

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

II. INORGANIC EXCRETION AND IONIC REGULATION

By M. J. BERRIDGE

*Department of Zoology, University of Cambridge**

(Received 20 May 1965)

INTRODUCTION

Buxton (1933) has classified insects into 'spenders' and 'savers'. The 'savers' (e.g. *Cimex*, *Tenebrio*) excrete a dry urine and are extremely resistant to desiccation. *Lucilia*, *Calliphora*, *Glossina* and *Musca* are given as examples of typical 'spenders' for they produce large volumes of liquid urine, the accompanying water loss being compensated by oral intake. The amount of water lost through the excretory system is, however, dependent not only on the availability of water, but also on the excess of inorganic ions which have to be excreted. Excretion in *Dysdercus* is unusual because relatively large volumes of urine are excreted (Berridge, 1965). The insect does not derive excess water from its diet; the possible influence of excess inorganic ions on this copious production of urine was, therefore, investigated.

MATERIAL AND METHODS

Female fifth-instar larvae have been used throughout these studies; the insects were cultured as described earlier (Berridge, 1965). Initial studies on excretion in *Dysdercus* showed that there were two distinct phases of excretion. It was necessary, therefore, to devise methods of collecting urine which took into account these two phases. Urine from insects in the excretory phase was collected by the method of blocking the anus (Berridge, 1965); this method was not only quicker, but it also obviated the difficulty of having to elute urine off filter paper. Animals were discarded after each collection. The amount of a substance excreted at daily intervals was determined, giving a typical rate curve. Urine was collected in the post-excretory phase without blocking the anus, because it is naturally retained during this period. Thirty-two animals were required for each experiment. On each day of this phase urine was removed from eight animals and the concentration and total content of any substance in this pooled urine was determined. The increase or decrease of the substance could thus be followed, in effect giving a cumulative curve.

There is, therefore, a fundamental difference in the method of collecting urine from animals in the two phases. It is important to remember this difference when attempting to interpret results of quantitative analyses of urine throughout the fifth instar.

pH was measured with a quinhydrone electrode modified for use on small volumes.

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

A sample of urine (1–2 μ l.) was drawn into a capillary tube containing a few crystals of quinhydrone. This capillary tube was slipped onto the end of a platinum electrode so that contact was made between platinum wire and urine sample; this assembly was gently lowered onto the agar plug in the vertical side-arm of a calomel electrode. Electrical contact was established between urine at the bottom of the capillary tube and the surface of the agar. The potential difference across the two electrodes was read and used to estimate the pH.

Inositol was identified chromatographically. Samples of untreated urine were run uni-dimensionally in iso-propanol/water (8:2, or ethyl acetate/pyridine/water/acetic acid (8:8:4:1). Inositol was detected with the ammoniacal silver nitrate method (Block, Durrum & Zweig, 1958); Fleury's reagent for polyols was also used as a more specific detection method (Block *et al.* 1958).

Chromatography was also used to identify inorganic phosphate in the haemolymph or urine. Samples of urine or haemolymph were run uni-dimensionally in acetone/25% trichloroacetic acid (3:1), or ethyl acetate/acetic acid/water (3:3:1) as solvents. The detection reagent used was that described by Burrows, Grylls & Harrison (1952).

Where quantitative analysis has been performed eight insects were used for each determination; the vertical lines on the graph represent \pm twice the standard error of each mean. Unless otherwise stated, analyses were performed on untreated urine and deproteinized haemolymph, the proteins in the haemolymph being precipitated by the addition of four volumes of absolute alcohol and subsequently removed by centrifugation. The clear alcoholic fraction was transferred to another tube and the precipitated proteins were washed twice with 80% alcohol. The total volume of alcoholic solution, including washings, could be used directly for quantitative analysis. All techniques used were tested by carrying out a series of determinations on a single sample; in all cases the error (standard deviation as a percentage of the mean) was within $\pm 5\%$.

Amino acid nitrogen was determined colorimetrically by the method of Rosen (1957). Total nitrogen in the urine was measured by the micro-Kjeldahl procedure of Shaw & Beadle (1949).

The cations sodium, potassium, magnesium and calcium were determined with a flame photometer. Samples of untreated urine or haemolymph were pipetted into 5 ml. of distilled water in capped Polythene vials. The approximate concentrations used for the different ions were sodium (0.5 p.p.m.), potassium (1.0 p.p.m.), magnesium and calcium (3.0 p.p.m.); at these concentrations no interference was recorded between the different ions. The apparatus was calibrated at the beginning of each series of determinations and this calibration was checked at frequent intervals.

Chloride was estimated by electrometric titration with 0.2% silver nitrate according to the first method of Ramsay, Brown & Croghan (1955). Determinations were performed on untreated haemolymph, because proteins do not interfere in these estimations.

A colorimetric method was used to determine the phosphate content of the urine (Filipowicz, Gross & Skoczylas, 1963). Preliminary chromatographic analysis indicated that only orthophosphate was present in the urine.

RESULTS

pH of the urine

The urine remains acid throughout the fifth instar (Table 1). The degree of acidity of the urine appears to be related to the osmotic pressure which is discussed in an earlier paper (Berridge, 1965). The more concentrated the urine the greater is its acidity. This relationship is shown most strikingly by the urine retained in the rectum. The osmotic pressure of this urine increases throughout the post-excretory phase as does the acidity (Table 1).

Table 1. *pH of urine excreted during the two phases of excretion*

Days	pH excretory phase (mean \pm 2 S.E.)	pH post-excretory phase (mean \pm 2 S.E.)
1	5.8 \pm 0.18	—
2	5.4 \pm 0.15	—
3	5.6 \pm 0.10	—
4	5.9 \pm 0.18	—
5	5.9 \pm 0.09	6.5 \pm 0.20
6	6.5 \pm 0.25	6.2 \pm 0.23
7	—	6.1 \pm 0.09
8	—	5.8 \pm 0.10

Inorganic excretion during the excretory phase

The excretion of potassium, magnesium, sodium, chloride and phosphate has been considered. Except in the case of phosphate, the concentration of these ions in the haemolymph has also been measured. In general, the inorganic ions identified in the urine of *Dysdercus* may be divided roughly into two groups. The first group (potassium, magnesium and phosphate) are considerably concentrated in the urine. The second group (sodium and chloride) are very much less concentrated in urine than haemolymph. Presumably, these ions are actively reabsorbed from the excretory system.

The ions of the first group have markedly similar excretory fates (Figs. 1–3). Both concentration and total amount excreted increase during the initial part of the fifth instar, but decline rapidly as soon as feeding ceases. The concentrations of potassium and magnesium in the urine, over the period 2–5 days, are considerably higher than the corresponding haemolymph values (Figs. 1, 2). Although the concentration of phosphate in the haemolymph has not been measured, a chromatographic comparison of equal volumes of haemolymph and urine clearly indicated that this ion is also concentrated in the urine.

The ions of the second group, sodium and chloride, are very much less concentrated in urine than haemolymph (Table 2). For the greater part of the excretory phase the concentration of these two ions in the urine is less than 1 mM/l. although the concentration does show a slight increase above this value during the beginning and closing stages of this phase.

Consideration has so far been restricted to the soluble constituents of the urine. However, insoluble calcium phosphate is present as lozenge-shaped crystals in the lumen of the Malpighian tubules and rectum throughout the two phases of excretion.

Unfortunately the quantity of this salt excreted has not been determined. The concentration of soluble calcium in the rectal fluid is very low.

Inorganic excretion during the post-excretory phase

During the post-excretory phase, the rectum contains a large volume of hypotonic urine, which functions as a water store (Berridge, 1965). The constant inorganic content of this retained urine (Fig. 4a) suggests that excretory products are no longer

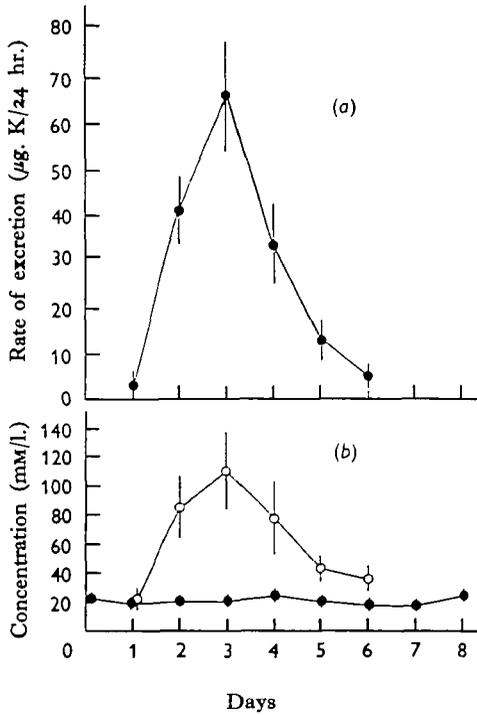


Fig. 1

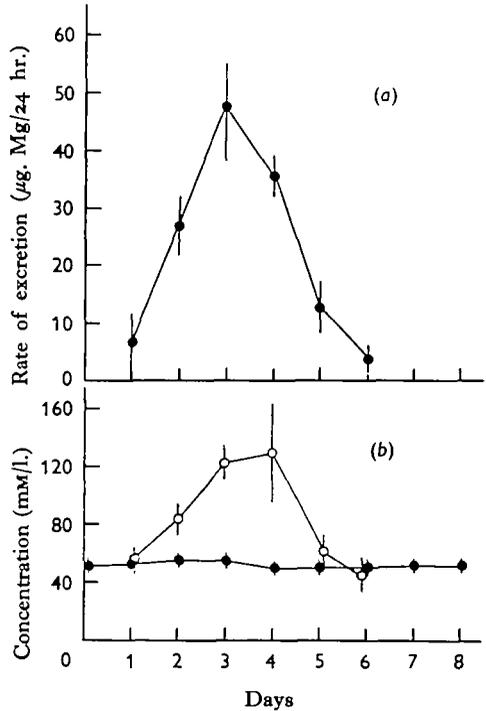


Fig. 2

Fig. 1. Potassium excretion. (a) Rate of potassium excretion. (b) Concentration of potassium in urine and haemolymph. O, Urine; ●, haemolymph.

Fig. 2. Magnesium excretion. (a) Rate of magnesium excretion. (b) Concentration of magnesium in urine and haemolymph. O, Urine; ●, haemolymph.

accumulating in the rectum during this phase. The total nitrogen content of this urine was also measured by way of comparison. There is a slight increase in the potassium, magnesium and total nitrogen content from the fifth to sixth day. In general, however, the inorganic and total nitrogen content of this retained fluid remains almost constant throughout this period.

The sodium and chloride content of this retained fluid declines regularly throughout this second phase. It appears that water reabsorption on successive days in this last phase is paralleled by the reabsorption of sodium and chloride, until on the 8th day, the concentration of these two substances (Fig. 4b) reaches a level similar to that in the urine of the excretory phase. The increase in the concentration of potassium, magnesium, and total nitrogen (Fig. 4b) probably reflects the decrease in

volume of this retained fluid as water is reabsorbed. The very low ionic concentration of the liquid retained in the rectum on the fifth day is probably a reflexion of the water storage function of this fluid.

These results suggest that, during the post-excretory phase, there is little further addition of either inorganic ions or nitrogenous compounds to the fluid stored in the rectum. This provides strong evidence for supposing that the Malpighian tubules are relatively inactive during this phase.

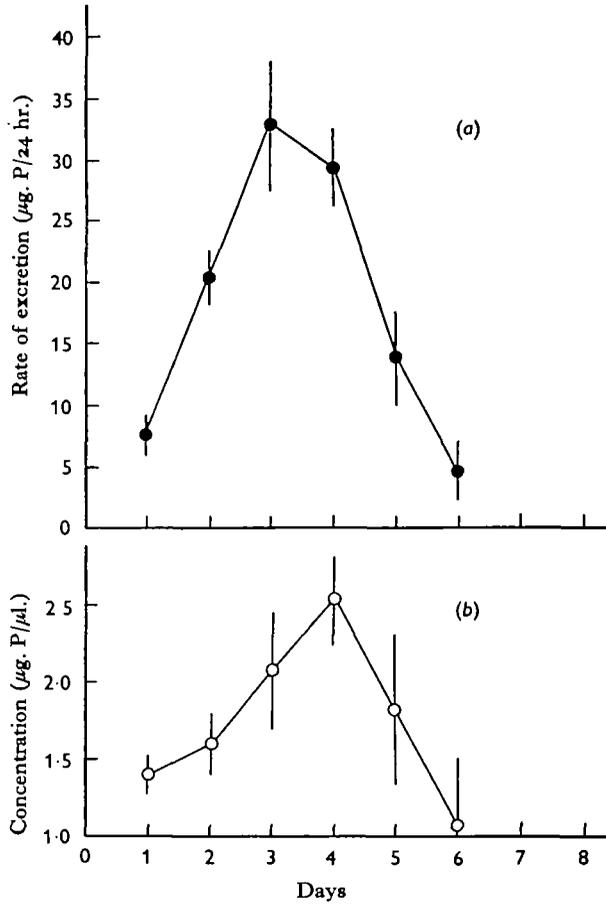


Fig. 3. Phosphate excretion. (a) Rate of phosphate excretion. (b) Concentration of phosphate in urine.

The influence of diet on inorganic excretion

In *Dysdercus* there is a close correlation between diet and inorganic excretion. A comparison of the ionic composition of the urine with that of the cotton seed shows that the rapid excretion of potassium, magnesium and phosphate is related to a high content of these ions in the diet (Table 3). Conversely, a low content of sodium and chloride in the seed is associated with the retention of these two ions by the excretory system.

This correlation between diet and inorganic excretion in *Dysdercus* is paralleled

Table 2. (a) *Sodium and chloride concentrations in urine and haemolymph during the excretory phase. (Concentration expressed as mM/l.)*

Days	Sodium		Chloride	
	Urine	Haem.	Urine	Haem.
0	—	20.8	—	19.2
1	2.63	24.6	2.40	20.2
2	0.79	30.8	0.65	33.5
3	0.57	31.3	0.45	37.5
4	0.57	26.1	0.60	36.7
5	4.33	24.6	0.53	32.5
6	7.54	22.3	1.68	28.4

Table 2. (b) *Sodium and chloride concentrations in urine and haemolymph during the post-excretory phase. (Concentration expressed as mM/l.)*

Days	Sodium		Chloride	
	Urine	Haem.	Urine	Haem.
5	14.2	24.6	5.0	32.5
6	12.9	22.3	4.4	28.4
7	5.6	22.1	3.6	21.5
8	2.0	22.9	1.6	18.6

Table 3. *The inorganic composition of cotton seed (mM/kg. dry weight) and of urine excreted by Dysdercus on the third day (mM/l.). Data on the inorganic content of cotton seed are taken from Bailey (1948)*

Element	Cotton seed (mM/kg.)	Urine (mM/l.)
Potassium	250.0	112.00
Magnesium	144.0	84.00
Phosphorus	178.0	67.00
Sodium	43.0	0.57
Chloride	14.2	0.45

Table 4. *The vitamin B content of cotton seed (data from Cheldelin & Lane, 1943)*

Vitamin	γ /g. on dry weight basis
Thiamin	3.20
Riboflavin	2.30
Nicotinic acid	16.00
Pantothenic acid	11.00
Pyridoxine	0.91
Biotin	0.29
Folic acid	3.80
Meso-inositol	3400.00

by the observation that the urine contains large amounts of the polyhydric alcohol, meso-inositol, which is present in high concentration in cotton seeds (Table 4). The meso-inositol was identified chromatographically; the unknown spot had identical R_f values to that of standard meso-inositol, when run in a wide variety of solvents. The unknown spot also gave a positive reaction when tested with Fleury's reagent.

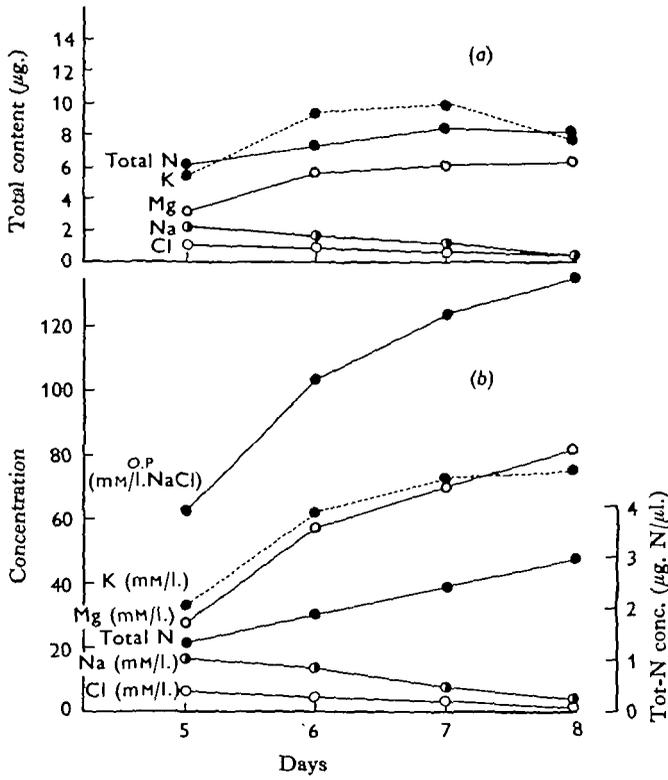


Fig. 4. Composition of the urine retained in the rectum during the post-excretory phase. (a) Total content of inorganic ions and nitrogen. (b) Concentration of inorganic ions (mM/l.) and nitrogen ($\mu\text{g. N}/\mu\text{l.}$) in the retained urine. The osmotic pressure of the urine (mM/l. NaCl) is also included.

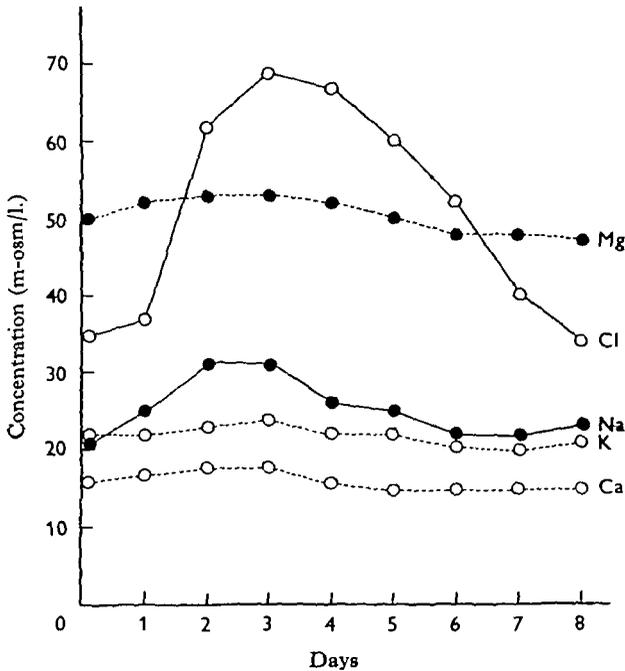


Fig. 5. Changes in the concentrations of inorganic ions in the haemolymph.

The meso-inositol content of cotton seeds is enormously larger than that of any other B-vitamin (Cheldelin & Lane, 1943). Meso-inositol forms the basis of a large complex molecule commonly known as phytin, which is composed of the calcium, magnesium and potassium salts of the hexaphosphoric acid ester of meso-inositol (*viz.* phytic acid, $C_6H_8(H_2PO_4)_6$). Phytin is believed to act as a phosphate reserve for seeds; when these seeds germinate they produce an enzyme, phytase, which hydrolyses phytin to liberate phosphate and meso-inositol. In *Dysdercus* this meso-inositol, together with its associated ions, is eliminated *via* the excretory system.

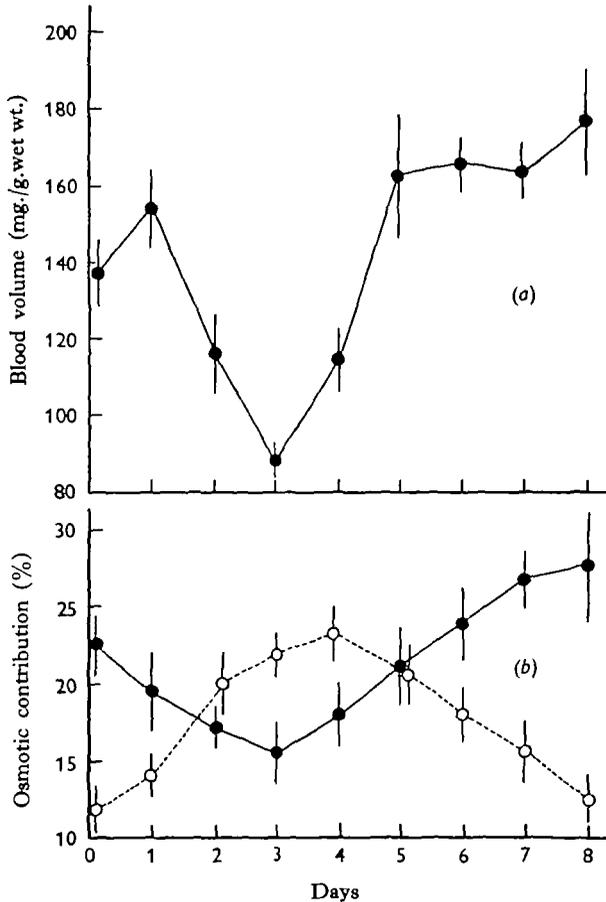


Fig. 6. (a) Changes in blood volume throughout the fifth instar. (b) Contribution of amino acids and chloride to the osmotic pressure of the haemolymph. O, chloride. ●, Amino acids;

Ionic regulation

Ionic regulation in *Dysdercus* has not been dealt with in detail, but certain aspects, which have emerged from the study of inorganic excretion, warrant further attention. Variation in the ionic composition of the haemolymph throughout the fifth instar is illustrated in Fig. 5. The concentration of calcium, potassium and magnesium remain relatively constant throughout the instar; but sodium and chloride, especially

the latter, show a marked increase in concentration during the initial part of the instar. The concentrations of these two ions return to their original levels in the latter part of the instar.

The excretory system plays a major role in maintaining constant calcium, potassium and magnesium concentrations in the haemolymph by rapidly removing excesses of these ions. The concentrations of sodium and chloride are not controlled in this way, these ions being retained by the excretory system even though their concentrations in the haemolymph increase. As there is little loss of these ions *via* the excretory system, their total content in the haemolymph presumably remains relatively constant. If it is assumed that the sodium and chloride contents of the haemolymph show little change, then the observed shifts in the concentration of these ions would most likely reflect a change in the blood volume. This possibility was investigated first; blood volume was measured by determining the loss of weight after cutting open the abdomen and thorax and removing as much as possible of the blood by gentle squeezing and swabbing with filter paper. These values must be regarded as minimum estimates as it was impossible to achieve complete removal of blood, but this method was accurate enough to detect the large changes which occur (Fig. 6*a*). A high concentration of sodium and chloride, particularly the latter, appears to be correlated with low blood volume and vice versa.

Because the change in the sodium concentration of the haemolymph is small, more attention was paid to the change in chloride concentration in the haemolymph. The change in the concentration of chloride, if uncompensated, would have an appreciable effect on the osmotic pressure of the haemolymph. However, this change in chloride concentration is partially compensated by the amino acid concentration of the haemolymph, which is inversely correlated with chloride concentration (Fig. 6*b*).

DISCUSSION

Dysdercus, by virtue of its peculiar diet, ingests a lot of unwanted ions, mainly magnesium, calcium, potassium and phosphate; in order to get rid of these ions it exhibits extensive diuresis during the process of feeding (Berridge, 1965). This diuretic period is considerably exaggerated by the inability of the rectal epithelium to reabsorb water against an osmotic gradient (Berridge, 1965). A similar effect of excess inorganic ions on water excretion has already been noted in the locust, *Schistocerca gregaria*. The rectal fluid of water-fed individuals is small in volume and has a low ionic concentration, whereas saline-fed locusts produce a large volume of urine of high ionic concentration (Phillips, 1964*b*). In this latter case the need to remove excess inorganic ions, in effect, converts these animals into fluid-excreting insects. Further reabsorption of water is impossible, because the concentration of the rectal fluid has reached the maximum limit, above which water can no longer be reabsorbed (Phillips, 1964*a, b*). Although these changes were induced experimentally, they serve to stress the importance of diet, as well as the availability of water, in determining the degree of water retention which can be achieved by the excretory system.

The marked decrease in blood volume which occurs during the feeding period cannot be accounted for solely by a blood/tissue exchange, because the total water content is also greatly reduced during this period (Berridge, 1965). The rapid rate

of excretion is a more likely explanation for the decrease in blood volume; it appears that water ingestion does not keep abreast with losses *via* the excretory system. This change in blood volume may be concerned in the mechanism controlling water ingestion; Dethier & Evans (1961) have shown that the acceptance or rejection of water by *Phormia regina* is dependent on blood volume.

A change in blood volume has a marked effect on the concentration of certain ions; since sodium and chloride are reabsorbed from the rectum a reduction in blood volume during the initial part of the instar is associated with an increase in the concentration of these ions in the haemolymph. If the concentration of sodium and chloride remained constant the insect could not counteract dilution of these ions when the blood volume increased again, because the sodium and chloride content of the diet is low. Teleologically it may be said that, by concentrating these ions in the haemolymph, the animal prepares itself for an increase in blood volume during the last part of the instar. There is no such problem with ions such as potassium, magnesium and phosphate, because any dilution in the haemolymph can be prevented by the large excess of these ions in the diet.

In spite of changes in sodium and chloride concentrations the osmotic pressure of the haemolymph remains constant because amino acids compensate for these ionic changes. The role of non-protein nitrogen in the osmoregulation of aquatic insects is well known (Beadle & Shaw, 1950; Schoffeniels, 1960). In *Dytiscus marginalis*, the total concentration of free amino acids in the haemolymph is inversely correlated with chloride concentration (Schoffeniels, 1960). For terrestrial insects, however, there is only circumstantial evidence for an osmoregulatory function of amino acids (Barton-Browne, 1964). *Dysdercus* is probably the first example of a terrestrial insect which employs amino acids to compensate for changes in chloride concentration.

SUMMARY

1. Inorganic excretion has been studied during both phases of excretion.
2. Large amounts of potassium, magnesium and phosphate are excreted during the first phase, but sodium and chloride losses are reduced to a minimum.
3. There is a close correlation between inorganic excretion and the relative abundance of these ions in the diet.
4. The inorganic ion and total nitrogen content of the urine retained in the rectum during the post-excretory phase shows little change. This provides strong evidence for assuming that the Malpighian tubules are inactive during this period.
5. Magnesium, potassium and calcium concentrations in the haemolymph remain relatively constant; sodium and chloride concentrations, however, show a marked increase during the middle of the instar. Amino acid concentration compensates for these changes, so ensuring a constant osmotic pressure in the haemolymph.
6. The high rate of liquid excretion in *Dysdercus* probably results from an excessive intake of inorganic ions.

This work is part of a thesis submitted to the University of Cambridge for the degree of Ph.D. I am grateful to Prof. Sir Vincent Wigglesworth for his advice and supervision and to Dr. E. Bursell for reading and criticizing the manuscript. I thank the Commonwealth Scholarship Commission for generous financial support.

REFERENCES

- BAILEY, A. E. (1948). *Cottonseed and Cottonseed Products, their Chemistry and Chemical Technology*. New York: Interscience Publishers, Inc.
- BARTON-BROWNE, L. (1964). Water regulation in insects. *Annu. Rev. Ent.* **9**, 63-82.
- BEADLE, L. C. & SHAW, J. (1950). The retention of salt and the regulation of the non-protein nitrogen fraction in the blood of the aquatic larva, *Sialis lutaria*. *J. Exp. Biol.* **27**, 96-109.
- BERRIDGE, M. J. (1965). The physiology of excretion in the cotton stainer, *Dysdercus fasciatus* Signoret. I. Anatomy, water excretion and osmoregulation. *J. Exp. Biol.* (in the Press).
- BLOCK, R. J., DURRUM, E. L. & ZWEIG, G. (1958). *A Manual of Paper Chromatography and Paper Electrophoresis*, 2nd. ed. New York: Acad. Press.
- BURROWS, S., GRYLLES, F. S. M. & HARRISON, J. S. (1952). Paper chromatography of phosphoric esters. *Nature, Lond.*, **170**, 800-1.
- BUXTON, P. A. (1933). The effect of climatic conditions upon populations of insects. *Trans. Roy. Soc. Trop. Med. Hyg.* **26**, 325-64.
- CHELDELIN, V. H. & LANE, R. L. (1943). B Vitamins in germinating seeds. *Proc. Soc. Exp. Biol. Med., N. Y.*, **54**, 53-5.
- DETHIER, V. G. & EVANS, D. R. (1961). The physiological control of water ingestion in the blowfly. *Biol. Bull., Woods Hole*, **121**, 108-16.
- FILIPOWICZ, B., GROSS, M. & SKOCZYLAS, B. (1963). Micromethod for simultaneous determination of nitrogen and phosphorus in single samples of nucleoproteins and their derivatives. *Anal. Biochem.* **5**, 187-94.
- PHILLIPS, J. E. (1964*a*). Rectal absorption in the desert locust, *Schistocerca gregaria* Forskål. I. Water. *J. Exp. Biol.* **41**, 15-38.
- PHILLIPS, J. E. (1964*b*). Rectal absorption in the desert locust, *Schistocerca gregaria* Forskål. III. The nature of the excretory process. *J. Exp. Biol.* **41**, 69-80.
- RAMSAY, J. A., BROWN, R. H. J. & CROGHAN, P. C. (1955). Electrometric titration of chloride in small volumes. *J. Exp. Biol.* **32**, 822-9.
- ROSEN, H. (1957). A modified ninhydrin colorimetric analysis for amino acids. *Archs. Biochem. Biophys.* **67**, 10-15.
- SCHOFFENIELS, E. (1960). Rôle des acides aminés dans la régulation de la pression osmotique du milieu intérieur des Insectes aquatiques. *Archs. int. Physiol.* **68**, 507-8.
- SHAW, J. & BRADLE, L. C. (1949). A simplified ultra-micro Kjeldahl method for the estimation of protein and total nitrogen in fluid samples of less than 1.0 μ l. *J. Exp. Biol.* **26**, 15-23.