual volume excreted, however, was 15·9 μl. (Berridge, 1965*a*), from which it can be estimated that only 28% of the primary Malpighian tubule fluid is reabsorbed by the rectum.

The validity of this figure can be checked by using it to calculate the final concentration of allantoin in the urine. The initial concentration of allantoin in the urine was estimated to be 1·11 μg. N/μl.; if 28% of this primary fluid is reabsorbed by the rectum, then the final concentration of allantoin will be 1·54 μg. N/μl. The observed value for the allantoin concentration of the urine excreted on the third day is 1·52 μg. N/μl. (Fig. 2); this very close agreement between observed and calculated values suggests that the estimate of 28% for the amount of water reabsorbed by the rectum from the initial tubule fluid is, in fact, valid.

Excretion of urea and amino acids

The course of urea excretion is essentially similar to that of allantoin, except that the amount excreted is considerably less (Fig. 4). The excretion of amino acids, however, differs from that of allantoin and urea in that the peak of excretion occurs earlier in the excretory phase. The amount excreted reaches a maximum on the second day, but then shows a fairly regular decrease during the remainder of the phase (Fig. 5). The concentration in the urine also shows a peak on the second day followed by a gradual decline until the sixth day.



Fig, 4. Urea excretion. (*a*) Rate of urea excretion. (*b*) Concentration of urea in the urine.

The significance of these differences is not fully understood, but they may reflect the possible role of the excretory system in the regulation of amino acid concentration of the haemolymph, which shows a significant decrease in the first part of the instar, to a minimum of 0·65 μg. N/μl. on the third day (Fig. 5). There is a subsequent increase in concentration during the latter part of the instar. These changes directly compensate for the opposite changes in chloride concentration of the haemolymph (Berridge, 1965*b*). The peak in amino acid excretion on the second day could well account for the initial fall in amino acid concentration of the haemolymph. Conversely, the gradual decline in amino acid excretion after the second day would facilitate the increase in concentration in the latter part of the instar.

Storage excretion

During the last part of the instar the excretory system no longer functions as a vehicle for the elimination of waste nitrogen (Berridge, 1965*b*). This raises some interesting questions, especially with regard to possible alternative mechanisms for removing waste nitrogen. By analogy with other insects the storage excretion of uric acid in the fat body would be the most likely mechanism for dealing with waste nitrogen during the post-excretory phase. Exhaustive qualitative analyses of the fat body, however, failed to reveal the presence of uric acid, but large quantities of

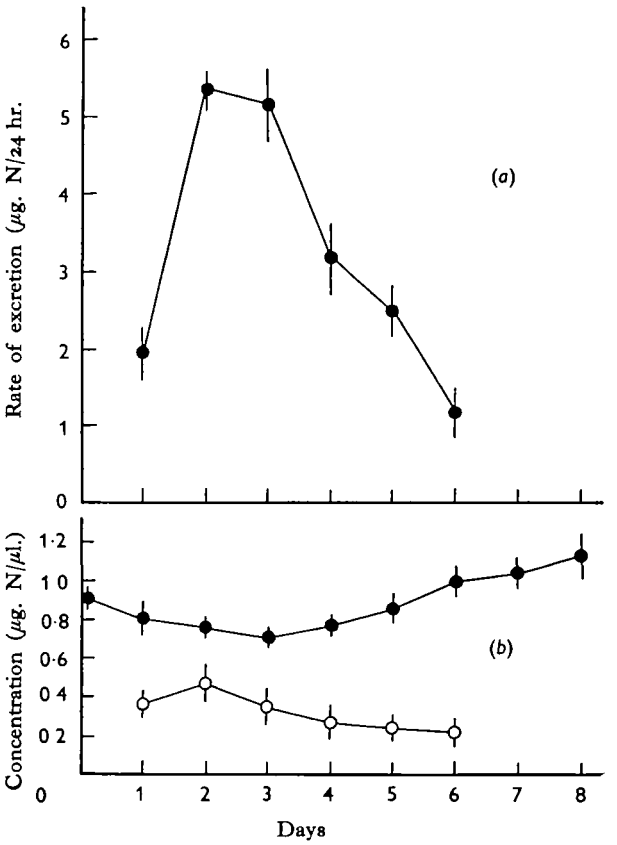


Fig. 5. Amino acid excretion. (*a*) Rate of amino acid excretion. (*b*) Concentration of amino acids in the haemolymph and urine. ○, Urine; ●, haemolymph.

this substance, together with a number of pteridines, were found in extracts prepared from the epidermis. The uric acid extracted from the epidermal cells had an identical absorption spectrum with that of standard uric acid. The pteridines have been tentatively identified as erythropterin, isoxanthopterin, leucopterin, and 2-amino-4-hydroxypteridine. The identification was based on a comparison of their R_f values and fluorescence characteristics with those of standard substances.

Uric acid was absent from all other parts of the body, but small quantities of pteridine were found in most tissues, especially the ovaries and testes. The abdomen

of *Dysdercus*, especially the ventral surface, has very distinct red and white bands running transversely across the body. By careful dissection these red and white areas can be separated from each other so that the pigments in each part can be identified. Uric acid was only found in the white areas, whereas pteridines were present only in the red parts. Electron microscopy has revealed that uric acid granules are restricted to the epidermal cells. No inclusions were apparent in the large oenocytes or cuticle.

Increase in the total uric acid content of the whole body has been used as an approximate measure of the rate at which uric acid is deposited in the epidermis. This was thought justifiable because of the absence of uric acid, at least in appreciable quantity, from the remaining tissues of the body. Another reason for this approach was the difficulty of separating epidermis from underlying fat body. This becomes increasingly difficult in the latter part of the fifth instar. The uric acid content of the whole body throughout the fifth instar is shown in Table 2. Contrary to expectation, the most rapid rate of deposition in the epidermis occurs during the feeding period (1–4 days).

Table 2. *Total uric acid synthesized throughout the fifth instar*

Day...	1	2	3	4	5	6	7	8
Uric acid N deposited in epidermis (μg. N)	3·76	18·3	26·7	24·0	3·4	8·0	3·0	2·7
Uric acid N excreted as allantoin (μg.N)	3·7	16·2	24·2	19·9	13·6	0·6	0	0
Total (μg. N/24 hr.)	7·46	34·5	50·9	43·9	17·0	8·6	3·0	2·7

The storage excretion of uric acid thus appears to be of little significance in the post-excretory phase. The mechanism for dealing with waste nitrogen in this phase is, therefore, not yet apparent. These studies on storage excretion in *Dysdercus*, however, were initiated on the assumption that the rate of uric acid production remained constant throughout the instar. An estimate of the rate of uric acid synthesis can be obtained by combining the information on allantoin excretion with that on the uric acid content of the body (Table 2). The rate of uric acid synthesis, shown graphically in Fig. 6, is most rapid in the first part of the instar, but decreases quite considerably in the period prior to the final moult. The original assumption of a constant rate of uric acid synthesis is, therefore, quite invalid.

The decreased nitrogen excretion in the post-excretory phase, therefore, results from a decline in uric acid synthesis. The small amounts of uric acid produced in this latter period, however, are stored in the body. Therefore this alternative mechanism for dealing with waste nitrogen does operate, albeit to a limited extent, in this last phase while the Malpighian tubules are temporarily inactive.

From the figures in Table 2 it is clear that, in the excretory phase, the storage excretion in the epidermis is as important a mechanism for the elimination of waste nitrogen as is the excretory system. This does not necessarily reflect an inefficiency of the excretory system, because uric acid deposition in the epidermis is functionally important as a form of pigmentation. Storage excretion in the epidermis is certainly not a temporary mechanism for storing waste nitrogen, since the uric acid granules are passed on from one instar to the next. This is clearly shown by the relative increase in area of the white patches of the body throughout the larval instars.

Excretory Metabolism

The most unusual aspect of nitrogen excretion in *Dysdercus* is the complete absence of uric acid from the urine. The enzyme uricase, which converts uric acid into allantoin, has been found in the Malpighian tubules of *Dysdercus*. A manometric test for uricase was applied to the following organs: fat body, abdominal epidermis, gonads, heart, gut and Malpighian tubules. The haemolymph, and the head and thorax, which remained after removing the above tissues, were also tested. Ten animals were dissected and the various tissues were pooled so as to provide sufficient material for analysis.

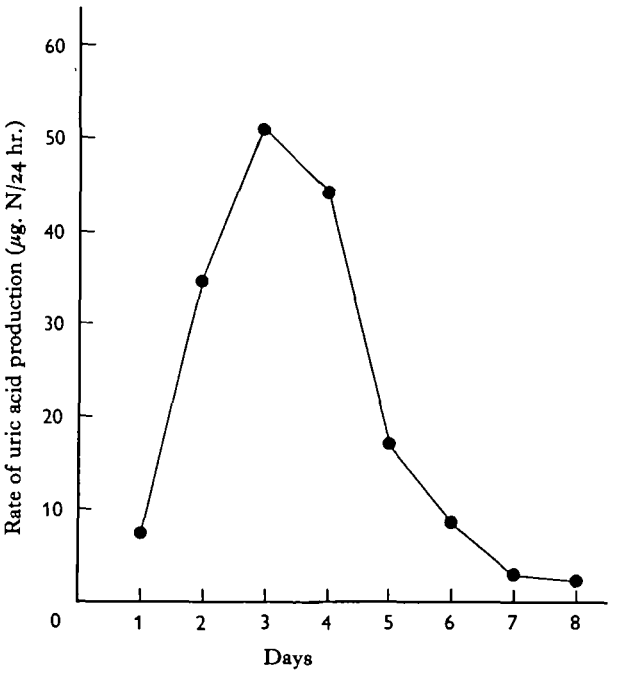


Fig. 6. Rate of uric acid synthesis throughout the fifth instar.

Table 3. *Distribution of uricase activity in the gut and Malpighian tubules*

Tissue	O_2 consumed (mm.³/hr./tissue)
m_1	0·60
m_2	0·83
m_{3+4}	0·61
Malpighian tubule	3·25
Hindgut (rectum + ileum)	0·06

The results of this survey indicated that the greatest uricase activity occurs in the Malpighian tubules. Apart from a little enzyme activity in the gut, uricase appears to be completely absent from the other tissues of the body. No attempt was made to purify the crude enzyme extract. Hence, various substances might have interfered with possible uricase activity, especially lipids in the case of the fat body. This objection was removed by adding small quantities of the Malpighian tubule extract

to the extracts of those tissues which showed no uricase activity. In all cases the enzyme activity of the Malpighian tubule extract was unimpaired.

The distribution of uricase activity along the length of the gut is shown in Table 3, that for the Malpighian tubules is included for comparison. It is clear from these results that only Malpighian tubules are responsible for the conversion of uric acid to allantoin. The uricase in the gut is probably of limited importance. If the disparity in size between the Malpighian tubules and the other tissues tested was taken into consideration, the differences in Table 3 would be considerably larger, because the Malpighian tubules are so much smaller than the other tissues. The localization of uricase activity in the Malpighian tubules adequately accounts for the absence of uric acid from the urine, but the high concentration of allantoin in the haemolymph requires further explanation.

The concentration of allantoin in the haemolymph is maintained at a high level in spite of its rapid removal by the excretory system. It seems unlikely that allantoin is reabsorbed from the tubule lumen or from the rectum, because the concentration in the latter is always less than that in the haemolymph. Another possibility exists, however, whereby the tubules may function to maintain a high concentration of allantoin in the haemolymph.

During the process of urine formation substances have to traverse two membranes in passing from haemolymph to urine. The active processes involved in the secretion of urine are presumably situated on the apical membrane, whose surface area is greatly increased by the presence of an elaborate 'brush-border' (Berridge, 1964). The basal membrane is probably freely permeable to low molecular weight substances in the haemolymph. On such an hypothesis allantoin and uric acid will enter the cells of the Malpighian tubule by simple diffusion; however, the active uptake of uric acid by the tubule cells cannot be excluded, especially since this substance is actively transported through human erythrocyte membranes (Overgaad-Hansen & Lassen, 1959). Uric acid will be converted to allantoin by the uricase present in the cells, this will increase the diffusion gradient, thus allowing more uric acid to enter the cell. The continuation of this process will result in an increase in the allantoin content within the cell and thus to the diffusion of allantoin back into the haemolymph. Allantoin, of course, also leaves the cell in the urine which is passing through the apical membrane.

There is some experimental evidence to suggest that such a mechanism could be operating to maintain a high concentration of allantoin in the haemolymph. Use has again been made of isolated Malpighian tubules, which were set up in serum of increased uric acid concentration. This was achieved by adding an equal volume of serum to Ringer containing sodium urate at a concentration of 300 mg.%. Disregarding the original urate content of the serum, the final concentration of uric acid in the serum was approximately 150 mg.%. For the purpose of this experiment a knowledge of the exact concentration of uric acid was not required. The increased osmotic pressure produced by the increased uric acid level was adjusted to normal by the addition of distilled water. As a control, tubules were set up in serum which had been diluted with Ringer containing no uric acid. Streptomycin and penicillin were added to serum to prevent possible bacterial contamination.

At the start of the experiment a small sample of serum was removed and placed

alongside its respective tubule preparation. At the end of 12 hr. the allantoin concentration of this drop, which therefore gave the initial concentration, was compared with the allantoin concentration of the serum which had been in contact with the Malpighian tubules for the entire experiment. There was a significant increase in the allantoin concentration of the serum which had a high uric acid content (Table 4). The slight increase observed in the control serum was not significant. It is apparent from the evidence here that, *in vivo*, the intact Malpighian tubules could well account for the high allantoin concentration of the haemolymph.

Table 4. *The conversion of uric acid to allantoin by isolated Malpighian tubules*

	Concentration of allantoin-N in serum (μg. N/μl.). Mean ± S.E.		Concentration of allantoin-N in urine (μg. N/μl.)
	0 hr.	12 hr.	
Serum + uric acid	1·83 ± 0·02	2·1 ± 0·04	1·61
Control	1·94 ± 0·03	1·98 ± 0·04	1·04

The ability of the intact tubule to convert uric acid to allantoin is shown even more strikingly by the increased allantoin content of the urine produced from serum with an increased uric acid content (Table 4). If the final concentration of allantoin in the serum is used, the U/P ratio obtained from Malpighian tubules in normal serum is 0·52, whereas this increases to 0·77 for the tubules set up in serum with a high uric acid content. This very large increase in the U/P ratio is of interest because it suggests that the concentration of allantoin within the cells of the tubules is considerably larger than that in the serum, which is precisely what was originally predicted. Clearly, this ability of the intact tubule to convert uric acid to allantoin does provide an adequate explanation both for the absence of uric acid in the normal urine of *Dysdercus* and for the maintenance of a high allantoin concentration in the haemolymph.

DISCUSSION

One of the difficulties involved in studying the excretion of insects is that it is often impossible to decide whether the various components of the excreta originate from the Malpighian tubules or are contaminants from the gut. In *Dysdercus* this latter possibility can be definitely excluded, because the gut is discontinuous during the larval instars (Berridge, 1965*a*). The nitrogenous compounds which have been identified in the urine of this animal are of special interest because they all arrive *via* the excretory system. In this respect the high concentration of amino acids in the urine is of particular relevance. Amino acids have been identified in the excreta of a number of insects, but contamination of the urine by gut contents was not excluded (Brown, 1937; Yoshitake & Aruga, 1950; Powning, 1953; Irreverre & Terzian, 1959; Harington, 1961; Bursell, 1963; Mitlin *et al.* 1964).

The presence of amino acids in the urine of *Dysdercus* is not surprising, especially in the light of Ramsay's studies on the passage of these molecules through the Malpighian tubules of *Dixippus morosus* (Ramsay, 1958). The amino acids alanine, arginine, glycine, lysine, proline and valine were all secreted by the Malpighian

tubules, even though these substances are of metabolic importance. Presumably there is considerable reabsorption of these 'useful substances' by the rectum. An analogous situation probably exists in *Dysdercus*; the Malpighian tubules produce urine, which contains a certain percentage of the amino acids of the haemolymph. When the urine enters the rectum, some of these amino acids are reabsorbed, but this process is by no means complete, as witnessed by the high amino acid content of the urine. The amount of amino nitrogen in the excreta of the grasshopper *Melanoplus bivittatus* is also considerable: 'It would appear that insects, like echinoderms and Crustacea, possess an excretory mechanism that is deficient with respect to amino acid retention' (Brown, 1937).

The mucopolysaccharide in the urine of *Dysdercus* may possibly contain free anion groups and so contribute to the large anion deficit in the urine. A mucopolysaccharide is also thought to provide anion groups to balance the large excess of cations in the extracellular fluid of cockroach abdominal ganglia (Treherne, 1962). A glycoprotein has also been reported in the excreta of three species of mosquitoes (Irreverre & Terzian, 1959), although the possibility that it represented a contamination from the gut was not excluded. This is an important consideration, because proteolytic enzymes have been found to persist in the excreta of blowfly larvae (Hobson, 1931). In *Dysdercus*, the mucopolysaccharide must have been formed by the excretory system because the gut is discontinuous. In this respect it is interesting to note that Martoja (1959) has reported the presence of mucous cells amongst the normal Malpighian tubule cells of the locust *Locusta migratoria*. The Malpighian tubules of spittle-bug nymphs also have mucous cells from which a mucocomplex is extruded (Marshall, 1964).

It is now well established that allantoin is fairly common in the excreta of insects (Brown, 1938*a*; Robinson, 1935; Razet, 1961), but in most cases it is a minor constituent. In larvae of the blowfly *Lucilia sericata* it only represents a very small percentage of the total nitrogen excreted, most of which is in the form of ammonia (Brown, 1938*a*,*b*). Recently, however, Razet (1961) has shown that in certain species of Hemiptera, Diptera, and Coleoptera, allantoin may represent a very considerable percentage of the nitrogen excreted. Allantoin is certainly the most important nitrogenous excretory substance produced by *Dysdercus*.

Observations on other insects suggest that uric acid is synthesized in the fat body by a similar pathway to that in vertebrates (Anderson & Patton, 1955; Desai & Kilby, 1958; McEnroe & Forgash, 1957; Heller & Jezewska, 1959). By analogy, therefore, uric acid is probably synthesized in the fat body of *Dysdercus* and released into the haemolymph to be subsequently dealt with by either of the two mechanisms responsible for nitrogen excretion. Some of this uric acid is deposited in the epidermal cells, while the remainder is converted to allantoin and excreted.

The enzyme uricase is widely distributed in insects (Leifert, 1935; Brown, 1938*a*; Desai & Kilby, 1958; Lisa & Ludwig, 1959; Razet, 1953, 1961). The localization of uricase activity within the body appears to vary considerably. It is found in the fat body of *Antheraea pernyi* (Leifert, 1935); the cockroach *Leucophaea maderae* (Lisa & Ludwig, 1959), and the larvae of *Calliphora* (Desai & Kilby, 1959). Certain insects, *Gryllus bimaculatus*, *Dytiscus semisulcatus* and *Hadrocarabus problematicus*, as in *Dysdercus*, have uricase activity concentrated in the Malpighian tubules (Razet, 1961).

Not only are the Malpighian tubules responsible for the production of urine, but they also appear to play a fundamental part in the excretory metabolism of certain insects. In fact Malpighian tubules probably play an important part in the general metabolism of insects. In *Periplaneta americana* the enzyme amine oxidase is almost entirely concentrated in the Malpighian tubules (Blashko, Colhoun & Frontali, 1961). The Malpighian tubules of locusts can perform various transaminations (Kilby & Neville, 1957), and those of the silkworm *Bombyx mori* contain xanthine dehydrogenase (Hayashi, 1960). The occurrence of transaminases in the Malpighian tubules of locusts is consistent with the observation that valine is metabolized during its passage through the Malpighian tubules of *Dixippus morosus* (Ramsay, 1958).

The amount of water reabsorbed from the rectum of *Dysdercus* is surprisingly low when compared with that of other terrestrial insects, which recycle water through the excretory system. It has been calculated that *Rhodnius prolixus* excretes approximately 0·5 mg. uric acid/day, but the amount of water lost *via* the excretory system after the initial diuretic period is very small indeed (Wigglesworth, 1931*a*). Nearly all the water delivered by the Malpighian tubules to excrete this large amount of uric acid must therefore be reabsorbed by the rectum. A similar situation exists in *Dixippus morosus* where, although urine is produced at a rate of 6 mm.^{3}/hr. (Ramsay, 1955), the excreta finally voided from the body are practically dry. In such insects the recycling of water through the excretory system permits the gradual accumulation of uric acid in the rectum with a minimal loss of water.

In *Dysdercus*, however, the need to excrete excess inorganic ions derived from the diet (Berridge, 1965*b*), coupled with an inability of the rectum to concentrate the urine (Berridge, 1965*a*), results in a large output of fluid. Excretion is achieved by maintaining a continuous flush of water through the excretory system, with little or no attempt at water retention by the rectum. In fact it has been calculated that on the third day of the instar only 28% of the urine produced by the Malpighian tubules is reabsorbed by the rectum. Nitrogen excretion therefore has to be achieved without recourse to the filtration/reabsorption mechanism employed by other insects. For this reason, the significance of allantoin as the main nitrogenous excretory product in *Dysdercus* is now apparent.

Allantoin is much more soluble than uric acid, therefore its concentration in the haemolymph can be increased to a very high level. The concentration of allantoin on the third day of the instar is 2·46 μg. N/μl., which represents 13·4% of the osmotic pressure of the haemolymph. This means that, although the whole emphasis of excretion is on 'filtration', a high concentration of allantoin in the urine can be rapidly achieved. If the concentration of allantoin in the haemolymph was low, large quantities of allantoin could be excreted only by filtering very large volumes of liquid or by the active transport of allantoin. Neither of these possibilities is feasible. The increase in concentration of waste nitrogen in the body fluid represents a novel method of eliminating large quantities of nitrogen through an excretory system where there is little recycling of water.

SUMMARY

1. The nitrogenous components of the urine are: allantoin, urea, amino acids, kynurenine, and a mucopolysaccharide. The main nitrogenous excretory product is not uric acid, as in other terrestrial insects, but allantoin.

2. The excretion of total nitrogen, allantoin nitrogen, urea nitrogen and amino acid nitrogen have been followed throughout the excretory phase. The concentration of allantoin in the urine is always less than that in the haemolymph, and is a result of the low permeability of the Malpighian tubules to this substance. When isolated Malpighian tubules were used the average U/P ratio was 0·45.

3. Waste nitrogen is also excreted by storage excretion of uric acid in the epidermal cells; the rate of deposition is most rapid during the excretory phase.

4. Low allantoin excretion and a low rate of uric acid deposition in the epidermis during the post-excretory phase suggest that uric acid synthesis is markedly reduced during this phase.

5. Uricase is concentrated in the Malpighian tubules, where it accounts not only for the absence of uric acid in the urine but also for the high concentration of allantoin in the haemolymph.

6. Calculations based on allantoin excretion show that only 28% of the primary tubule fluid is reabsorbed from the rectum. Since allantoin is more soluble than uric acid, it is better suited as a nitrogenous waste substance in such an excretory system, which recycles little water.

7. The method of excreting waste nitrogen is compared with that of other insects.

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