EXCRETION BY THE MALPIGHIAN TUBULES OF THE STICK INSECT, *DIXIPPUS MOROSUS* (ORTHOPTERA, PHASMIDAE): CALCIUM, MAGNESIUM, CHLORIDE, PHOSPHATE AND HYDROGEN IONS

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INTRODUCTION

In earlier papers (Ramsay, 1954, 1955*b*) an account was given of the excretion of sodium, potassium and water by the Malpighian tubules of the stick insect. The present paper describes the extension of this work to the more important of the remaining inorganic substances: calcium, magnesium, chloride, phosphate and hydrogen ions. An indication of the manner in which the first four are excreted is given by the analyses reported in another paper (Ramsay, 1955*a*) from which Table 1 is taken. It is seen that the concentration of phosphate is greater and the concentrations of calcium, magnesium and chloride are less in the urine than in the haemolymph or serum. (Serum is haemolymph from which most of the protein has been removed by heat-coagulation.) These analyses were made upon urine excreted by tubules bathed in haemolymph with the various components of the haemolymph at about their normal levels of concentration. It was felt that for the sake of completeness some investigation should be made of the excretion of these substances over a range of concentration.

Malpighian tubules will not survive in any known artificial medium, and hitherto it has been necessary to add serum in the proportion of 1 part to 3 parts of artificial medium. This requirement for the use of serum introduces two practical difficulties. The first is that the limitation upon dilutions greater than 1 part to 3 sets a limit to the changes in concentration of any given component which can be brought about by mixing serum with isotonic salines. This difficulty has now been overcome, as will presently be described. The second difficulty is that chronic shortage of serum makes it necessary to work with single tubules isolated in small drops of medium; and this in turn has made it necessary to adapt methods of analysis to volumes of the order of 0·1 mm.^{3} or less.

MATERIAL AND METHODS

All the results described in this paper have been obtained from the superior tubules (Ramsay, 1955*a*) of the adult female stick insect fed on privet. The preparation of the tubule isolated in a drop of medium under liquid paraffin and the collection of urine from it are described elsewhere (Ramsay, 1954).

No progress whatever has been made with the problem of separating from serum the 'active principle'—whatever it be—that enables the Malpighian tubules to function. Fortunately it has been found possible to replace the original inorganic ions (see Table 1) with others, using the method of electromigration, since the 'active principle' appears to be uncharged. The apparatus is shown in Fig. 1. It is constructed of Perspex, the main considerations in design being to combine the maximum rate of electromigration with the minimum mixing by convection and diffusion.

Table 1

	Haemolymph	Serum	Urine
pH	—	6·6	6·8–7·5
Δ	160 mM./l. NaCl	171 mM./l. NaCl	171 mM./l. NaCl
Na	8·7 m.equiv./l.	11 m.equiv./l.	5 m. equiv./l.
K	27·5 m.equiv./l.	18 m.equiv./l.	145 m. equiv./l.
Ca	16·2 m.equiv./l.	7 m.equiv./l.	2 m. equiv./l.
Mg	145 m.equiv./l.	108 m.equiv./l.	18 m. equiv./l.
Cl	93 m.equiv./l.	87 m.equiv./l.	65 m. equiv./l.
PO_4^{3-}	120 m.equiv./l.	39 m.equiv./l.	51 m. equiv./l.
Uric acid	10·4 mg./100 ml.	4·5 mg./100 ml.	43 mg./100 ml.

Note. Phosphate was expressed as trivalent is this table to emphasize the anion deficit. It is more probably present as monovalent and divalent ions in accordance with pH, from which it can be calculated that there will be 19 m.equiv./l. in serum and 30 m. equiv./l. in urine at pH 7·2.

The saline solution, say, 150 mM./l. NaCl, which is to replace the ions present in the serum, is set in agar in two tubes *a* and *b*, $\frac{3}{8}$ in. internal diameter, which connect with the two electrode vessels *c* and *d*. A continuous flow of 150 mM./l. NaCl through these vessels serves to remove the products of electrolysis, and as a check on this indicators are added to the agar in the tubes. The sample of serum, about 1 ml. in volume, is placed in the central chamber *e*. If the serum is set in agar the 'active principle' cannot be recovered, and it is therefore necessary to take other steps to reduce convection. Cellulose pulp, made from filter-paper in a Waring blendor, is squeezed dry and enough is added to the serum to make a thick pulp which will not separate out under gravity. This pulp is then placed in *e* and the whole apparatus is immersed in an ice-bath since the limit upon the current (60 mA.) is its heating effect which tends to melt the agar. After the current has passed for about 1 hr. the pulp is removed to a glass syringe and the serum is squeezed out. Replacement of ions by this method is shown by analysis to be about 95 % effective. In this way the following sera were prepared: 150 mM./l. NaC. serum; 100 mM./l. $CaCl_2$ serum; 100 mM./l. $MgCl_2$ serum; 100 mM./l. Na_2HPO_4–NaH_2PO_4 serum. Osmotic pressure and pH were adjusted if necessary to normal values. It is of course impossible to vary the concentration of one ion without at the same time varying the concentration of another. The method adopted was to study calcium and magnesium when replacing sodium, and phosphate when replacing chloride. Details are given below in the appropriate context.

The methods of analysis used were as follows. Calcium, precipitation (twice) as oxalate and titration with ceric sulphate (Kirk, 1950) and labelling with ^{45}Ca.

Chloride; electrometric titration (Ramsay, Brown & Croghan, 1955). Phosphate; ammonium molybdate method as described by Delory (1949) and labelling with ^{32}P. In the case of magnesium it was hoped to make use of ^{28}Mg but it did not prove possible to prepare this isotope free from poisonous impurities. The method eventually used was to precipitate the magnesium as magnesium ammonium phosphate using ^{32}P.

The precipitation is carried out in a droplet resting upon a cover-slip under liquid paraffin and if the conditions are suitably adjusted the precipitate separates as crystalline needles. The mother liquor can be sucked away almost completely and the cover-slip is then heated to drive off the liquid paraffin before being counted in the usual way. This method is simple and convenient but cannot be used in the presence of calcium or phosphate.

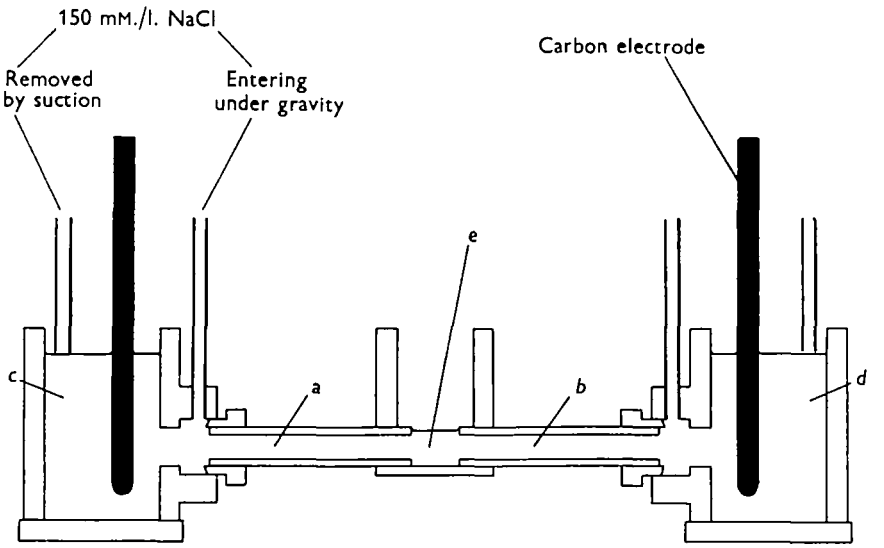

Fig. 1. Apparatus for changing the ionic content of serum by electromigration; in the illustration the ions of serum are to be replaced by 150 mM./l. NaCl. *a*, *b*, tubes containing 150 mM./l. NaCl set in agar; *c*, *d*, electrode vessels flushed out with 150 mM./l.; *e*, vessel containing serum in cellulose pulp. For further explanation, see text.

Measurements of the rate of urine production were also made, the volume of urine being found from the diameter of the droplet sinking through liquid paraffin.

It would be difficult to give precise figures for the accuracies of the foregoing methods without endless qualification as to conditions. It should be sufficient to state that the errors are nowhere greater than $\pm$ 10% and that this degree of accuracy is sufficient to support the conclusions which are drawn from the results.

The measurement of pH presented the greatest difficulty. The electrode system most readily adaptable to small volumes is the quinhydrone electrode. Pierce & Montgomery (1935) developed a method capable of working on 0·1 mm.^{3} which gave very close agreement when compared with the large-scale method, but their pH values do not seem to have been checked against any other electrode system.

The quinhydrone electrode is of course notoriously unreliable in the presence of substances such as proteins which react with quinone, and under such conditions a stable potential is not obtained. In the present work in which the pH of serum had to be measured the drift of potential was slow and it was possible to achieve consistency by taking measurements at a fixed time after the addition of the quinhydrone. Checks were made with a conventional glass electrode whenever possible, e.g. on stock serum. Attempts were also made to develop a small-scale glass electrode. A glass electrode system capable of being used with 5 mm.^{3} of fluid

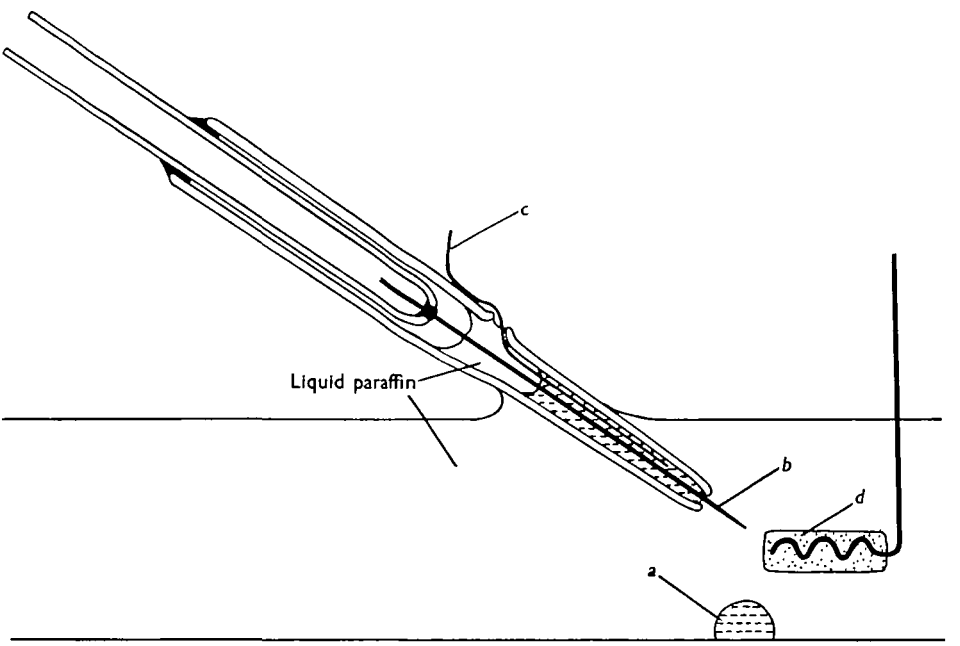

Fig. 2. Small-scale glass electrode. The droplet of solution to be tested, *a*, is kept under liquid paraffin. Some of this solution is sucked up to fill the capillary tube *b* which is of Corning 015 electrode glass. The capillary is surrounded with N-HCl with which electrical contact is made through the silver wire *c*. The tip of the capillary is allowed to come into contact with the reference electrode *d* which is a silver wire embedded in a cake of saturated KCl-agar. This electrode has been used with a Pye Universal pH meter.

has recently been described by Bishop, Hartree & McConachie (1956); the electrode is in the form of a sealed capillary which dips into a wider capillary containing the solution to be tested. Combining the capillary technique of these authors with the arrangement devised by Hartree (1952) in which the solution to be tested is inside the capillary and the standard acid is outside, an electrode suitable for working under liquid paraffin was designed (see Fig. 2). One advantage of this arrangement is that the oil does not allow the surface of the glass to develop leakage paths whereby the potential of the electrode might be short circuited. Several electrodes of this type were prepared, of from 0·3 to 0·5 mm.^{3} capacity, but (as is the usual experience) very few were of sufficiently low resistance and only one fully retained

its calibration when retested after a period of use; but this particular electrode provided an important check upon the pH of urine of which there was insufficient for use with a conventional glass electrode. For routine measurements the quinhydrone system was used throughout. A platinum wire 0·005 in. in diameter and an Ag/AgCl capillary reference electrode were inserted into the droplet to be tested —about 0·2–0·5 mm.^{3}—under liquid paraffin. Crystals of quinhydrone were then added with a glass needle and the potential was read 2 min. later. The measurements of pH are believed to be accurate to $\pm 0{\cdot}2$ pH.

All experiments were carried out at toom temperature 14–17° C.

RESULTS

(1) *Phosphate/chloride.* 1 mc. of carrier-free ^{32}P as orthophosphate was added to 0·5 ml. of Na_2HPO_4–NaH_2PO_4 serum. (The amount of phosphate thus added was less than 1 % of that already present.) KCl and $CaCl_2$ were then added to this serum and to NaCl serum so as to bring the concentrations in each to 15 m.equiv./l. of potassium and 8 m.equiv./l. of calcium. Drops of these two sera mixed in varying proportions were then set out under liquid paraffin, tubules were prepared and collections of urine were made. The results of analysis are given in Table 2 and Fig. 3A. The pH of the urine was not measured in these experiments and there is some evidence to suggest that it was affected by the phosphate content of the urine; for this reason concentrations of phosphate are expressed not as m.equiv./l. but as mg.-atoms P/l. since the valence of the phosphate in the urine is not known. From the table and figure it can be seen that the concentration of phosphate is greater and the concentration of chloride is less in the urine than in the

Table 2

Medium		Urine		Rate	
Phosphate (mg.-atoms P/l.)	Chloride (m.equiv./l.)	Phosphate (mg.-atoms P/l.)	Chloride (m.equiv./l.)	mm.^{3} × 10^{-3}/min.	Averages
6	118	24	106	0·92	
9	122	25	107	1·11	
11	110	14	113	1·03	1·13
12	116	29	104	1·01	
14	116	40	92	1·58	
30	93	81	61	1·46	
31	94	79	64	0·96	1·06
32	92	57	81	0·75	
49	74	101	46	1·19	
51	77	115	35	1·43	
52	71	98	45	1·61	1·49
52	75	105	44	1·43	
54	71	112	39	1·78	
66	55	127	28	1·59	
70	54	138	23	2·06	1·68
70	56	120	31	0·93	
74	51	132	22	2·16	
84	36	140	14	2·46	
84	35	129	18	1·76	2·03
87	39	137	27	1·98	
88	35	139	13	1·91	

medium, and that this is true over the whole range of concentrations studied. It is also to be noted that as phosphate is increased at the expense of chloride in the medium there is a significent increase in the rate of urine flow.

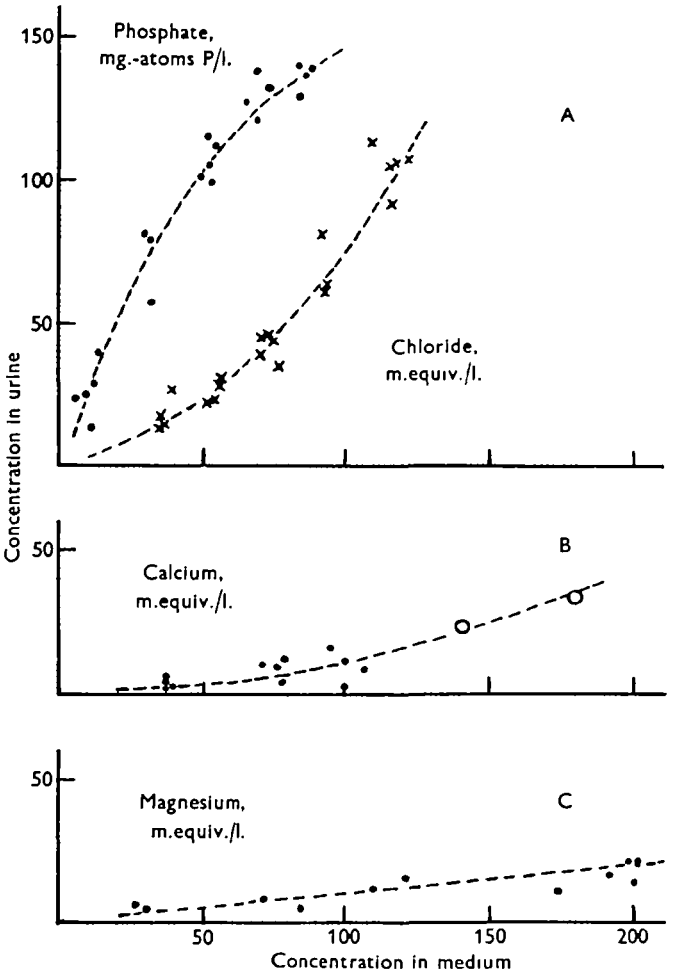

Fig. 3. The relation between concentration in urine and concentration in medium for phosphate, chloride, calcium and magnesium. Except for phosphate all concentrations are expressed as m.equiv./l. Since the pH of the urine was not measured in these experiments the valence of phosphate in the urine is uncertain, and for this reason the results have been expressed as mg.-atoms P/l.

(2) *Calcium/sodium.* 100 μc. of $^{45}CaCl_2$ (specific activity 970 μc./mg. and therefore containing about 0·1 mg. Ca) were added dry to 0·1 ml. of $CaCl_2$ serum (in which the concentration of $CaCl_2$ was about 100 mM./l. and which therefore contained about 0·4 mg. Ca). The freezing-point depression was checked and as anticipated it was necessary to add 25 mm.^{3} of distilled water to restore it to normal. KCl was then added to bring the concentration of potassium to 15 mM./l. This

labelled $CaCl_2$ serum was mixed with the NaCl serum used in the previous experiment (containing 8 m.equiv./l. of unlabelled calcium, for which correction was made). The rate of urine flow decreases as the concentration of calcium in the medium is increased, and at the higher concentrations it was necessary to pool the urine of four tubules to get enough for analysis. The results (Table 3 and Fig. 3 B) show that the calcium concentration of the urine, while increasing with the calcium concentration of the medium, is always much lower, by a factor of 5–10.

Table 3

(Concentrations in m.equiv./l. Rates in $mm.^3 \times 10^{-3}$/min.)

Calcium			Magnesium		
Medium	Urine	Rate	Medium	Urine	Rate
37	5·6	1·9	27	6.0	1·25
37	4·4	1·8	30	5·2	1·57
39	2·4	1·4	72	8·5	1·77
39	1·6	2·9	84	4·8	1·92
72	9·8	1·1	110	12·0	1·77
77	8·8	1·0	121	16·4	0·78
78	3·8	1·7	173	10·7	1·08
79	12·4	0·9	191	16·8	1·65
95	15·6	0·2	200	13·6	—
100	12·0	0·19	200	20·6	—
100	2·8	0·67	200	21·2	—
107	9·2	0·5			
*141	23·6	0·2			
*180	34·4	0·15			

* Four collections pooled.

Table 4

(Rate of urine flow in $mm.^3 \times 10^{-3}$/min.)

pH medium	pH urine	Rate	pH medium	pH urine	Rate	pH medium	pH urine	Rate
4·6	N.S.	—	6·3	7·1	—	6·5	7·1	1·7
5·0	N.S.	—	6·4	7·1	2·8	6·5	7·2	—
5·3	6·8	0·4	6·4	7·3	3·0	6·5	7·3	2·1
5·3	6·7	1·1	6·4	7·1	3·0	6·6	7·3	—
5·3	6·7	1·9	6·4	7·1	3·0	6·7	7·3	—
5·3	6·9	0·7	6·4	7·1	—	6·7	7·2	—
5·3	6·3	0·8	6·4	7·3	3·0	6·9	7·4	2·3
5·3	6·9	0·7	6·4	7·3	—	6·9	7·4	3·0
5·5	7·1	1·9	6·4	7·0	2·3	6·9	7·4	2·7
5·5	6·8	1·4	6·4	7·1	2·6	7·2	7·7	2·3
5·6	6·9	1·9	6·4	7·2	2·6	7·2	7·7	2·0
5·7	7·0	2·5	6·4	7·1	1·9	7·2	7·7	3·0
5·7	7·0	2·3	6·4	7·1	2·0	7·4	7·7	0·5
5·7	7·1	2·7	6·4	7·2	2·1	7·5	7·9	0·3
5·7	7·4	2·0	6·4	7·1	2·0	7·5	7·9	0·3
6·0	7·2	3·0	6·5	7·1	2·3	7·5	7·9	0·5
6·0	7·2	1·0	6·5	7·0	1·3	7·5	7·6	0·4
6·0	7·2	3·0	6·5	6·9	—	7·6	N.S.	—
6·3	7·2	—	6·5	7·2	—			
6·3	7·0	—	6·5	7·2	2·2			

N.S. = no secretion.

(3) *Magnesium/sodium.* The two sera mixed in this case were NaCl serum and $MgCl_2$ serum, to each of which 15 mM./l. of potassium but no calcium had been added. The results show that the concentration of magnesium in the urine is always

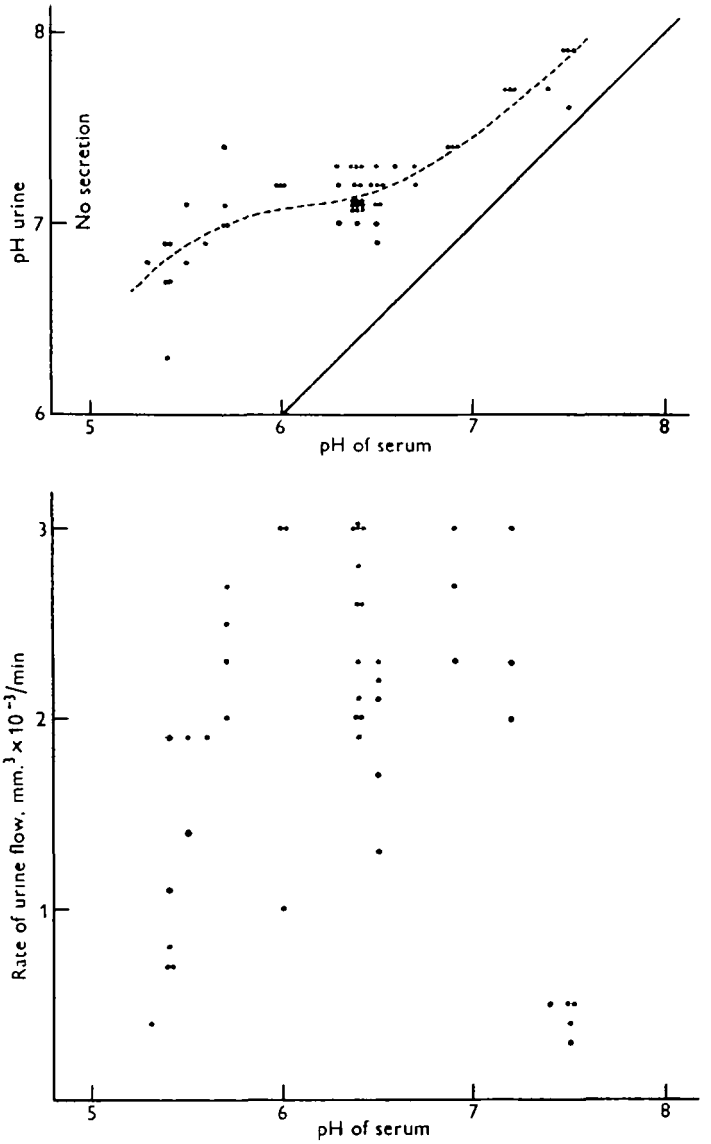

Fig. 4. The dependence of pH of urine and rate of urine flow upon the pH of serum.

low, even lower than the concentration of calcium, but high concentrations of magnesium in the medium do not seem to depress the rate of urine flow (Table 3 and Fig. 3 C).

(4) *pH*. The pH of stock serum was varied by adding N-HCl or N-NaOH. The tubules were left working until a sufficient volume of urine had accumulated and a sample of serum was taken immediately after the urine had been collected; pH was then measured without delay. The results obtained in this way are presented in Table 4 and Fig. 4. The difference in pH between urine and serum is somewhat variable, but in all cases the urine is alkaline to the serum. Furthermore, the difference in pH tends to increase in the more acid media, or in other words the pH of the urine tends to remain constant in the face of changes in the pH of the medium. The measurements of the rate of urine flow show so great a scatter that it is not possible to suggest any relationship between rate and pH. All that can be noted is the decrease in rate as the working limits of pH are approached. These limits appear to be pH 5·2 and 7·5.

It is appropriate to record at this point that six measurements were made of the pH of the rectal fluid expressed through the anus. This was found to be fairly strongly acid, varying between pH 3·5 and 4·5.

DISCUSSION

Now that the scope of this investigation has been extended to include a total of seven ions the whole problem has become vastly more complex. It may be presumed that the excretion of any one ion is affected in greater or less degree by the pattern of other ions present in the medium. A fairly complete investigation has been made of the mutual interaction of sodium and potassium (Ramsay, 1955*b*); to study all possible combinations of seven ions in the same detail is hardly to be contemplated.

The present survey has done little beyond confirm what was suggested by the earlier analyses—that the concentrations of calcium, magnesium and chloride are always less in the urine than in the medium and that the concentration of phosphate is always greater. No measurements of potential difference were made during the course of this work, and it is therefore not possible to reach a definite decision as to whether energy is required for the movements of the ions now under consideration. But this does not seem likely. Calculations based upon potential difference measurements recorded in the earlier papers already referred to and upon the differences in concentration recorded here do not provide any statistically significant evidence that active transport is involved. In the case of chloride it is abundantly clear that this ion moves with and not against the electrochemical gradient; as for the others it is admissible as a working hypothesis that their movements are brought about by passive diffusion. In the case of phosphate, however, there are certain other features of the results which cannot pass without comment. Under the conditions of the experiment the concentration of phosphorus in the medium cannot exceed 100 mg.-atoms/l., whereas concentrations of up to 140 mg.-atoms/l. have been recorded for the urine. This might lead one to suppose that the osmotic pressure of the urine must have been substantially greater than that of the medium. But such does not seem to be the case; in subsidiary experiments to test this point the urine was found

to be isotonic with the medium. There are various possible explanations of this anomaly, e.g. that non-electrolytes account for a substantial fraction of the osmotic pressure of serum. There is also the possibility that some of the phosphate present is in combination with organic molecules. From his studies on the neurophysiology of the stick insect Dr D. W. Wood (personal communication) has come to this view. The excretion of phosphates merit further investigation, but it cannot be effectively studied until it is possible to prepare a fully specified medium in which the tubules will survive and secrete.

This indeed applies not only to phosphate but to the problem as a whole. As has already been mentioned no progress has been made towards isolating the 'active principle' from serum. By the methods of chromatography and ion exchange it has not so far proved possible to recover any fraction which is better than Ringer solution. A large number of substances known to be biologically active have been tested, again without positive result. One substance only, when added to Ringer, enables the tubules to preserve a healthy appearance and to excrete urine for long periods. This substance is 3-hydroxykynurenin, suggested and kindly supplied to me by Dr M. G. M. Pryor. Unfortunately it is not offered on the market and the minute supplies available from private sources would not sustain a full programme of investigation.

There being no immediate prospect of advance along these lines it may be useful at this stage briefly to review the position now reached.

It appears certain that potassium is actively transported (i.e. against an electrochemical gradient) across the wall of the tubule in the stick insect and in certain other insects (Ramsay, 1953). It is probable that this active transport of potassium is fundamental to urine production in all insects. Sodium can be actively transported but it does not seem likely that this ion or any other ion so far studied is actively transported under normal circumstances. At one time it seemed possible that the secretion of potassium (together with some anion) into the tubule would set up an osmotic pressure which in its turn would promote a passive inward diffusion of water; but having found that the osmotic pressure of the urine was slightly lower than that of the haemolymph (Ramsay, 1954) I abandoned this conception. Subsequently it was pointed out to me that since there is a potential difference across the wall of the tubule there still remains the possibility that water moves by electro-endosmosis against the slight osmotic gradient. It did not prove possible to set up the experimental conditions required to test this suggestion. The original theory is therefore still in the field, namely, that the secretion of potassium is the prime mover in generating the flow of urine and that in consequence of this secretion conditions are created which enable water and other constituents of the urine to follow.

The tubules are remarkable for their ability to continue to function in media of grossly abnormal composition. The rate of urine flow is much reduced in the presence of high concentrations of calcium, but the other ions can be varied tenfold or more in concentration without impairment of the tubule's function. There is nothing in the response of the tubules to these variations which might suggest

that they are responsible for the maintenance of the normal composition of the haemolymph; rather it is that the activity of the tubules alone would radically alter the composition of the haemolymph were it not for the participation of the other important organs of the excretory system, the rectal glands.

In his work on the tubules of *Rhodnius* Wigglesworth (1931) has proposed a mechanism for the excretion of uric acid, namely, that this substance is excreted in alkaline solution as urate into the distal region of the tubule and is precipitated as uric acid by the subsequent acidification of the urine in the proximal region of the tubule. I am not aware that there is any other insect besides *Rhodnius* in which a histological and physiological division of the tubule into two regions has been described. In the case of the stick insect there is evidence of some gradation of properties along the length of the tubule, but nothing comparable with the abrupt transition which is seen in *Rhodnius*. Furthermore, the urine issuing from the proximal end of the stick insect tubule is alkaline to the haemolymph, whereas the rectal fluid is distinctly acid. If Wigglesworth's mechanism operates in the stick insect it seems that the process of acidification must take place in the gut. In the mosquito larva which has been allowed to ingest phenol red it is observed (Ramsay, 1950) that the intestinal fluid becomes acid about 1 min. after it has reached the rectum. One is tempted to suggest that in the majority of insects the process of acidification takes place in the gut, probably in the rectum, and that the arrangement in *Rhodnius* is an adaptation for dealing with the large volumes of urine which are produced immediately after a meal of blood.

SUMMARY

1. The excretion of calcium, magnesium, chloride, phosphate and hydrogen ions has been studied in preparations of single Malpighian tubules isolated in drops of serum under liquid paraffin.

2. The concentrations of calcium, magnesium and chloride are always lower in the urine than in the serum.

3. The concentration of phosphate is always greater in the urine than in the serum. As the concentration of phosphate in the serum increases, the rate of urine flow also increases.

4. The urine is always alkaline to the serum but becomes acid in the rectum.

5. The general problem of excretion by Malpighian tubules is briefly reviewed and discussed.

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