EXCRETION BY THE MALPIGHIAN TUBULES OF THE STICK INSECT, *DIXIPPUS MOROSUS* (ORTHOPTERA, PHASMIDAE): AMINO ACIDS, SUGARS AND UREA

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(*Received* 19 *July* 1958)

INTRODUCTION

In a series of papers (Ramsay, 1954, 1955*a*, *b*, 1956) the author has made a study of the excretion of inorganic ions by the Malpighian tubules of the stick insect. The only ion which is undoubtedly actively transported from haemolymph to urine under normal conditions is potassium, and on the basis of this fact it has been suggested that the secretion of potassium is, as it were, the prime mover in the process or urine formation, water and other ions following passively. It now becomes of interest to study the passage of organic molecules across the wall of the tubule. For organic substances information is available only in the case of uric acid, for which it is known that in the stick insect the concentration in the urine is greater than in the haemolymph (Ramsay, 1955*a*) and in respect of which an excretory mechanism has been proposed by Wigglesworth (1931) for *Rhodnius*.

Where the primary process of urine formation is ultrafiltration, as in the normal vertebrate nephron, the glomerular urine has the same composition as an ultrafiltrate of the blood and one would expect all solutes of low molecular weight present in the blood to appear in the urine unless they are specifically reabsorbed in the tubule. But in an aglomerular nephron, in which urine is formed by a process of secretion, one would not expect to find substances in the urine unless they are specifically excreted, and one would not expect to find excretory mechanisms specifically evolved in respect of metabolically useful constituents of the blood.

The Malpighian tubules of insects appear to have more in common with aglomerular than with glomerular nephrons of vertebrates, and for this reason one would not expect to find that they allow metabolically useful substances to pass into the urine. But, to anticipate, it is found that they do. One must therefore be prepared to readjust one's first conceptions of the role of the Malpighian tubules in the insect's physiological economy.

PRELIMINARY EXPERIMENTS

Very little is known about the uptake and translocation, let alone excretion, of organic substances in insects. It was therefore considered necessary to undertake certain preliminary investigations. The method used was a simple form of one-dimensional paper chromatography, with development in 80% propanol, and it is

to be understood that 'amino acids' refers to substances reacting with ninhydrin and that 'sugars' refers to substances reacting with aniline phthalate.

Gut contents. Amino acids were detectable in the fore-gut, present in quantity in the mid-gut and absent from the hind-gut. The contents of the fore-gut and mid-gut, but not of the hind-gut, gave a positive reaction with aniline phthalate, but separate spots were not recognizable on the chromatogram. After massive injection into the haemolymph glucose, but not glycine, was detectable in the faeces.

Gut exudate. Several experiments were carried out in which the gut of a well-fed insect was dissected out, tied off at mouth and anus and allowed to remain for some hours in a small volume of Ringer (for composition, see Ramsay, 1955*b*). The Ringer was then examined by chromatography. In some cases the fore-, mid- and hind-gut were tied off separately and studied in isolation.

Amino acids were easily detectable in the Ringer surrounding the whole gut, and when separate regions were studied it was found that nearly all the amino acids came from the mid-gut. In further experiments the separate regions of the gut were filled with Ringer containing 70 mM./l. glycine (made up with indian ink to reveal any gross perforation of the gut wall) and similarly tested. It was found that there was slightly more output of glycine from the mid-gut than from the fore-gut; the greatest output was from the hind-gut and was associated with an obvious decrease in the volume of the hind-gut contents. Glucose was similarly reabsorbed from the hind-gut.

Haemolymph. The haemolymph has a relatively high concentration of protein and peptides and easily detectable concentrations of such amino acids as alanine, glycine and valine. The amino acid pattern as seen in chromatograms from different insects is by no means constant, and it might be thought that the pattern corresponded to a steady state between absorption from the gut and assimilation by the tissues. To some extent this is true. In the haemolymph taken from an insect which has starved for a week alanine and valine are undetectable, but glycine and other amino acids as yet unidentified are scarcely diminished in concentration. The amino acids of the haemolymph are apparently maintained in balance with the proteins of the haemolymph and of the tissues, while also being affected by current absorption from the gut.

After the preliminary investigations had been completed Treherne (1958) showed that in the locust ^{14}C-labelled glucose absorbed from the gut appears in the haemolymph as the disaccharide trehalose, originally reported in insect blood by Wyatt & Kalf (1956). This matter was then investigated on the stick insect and the haemolymph was found to contain a substance which corresponded with trehalose on the chromatogram. ^{14}C-labelled glucose was injected into the haemolymph, which was collected about 12 hr. later, and it was found that practically all the activity was concentrated at the trehalose spot. The inadequate chemical characterization of trehalose in these preliminary investigations makes it impossible to speak with certainty; but it seems likely that rapid conversion of glucose to trehalose occurs in the stick insect as in the locust.

Urine. Urine was obtained from Malpighian tubules isolated singly or in pairs,

in drops of haemolymph as described elsewhere (Ramsay, 1954). Normal urine contains at least one amino acid but this has not been identified, nor its presence in haemolymph confirmed. Glycine, although present in haemolymph at a concentration of about 10 mM./l., is not detectable in normal urine by chromatography, but it can be detected in the urine if its concentration in the haemolymph is raised to about 70 mM./l. Glucose is not detectable even in haemolymph, but when added to haemolymph to give a concentration of about 70 mM./l. it is readily detectable in the urine. Trehalose is not detectable in the urine by chromatography.

These preliminary investigations indicate the following general conclusions, which must of course be regarded as provisional.

Sugars such as glucose are so rapidly metabolized that their concentrations in the haemolymph are normally very low; they are not presented to the excretory system in measurable concentration. When its concentration in the haemolymph is artificially increased, glucose is fairly rapidly excreted by the Malpighian tubules. Some of this glucose leaves the body with the faeces but most of it is reabsorbed from the hind gut.

Amino acids are present in the haemolymph in varying concentrations which in some cases can be related to the state of digestion. Most of these are not detectable in the urine, but glycine appears in the urine if its concentration in the haemolymph is raised. Like glucose it is reabsorbed from the hind-gut.

It therefore appears that, contrary to expectation, metabolically useful substances can pass into the urine; and this at once raises further questions. Is glycine, for example, normally present in urine but at a concentration too low to be detected by simple methods, or is its appearance in the urine a 'threshold' phenomenon related to a certain critical concentration in the haemolymph? Does the tubule simply allow a certain proportion of the glycine in the haemolymph to escape or does it act in such a way as to regulate the concentration of this substance in the haemolymph?

Answers to these and other questions are sought in the work now to be described.

MATERIAL AND METHODS

All the experiments herein to be described were carried out on the 'superior' tubules (de Sinéty, 1901). These were mounted as described earlier either singly or in pairs in small drops (about 25 μl. volume*) of medium under liquid paraffin. Collection was continued until sufficient volume of urine had been produced; after 12 hr. volumes of the order of 1 μl. were obtainable.

Although quantitative chemical techniques have been worked out for most of the substances studied in the present work, these techniques are generally more laborious than techniques involving the use of radioactive labelling. Many ^{14}C-labelled organic compounds of physiological interest are now available but at such cost that some restriction must be placed upon full freedom of choice. From among the available amino acids six were selected—alanine, arginine, glycine, lysine, proline

* In earlier papers volumes have been given in 'mm.^{3}' and 'mm.$^3 \times 10^{-3}$'. In the present paper these measures will be written 'μl.' and 'm μl.' respectively.

and valine—largely on account of their high solubility in water which made it possible to test them over a wide range of concentration. From among the carbohydrates, glucose, fructose and sucrose were selected. In additon to these two classes of compound urea was also included in the investigation.

If it is desired to obtain a relatively high concentration of the organic substance in the medium without at the same time raising the osmotic pressure it follows that the other constituents of the medium must be correspondingly diluted. The possibility therefore exists that observed effects upon the secretion of urine, which might be attributed to the presence of the specified organic substance, could equally be due to the dilution of some other constituent normally present. This consideration applies with special force to potassium, the rate of urine flow being greatly affected by the concentration of this ion in the medium. For this reason the concentration of potassium was kept approximately constant in the various media used (except where stated otherwise) and the other constituents were allowed to suffer dilution.

The standard method of making up media can be most easily described by taking a particular example. The Ringer used for making up media differed from the usual mixture in that no sucrose was added; the other constituents were uniformly concentrated so as to give a freezing-point depression of $\Delta = 0{\cdot}55°$ C. and a potassium concentration of 21 m.equiv./l. (solution A). 1·06 mg. of ^{14}C-alanine, having an activity of 0·05 mc., was dissolved in 1 ml. of solution A, giving a concentration of alanine of approximately 12 mM./l. (solution B). Solution C contained 262 mM./l. of carrier alanine and 20 mM./l. KCl, and had a freezing-point depression of $\Delta = 0{\cdot}56°$ C. The stick insect serum used in this work contained approximately 10 mM./l. alanine and 18 m.equiv./l. potassium, and will be referred to as solution D. These solutions were mixed as shown in Table 1.

Table 1

Solution A: Ringer; K, 21 m.equiv./l.
B: Ringer; K, 21 m.equiv./l., ^{14}C-alanine, 12 mM./l.
C: Carrier; K, 20 m.equiv./l., alanine, 262 mM./l.
D: Serum; K, 18 m.equiv./l., alanine, 10 mM./l.

Medium	A (ml.)	B (ml.)	C (ml.)	D (ml.)	Nominal concentrations	
					Alanine (mM./l.)	Potassium (m.equiv./l.)
I	0·3	0·1	0·0	0·1	4·4	20
II	0·2	0·1	0·1	0·1	56	20
III	0·1	0·1	0·2	0·1	109	20
IV	0·0	0·1	0·3	0·1	161	20

In this way four media were prepared having different concentrations of alanine but having the same concentration of potassium and the same activity, this latter being the lowest activity convenient for conventional counting procedures with the volumes of urine available.

For experiments of 24 hr. duration it was found necessary to add an antibiotic to the medium, and for this terramycin was used, penicillin having been found

to be ineffective. The pH of all media was adjusted to 6·7 by addition of NaOH and was checked at the beginning of each experiment.

The design of experiments—and, even more, their interpretation—is complicated by the fact that many organic substances are metabolized as they pass through the tubule. When chromatographic separation was performed on the urine it was sometimes found that the activity was not exclusively associated with the spot given by the labelled substance. This was more noticeable, as one would expect, when the labelled substance was present in low concentration. When the medium was tested in the same way the activity was found to be confined to the area of the spot, which is again as one would expect, since the total amount of labelled substance present in the medium was very much greater than the total amount present in the urine and a smaller proportion of the amount in the medium—if, indeed, any—would be metabolized. Nevertheless, some doubt was felt about this interpretation since in chromatograms of urine the activity not associated with the spot was sometimes rather diffusely spread as if something was present in urine which interfered with the normal running of the chromatogram; and this, rather than metabolization, might account for the spread of the activity.

To put this to the test the following experiment was carried out using valine. Eight tubules were set up in a medium (*X*) containing labelled valine in low concentration but high specific activity and eight tubules were set up in normal serum (*Y*). Urine collected from *X* and from *Y* were pooled separately. To urine *X* inactive carrier valine was added. To urine *Y* inactive carrier valine and labelled valine were added to bring the total concentration and the activity to approximately the same values as in *X*. *X* and *Y* prepared in this way both contained labelled valine; but in *X* the labelled valine had passed through the tubule, whereas in *Y* it had not. If the spread of activity on the chromatogram were due to interference it should occur equally in *X* and *Y*, but if due to metabolization it should appear in *Y* only. In the event 74% of the total activity in *X* was concentrated at the valine spot, and 97% in *Y*. It was therefore concluded that the spread of activity in *X* could not be attributed to interference from substances present in urine but must be due to metabolization of the labelled valine during this passage through the tubule.

It is possible to assess the excretion of a labelled substance in two ways: (1) by counting the urine directly, which will give the total activity and a measure of the amount of labelled substance removed from the medium: and (2) by performing a chromatographic separation on the urine and then counting the activity associated with the appropriate spot, which will give a measure of the amount of labelled substance finally present in the urine. For most organic substances studied in this investigation both methods were used, and of course where mixtures were involved only the second method was practicable. Chromatograms were developed with 80% propanol–water or with butanol–acetic acid. The strips were first scanned under the counter and the appropriate active areas were cut out; each area was then eluted and eluate was evaporated to dryness on a counting planchette. In all experiments the count rate of a certain volume of urine was compared with the count rate of

the same volume of medium and, following the convention of vertebrate physiology, was expressed as the U/P ratio for the substance in question, where

$$\frac{U}{P} = \frac{\text{count rate of urine}}{\text{count rate of medium}}.$$

By reason of the cost of ^{14}C-labelled compounds the experiments were planned to give the lowest counting rates consistent with the required accuracy. With the direct method (i.e. not involving chromatographic separation) relatively small samples were taken with a pipette of approximately 0·35 μl. capacity and were transferred directly to counting planchettes, evaporated to dryness and counted. The error of the whole process of sampling and counting was found to be of the order of $\pm 7\%$ (standard deviation as % of the mean). Since the corresponding figure for the variation between urine samples taken under identical experimental conditions was of the order of $\pm$ 15% the analytical error was considered acceptable. In these experiments the rate of urine flow was also recorded, the volume of urine produced being found by measuring the diameter of the droplet while it was allowed to sink through liquid paraffin. The error of this method is within $\pm$ 10% of volume, which is again acceptable in view of variations in the observed rates of flow. When chromatographic separation, involving scanning of the paper, was carried out it was necessary to use larger quantities. Eight tubules were set up in each medium and the urine collected over a period of about 12 hr. was pooled. Samples of urine and medium were taken with a pipette of approximately 5 μl. capacity. When the active areas were subsequently eluted and counted the count rates were of course much higher and the counting error correspondingly reduced. It is estimated that the total analytical error in these determinations was of the order of $\pm 4\%$.

RESULTS

Amino acids

In the first series of experiments the direct method was used. The results of these experiments are summarized in Table 2, upon which the following comments may be made.

(i) All these substances appear in the urine; since they are all to be regarded as metabolically useful, this is in itself mildly surprising.

(ii) The U/P ratio is virtually independent of the concentration of the substance in the medium; this statement will appear to conflict with the evidence in Table 2 and must receive further explanation. The figures show that for all the amino acids (except lysine, for which no urine was produced at the higher concentrations) there is a tendency for the U/P ratio to increase with increasing concentration in the medium; this tendency is statistically highly significant ($P < 0{\cdot}01$) for alanine, glycine and proline and is not significant ($P > 0{\cdot}05$) for arginine and valine. But the increase in the U/P ratio is in all cases very small compared with the increase in the amino acid concentration in the medium. In the case of glycine the range of concentration in the medium is represented by a factor of 37, whereas the range of the U/P ratio is represented by a factor of 3·2; the factor for the medium is more than

Table 2

(Mean ± standard deviation (no. of observations). Amino acids as specified in catalogue of Radiochemical Centre, Amersham. *P*, concentration in medium, mM./l.; *U*, concentration in urine, mM./l.; *R*, rate or urine flow, mμl./min.)

Amino acid	*P*	*U/P*	*R*
DL-Alanine-1-^{14}C	4·4	0·23 ± 0·029 (9)	1·69, 0·24 (9)
	56	0·25 ± 0·048 (10)	1·45 ± 0·41 (10)
	109	0·32 ± 0·041 (6)	1·11 ± 0·43 (6)
	161	0·37 ± 0·061 (9)	1·19 ± 0·30 (9)
L-Arginine-^{14}C(G)	0·34	0·39 ± 0·07 (4)	1·34 ± 0·14 (4)
	53	0·35 ± 0·09 (4)	1·29 ± 0·21 (4)
	105	0·40 ± 0·19 (4)	1·09 ± 0·29 (4)
	158	0·62 ± 0·29 (4)	0·40 ± 0·01 (4)
Glycine-^{14}C(G)	4·5	0·14 ± 0·03 (4)	Not recorded
	43	0·17 ± 0·05 (7)	Not recorded
	82	0·22 ± 0·03 (6)	Not recorded
	121	0·27 ± 0·05 (5)	Not recorded
	166	0·45 ± 0·07 (4)	Not recorded
L-Lysine-^{14}C(G)	0·15	0·13 (1)	Not recorded
L-Proline-^{14}C(G)	0·16	0·22 ± 0·03 (4)	1·22 ± 0·54 (4)
	26	0·55 ± 0·08 (4)	2·25 ± 0·07 (4)
	52	0·59 ± 0·04 (4)	1·43 ± 0·54 (4)
	105	0·58 ± 0·13 (7)	1·94 ± 0·57 (7)
	157	0·63 ± 0·09 (4)	1·21 ± 0·75 (4)
DL-Valine-1-^{14}C	6·3	0·24 ± 0·04 (8)	2·72 ± 0·42 (8)
	59	0·24 ± 0·04 (8)	2·36 ± 0·64 (7)
	111	0·23 ± 0·04 (8)	2·44 ± 0·61 (8)
	163	0·25 ± 0·05 (8)	2·40 ± 0·53 (7)

10 times greater than the factor for the *U/P* ratio. In all the other cases the contrast is even greater. When it is remembered that in order to achieve the highest concentrations of amino acid it has been necessary drastically to lower the concentrations of other constituents of the medium it would seem unlikely that the apparent increase in the *U/P* ratio is attributable to a direct effect of the concentration of the amino acid in the medium. The more striking fact is that not withstanding the very high and unphysiological concentrations which have been used one does not seem to have approached any limit to the capacity of the tubule to remove the amino acid from the medium—there appears to be no T_m value such as can be found for the transport of substances by the vertebrate nephron. One must also conclude that there is no evidence for any 'threshold' concentration below which these substances are not excreted and no evidence that the tubule acts so as to regulate their concentrations in the haemolymph.

(iii) The *U/P* ratios are different for the different amino acids being notably higher for proline than for the others.

(iv) There is a tendency for the rate of urine flow to decrease as the concentration of the amino acid in the medium increases. This tendency is statistically significant in the case of alanine and arginine, but not in the case of proline and valine.

The second series of experiments was designed to reveal any competition or other interaction between amino acids when presented to the tubule in a medium

containing three amino acids. Two such mixtures were made up, having regard to ease with which the components could be separated on a chromatogram; these were (*a*) arginine, proline and valine, and (*b*) alanine, glycine and valine. The mixtures were made from equal volumes of type IV media and thus contained each of the amino acids in 50–56 mM./l. concentrations. As controls the same three amino acids were tested in type II media in which they were present alone and in the same range of concentration. In each experiment four drops of each of the four media (mixture and three separates) were used and two tubules were set up in each drop, thirty-two tubules in all. Urines from all eight tubules in each medium were pooled and aliquots of 5 μl. were spotted on paper, developed, eluted, dried and counted as described under Methods. The results of these experiments are given in Table 3. From these results it appears that the *U/P* ratios for the amino acids

Table 3

		U/P (mixture)	*U/P* (separate)
Group 1.	Arginine	0·30	0·25
	Proline	0·76	0·56
	Valine	0·29	0·20
2.	Alanine	0·22	0·24
	Glycine	0·32	0·19
	Valine	0·24	0·21

presented separately are very much the same as those seen in Table 2. In the case of the mixtures the *U/P* ratios are in general higher, notably in the case of glycine and proline. While this may be taken as evidence of interaction it may equally be related to the observation discussed above, namely, that the *U/P* ratio tends to increase with increasing amino acid concentration in the medium. There is certainly no evidence of competitive inhibition affecting the excretion of one amino acid in the presence of another.

Since for all the amino acids here studied the concentration in the urine is less than that in the medium, it is unnecessary to postulate active transport of the unionized molecules. At pH 6·7 alanine, glycine, proline and valine are on the alkaline side of their iso-electric points and therefore will be negatively charged; since the interior of the tubule is positive with respect to the exterior (Ramsay, 1955*b*) it follows, *a fortiori*, that it is unnecessary to postulate active transport of the ions. The reverse is true of arginine, and from a purely thermodynamic stand point its transport may be active; but one may note that its *U/P* ratio is not widely different from the *U/P* ratios of alanine, glycine and valine, which suggests that the same type of transport mechanism is operating in all cases.

If we are to take the view that all these amino acids are transported independently, each by its own specific transport mechanism, then we must concede that these transport mechanisms have capacities far in excess of those which are normally exercised in the body of the insect. The simpler view is that amino acids enter the urine by a passive, non-specific process.

Sugars and urea

The sugars tested were glucose, fructose and sucrose, and the experiments were carried out on the same lines as those just described for amino acids. The results obtained with single sugars are presented in Table 4, and the results obtained with urea are included in this table for convenience. Once again it is seen that all these substances appear in the urine. For the sugars the U/P ratios at the lowest values of P are significantly lower than at the three higher concentrations and this discrepancy is tentatively ascribed to metabolization. For glucose and fructose the U/P ratio does not change significantly with concentration in the range 53–158 mM./l. In the case of sucrose there is a small but significant increase in the U/P ratio over the same range as with certain amino acids. In the case of urea at all concentrations the U/P ratio does not differ significantly from 1, which is not surprising in view of the relative ease with which this substance is known to penetrate animal cells.

Table 4

(Mean ± standard deviation (no. of observations). Labelled compounds as specified in catalogue of Radiochemical Centre, Amersham. P, concentration in medium, ml./l.; U, concentration in urine, mM./l.; R, rate of urine flow, mμl./min.)

Labelled Compound	P	U/P	R
D-Glucose (G)	0·17	0·32 ± 0·05 (5)	1·34, 1·46
	53	0·86 ± 0·18 (6)	2·04 ± 1·29 (4)
	105	0·90 ± 0·14 (6)	2·14 ± 1·04 (4)
	158	0·85 ± 0·07 (6)	1·59 ± 0·73 (4)
D-Fructose (G)	0·18	0·45 ± 0·06 (3)	1·50 ± 0·09 (3)
	53	0·61 ± 0·08 (3)	1·15 ± 0·47 (3)
	105	0·65 ± 0·07 (3)	0·87 ± 0·26 (3)
	158	0·56 ± 0·07 (3)	0·91 ± 0·47 (3)
Sucrose (G)	0·15	0·47 ± 0·07 (6)	2·63 ± 0·59 (6)
	53	0·53 ± 0·10 (6)	2·86 ± 0·52 (6)
	105	0·64 ± 0·03 (4)	2·08 ± 0·20 (4)
	158	0·71 ± 0·06 (5)	1·63 ± 0·09 (3)
Urea	3·1	1·03 ± 0·06 (6)	3·11 ± 1·90 (6)
	56	0·96 ± 0·06 (5)	3·95 ± 0·88 (5)
	108	0·99 ± 0·07 (6)	5·09 ± 2·04 (5)
	160	0·99 ± 0·03 (6)	5·62 ± 2·56 (6)

Experiments were also carried out with mixtures of glycine, glucose and urea, each substance being present in concentration of the order of 50 mM./l. The U/P ratios found in these experiments are presented in Table 5 together with the U/P ratios for the same susbtances when present alone. These latter figures are taken from Tables 2 and 4; in this part of the work the somewhat arduous task of simultaneous comparison, involving the rapid preparation of thirty-two tubules, was not attempted. Once again, the U/P ratio for each substance in the mixture is of the same order as the U/P ratio for the same substance alone. Urea appears to stimulate an increased rate of urine flow.

The general conclusions reached for amino acids appear to be equally applicable to the substances considered in the present section.

Table 5

(Mean ± standard deviation (no. of observations). The figures for U/P (separate) are taken from Tables 2 and 4, using values of P nearest to 50 mM./l.)

	U/P (mixture)	U/P (separate)
Glycine	0·24 ± 0·04 (3)	0·17 ± 0·05 (7)
Glucose	0·95 ± 0·10 (4)	0·86 ± 0·18 (6)
Urea	0·92 ± 0·09 (4)	0·96 ± 0·06 (5)

EXCRETION AS A PROCESS OF DIFFUSION

For all the substances here investigated it has been found that the U/P ratio remains fairly constant and independent of the value of P. This is to be expected if these substances enter the tubule by passive diffusion.

If a simple process of diffusion is at work these substances should be able to pass equally easily through the wall of the tubule in the reverse direction. Rapid diffusion in the reverse direction has been demonstrated for urea and for sucrose in experiments of the following type. The main part of the tubule was placed in a drop

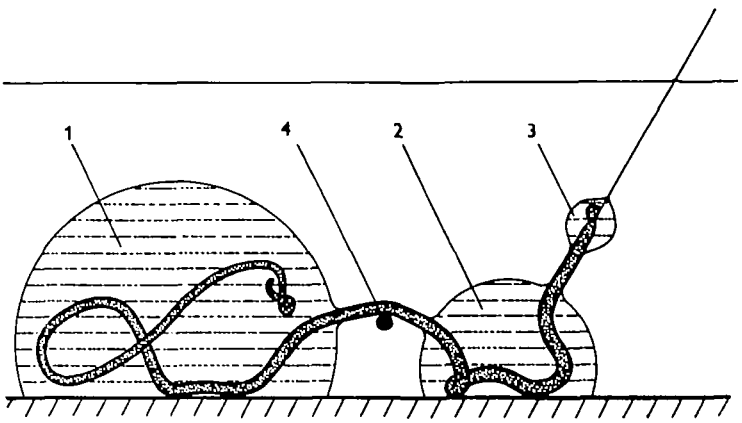

Fig. 1. Arrangement for demonstrating permeability of tubule in both directions. 1, large drop of medium containing labelled substance; 2, small drop of medium from which substance initially absent; 3, droplet of urine; 4, platinum wire support. All under liquid paraffin.

of medium containing the labelled substance, and the proximal end was arranged to pass through a second drop of medium in which the substance was not present (Fig. 1). After about 4 hr. the urine and the second drop of medium were collected entire and counted. From the results:

	Count of second drop (c.p.m.)	Count of urine (c.p.m.)
Urea	758	40
	691	39
Sucrose	524	17

it is obvious that most of the substance which might have been excreted in the urine has passed back through the wall of the tubule into the second drop. Both substances therefore can pass the tubule wall in both directions, although it has not been established that their rates in the two directions are the same. The other substances with which this paper is concerned were not tested.

The argument for the diffusion theory may be further tested by examining the effects of alterations in the rate of urine flow upon the U/P ratio. The rate of urine flow may be altered either (i) by altering the rate at which natural urine is formed by secretion through the walls of the tubule, or (ii) by perfusing the tubule with artificial urine. As will now be shown, the relationship between rate of flow and U/P ratio is different in the two cases.

We will represent the tubule as a tube of length L and radius r, closed at one end, and we will regard the wall of the tubule as a membrane of negligible thickness. We will suppose that water is actively pumped through unit area of wall into the lumen at a rate denoted by a. If R is the rate at which urine issues from the tubule, $R=2\pi rLa$.

We will further suppose that a substance dissolved in the plasma at concentration P diffuses into the lumen of the tubule where it attains concentration U. The rate of diffusion will be proportional to the area of the wall of the tubule and to the concentration difference. If X is the quantity of the substance which enters the tubule in unit time, we have

$$X=b.2\pi rL(P-U),$$

where b is constant. The concentration of the substance in the urine is

$$U=\frac{X}{R}=\frac{X}{2\pi rLa}.$$

Substituting for X we obtain

$$\frac{U}{P}=\frac{b}{a+b}. \tag{1}$$

Equation (1) shows that the U/P ratio is independent of P, as the experimental results have indicated. Since the same relationship could have been derived by considering an element of the tubule wall instead of the whole tubule it follows that U has the same value at all points in the lumen of the tubule and that the speed with which the stream of urine passes along the lumen is irrelevant. This equation defines the effect upon the U/P ratio of alterations in the rate of secretion of natural urine.

We will now consider a tubule which is perfused with artificial urine at a rate v and will first seek to establish the time taken for a particle of urine to traverse the length of the tubule. For this purpose we will assume that over any cross-section the speed is uniform. If the tubule is not secreting natural urine then the speed of the stream is

$$\frac{dl}{dt}=\frac{v}{\pi r^2},$$

and the time taken to traverse the tubule is

$$t=\frac{\pi r^2 L}{v}.$$

But if the tubule is secreting urine then over a distance l from the point of injection a volume of natural urine equal to $2\pi rla$ will be added to the artificial urine. The speed of the stream as it passes the cross-section at l is

$$\frac{dl}{dt}=\frac{v+2\pi rla}{\pi r^2},$$

and the time taken to traverse the tubule is

$$t'=\pi r^2 \frac{1}{2\pi ra}\log_e\left(\frac{v+2\pi rLa}{v}\right).$$

As v becomes very much greater than $2\pi rLa$, t' approximates to t and it can be calculated that if $v=10\times 2\pi rLa$ then $(t-t')/t\times 100=4{\cdot}8\,\%$, an error which may be accepted in relation to the errors of observation.

We will therefore assume that, as a result of perfusion, elements of volume traverse the tubule in time $t=\pi r^2 L/v$, during which time the substance diffuses into them. The same results would follow if the tubule were instantaneously filled with perfusion fluid, left for time t and then instantaneously emptied. The rate of entry of the substance will be proportional to the area of the wall of the tubule and to the concentration difference, so that

$$X=b.2\pi rL(P-U)$$

as before, or alternatively,

$$\frac{dQ}{dt}=b.2\pi rL(P-U).$$

Since $U=Q/\pi r^2 L$,

$$\frac{dU}{dt}=b\frac{2}{r}(P-U),$$

which on integration becomes

$$\frac{U}{P}=1-\exp\left[-\frac{2b}{r}t\right].$$

Putting $t=\pi r^2 L/v$,

$$\frac{U}{P}=1-\exp\left[-\frac{b.2\pi rL}{v}\right]. \qquad (2)$$

Before proceeding to test equations (1) and (2) against the observations it is first necessary to define the units to be used. Time will be measured in minutes and length in millimetres; volumes are therefore measured in μl. Rates of urine flow have been quoted in mμl./min. and must therefore be multiplied by 10^{-3}. The constants a and b have dimensions μl. mm.$^{-2}$ min.$^{-1}$. It is unnecessary to define units of concentration since concentrations appear as the ratio U/P. It is also necessary to define a standard tubule. Tubules differ in diameter in different regions; in the proximal region the external diameter is of the order of 100 μ and the internal diameter of the order of 50 μ in a normal state of distention. The theoretical

treatment considers the wall of the tubule as being of negligible thickness. As a bold approximation the standard tubule will be defined as having a length of 20 mm. and a radius of 0·04 mm.

In the theoretical treatment the assumption was made that over any cross-section of the tubule the concentration of the diffusing substance is uniform. But in fact the concentration will be greater near the walls because (i) the substance is diffusing inwards through the walls, and (ii) the speed of the stream is greatest at the axis. The case of diffusion into an infinite cylinder has been treated by Hill (1928), and his paper provides a graph from which it can be ascertained that 90% of the equilibrium value for the concentration within the cylinder is attained in a time given by

$$t=\frac{0\cdot34r^2}{k},$$

where r is the radius of the cylinder and k is the coefficient of diffusion in cm.²/min.

In what follows it will be seen that the worst case is that of an experiment with urea in which a rate of perfusion of 0·625 μl./min. was used. For urea in water the coefficient of diffusion is 0·97 cm.²/day (*Handbook of Physics and Chemistry*). Taking r as 0·004 cm. we find $t=0\cdot485$ sec. The volume of the standard tubule is 0·1 μl. so that under perfusion at 0·625 μl./min. the time taken to traverse the tubule is 9·6 sec. If the flow within the tubule is laminar then the speed on the axis is twice the average speed. Putting the worst case in the worst possible way we can say that the time taken to traverse the tubule cannot be less than 4·8 sec. This exceeds the time taken to reach 90% equilibrium by a factor of almost 10, and for this reason the assumption in question is considered to be justifiable.

(1) *Effect of altering the rate of secretion of natural urine.* The rate of secretion of natural urine may be altered by altering the concentration of potassium in the medium; but since the permeability of the tubule to other substances is likely to be altered by changes in the balance of monovalent and divalent cations, the method must be used with circumspection. Both increase and decrease of the normal rate can be achieved by this method and it follows that the substance to be studied should have a normal U/P ratio of 0·5 if the test is to be performed to best advantage. For this reason sucrose was chosen. Media were made up as indicated in Table 6, from which it can be seen that the total concentration of monovalent cations is approximately the same in all media.

Table 6

(Solution X: 1 part serum, 1 part 300 mM./l. labelled sucrose; Y: Ringer having potassium replaced with sodium; Z: Ringer having sodium replaced with potassium; R: Ringer.)

Medium	Sucrose (mM./l.)	Na (m.equiv./l.)	K (m.equiv./l.)	Na+K (m.equiv./l.)
(1) 1 pt. X, 1 pt. R	75	15	15	30
(2) 1 pt. X, 1 pt. Y	75	25	4·5	29·5
(3) 1 pt. X, 1 pt. Z	75	2·75	27	29·75

The results of experiments using these media are presented in Table 7. For each pair of observations the constant b has been calculated for the standard tubule and the average value of b is $3{\cdot}4 \times 10^{-4}$. Using this value of b the curve of equation (1) is plotted in Fig. 2 with the observed values for comparison. The observations are not incompatible with this curve, but it is clear that in relation to the variation between one tubule and another the range of R achieved is insufficient to test the fit of the observations.

Table 7

Medium	U/P	R	b
(1)	0·47	2·0	$3{\cdot}54 \times 10^{-4}$
	0·47	1·6	2·84
	0·50	2·3	4·58
	0·47	1·9	4·28
(2)	0·49	0·65	1·23
	0·84	0·43	4·52
	0·82	0·48	4·37
(3)	0·35	2·7	2·91
	0·38	2·5	3·04
	0·35	2·4	2·58
			Av. $3{\cdot}39 \times 10^{-4}$

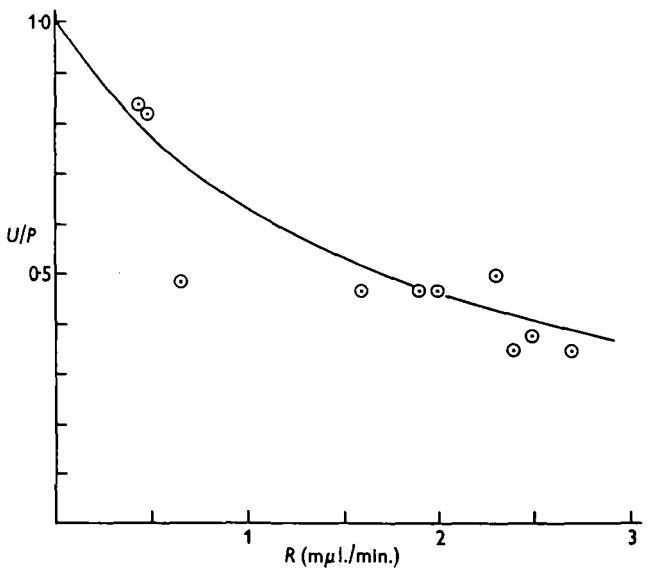

Fig. 2. The relation between the U/P ratio for sucrose and the rate of secretion of natural urine.

(2) *Effect of perfusing with artificial urine.* The injection of artificial urine into tubules at controlled rates of the order of mμl./min. presents certain technical problems. The obvious alternatives are either: (i) to use a mechanically driven syringe, or (ii) to use a variable head of pressure in conjunction with an appropriate resistance. The problems of design and construction arising under (i) appeared

formidable and effort was therefore directed towards the second alternative. A pipette which is suitable for penetrating the tubule has a relatively low resistance and the pressures necessary to produce the requisite rates of flow are comparable with the pressures necessary to overcome the effects of surface tension in the system. An adequate resistance was achieved by the use of liquid paraffin in a fine capillary, the paraffin acting as a piston to drive the artificial urine through the pipette. The

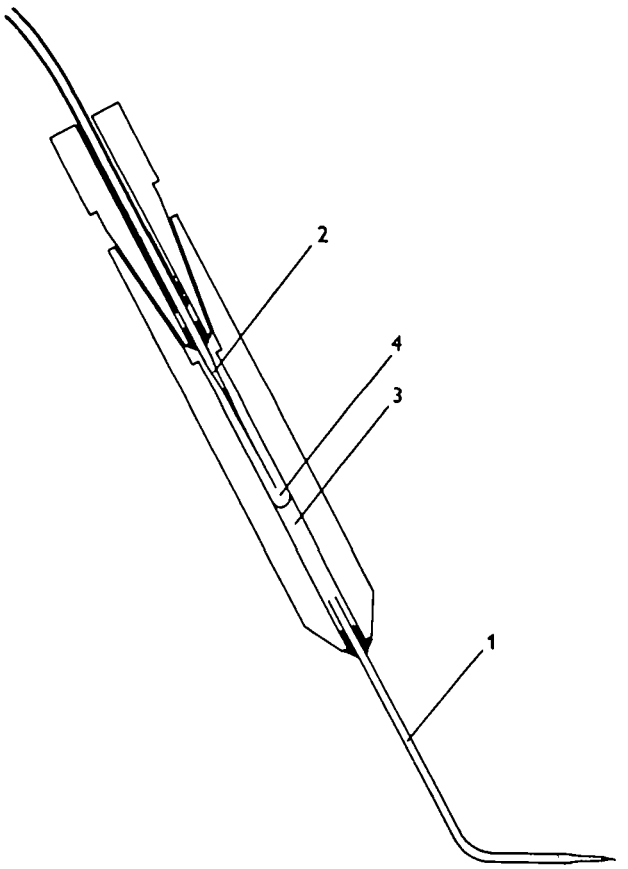

Fig. 3. Device for controlling rate of perfusion. 1, pipette for insertion into tubule; 2, fine capillary providing high resistance; 3, artificial urine; 4, liquid paraffin.

form of the injection apparatus is shown in Fig. 3. The silica pipette which penetrated the tubule was sealed into one end of a Perspex tube whose other end was provided with a tapered stopper. Into the stopper was sealed another silica tube, drawn out into a long fine point and connected to a nylon tube; these were filled with liquid paraffin. With precautions to exclude air bubbles the Perspex tube and pipette were partly filled with artificial urine; liquid paraffin was then added above the artificial urine and the stopper was placed in position. The other end of the nylon tube was connected to a screw-plunger device containing mercury and attached

to a mercury manometer. The Perspex tube was mounted on a micro-manipulator which is described elsewhere (Ramsay, 1953).

The artificial urine had the following nominal composition:

Sodium	5 m.equiv./l.	Phosphate	35 m.equiv./l.
Potassium	150	Chloride	150
Magnesium	30		

The tubule was set up in the usual way in a drop of medium containing the labelled substance, under liquid paraffin. The pipette was inserted at the distal end of the tubule and the perfusate was removed from the proximal end as it accumulated. After a suitable period of perfusion the volume of perfusate was estimated by measuring the diameter of the drop suspended in liquid paraffin, and the whole of the perfusate was taken for counting. The perfusion method has this advantage over the natural urine method that several observations at different rates of flow can be made on a single tubule.

Table 8. *Perfusion of tubule in medium containing labelled sucrose*

Tubule	Activity of medium, P, c.p.m./mμl.	Time of perfusion, t, min.	Volume of perfusate, V, mμl.	Rate of perfusion, $R=V/t$	Count rate of perfusate, N, c.p.m.	Activity of perfusate, $U=N/V$	U/P	b	Equation
1	1·17	120	310	2·58	195	0·63	0·54	$6·0\times10^{-4}$	(1)
		20	1190	59·5	53	0·044	0·038	$4·6\times10^{-4}$	(2)
		240	270	1·12	240	0·89	0·76	$7·1\times10^{-4}$	(1)
		30	2150	71·6	62	0·029	0·025	$3·6\times10^{-4}$	(2)

The substance first studied by this method was sucrose and the results of one experiment are shown in Table 8. High and low rates of perfusion were compared, but the low rates are within the range of the rates of natural urine flow and it is probable that there was no perfusion under these conditions. Values for b were therefore calculated using equations (1) and (2) as appropriate, and are given in the table. As will be seen, the values are of the same order of magnitude, but the agreement is not good.

To realize the full potentialities of the perfusion method one should use a substance which penetrates rapidly so that useful values of the U/P ratio correspond to rates of perfusion which are many times greater than the normal rate of secretion. Of the substances used in this investigation the choice obviously falls upon urea. Table 9 and Fig. 4 give the results of experiments with this substance. The point on the ordinate was obtained from natural urine; for all the other points the rate of perfusion was more than 20× the normal rate of secretion. The curves of equation (2) are drawn for the average value of b in each experiment. For a total of four tubules tested the mean value (± S.D.) for b was found to be $4·5\pm2·5\times10^{-2}$.

It is of passing interest to compare the wall of the tubule with other natural membranes in respect of permeability to dissolved sustances. Davson & Danielli (1952) give figures in terms of litres.μ^{-2}.sec.$^{-1}$ to which the present figures for b

may be converted by multiplying by $1{\cdot}67 \times 10^{-14}$. For urea the value of $b = 4{\cdot}5 \times 10^{-2}$ is used. For sucrose the value of $b = 6{\cdot}7 \times 10^{-4}$ can be derived from the data in Table 4, using equation (1). The comparison is set out in Table 10, from which it is seen that the wall of the tubule is among the most permeable of natural membranes.

Table 9. *Perfusion of tubule in medium containing labelled urea*

(In all cases the concentration of urea in the medium was 53 mM./l.)

Tubule	Activity of medium, P, c.p.m./mμl.	Time of perfusion, t, min.	Volume of perfusate, V, mμl.	Rate of perfusion, $R = V/t$	Count of perfusate, N, c.p.m.	Activity of perfusate, $U = N/V$	U/P	b
1	0·312	30	1965	65	621	0·316	1·01	—
		15	2850	190	615	0·216	0·69	$4{\cdot}4 \times 10^{-3}$
		5	3125	625	303	0·097	0·31	$4{\cdot}6 \times 10^{-3}$
2	2·50	145	108	0·75	250	2·31	0·92	—
		5	470	94	609	1·30	0·52	$1{\cdot}4 \times 10^{-3}$
		5	1020	204	824	0·808	0·32	$1{\cdot}6 \times 10^{-3}$
		5	2470	494	1034	0·419	0·17	$1{\cdot}8 \times 10^{-3}$
3	0·297	10	2060	206	521	0·253	0·85	$7{\cdot}8 \times 10^{-3}$
4	0·297	30	1980	66	568	0·287	0·96	$4{\cdot}2 \times 10^{-3}$

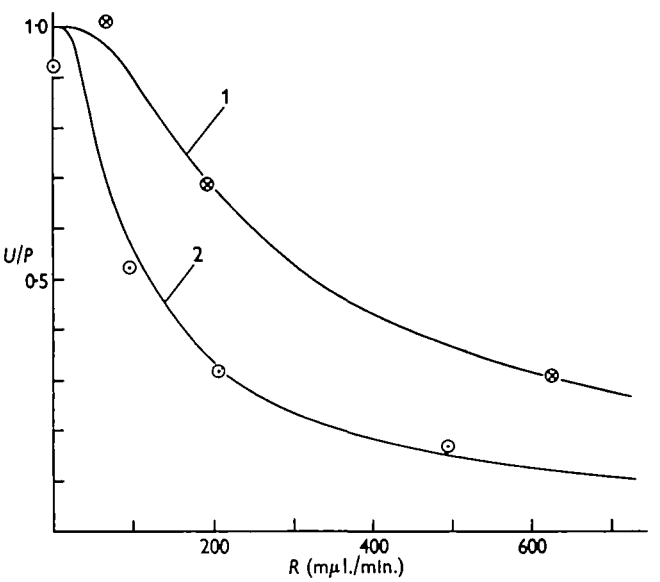

Fig. 4. The relation between the U/P ratio for urea and the rate of perfusion with artificial urine. Tubules 1 and 2 of Table 9.

Table 10. *Permeability of natural membranes of urea and sucrose*

(Units: litres.μ.$^{-2}$sec.$^{-1}$. All figures should be multiplied by 10^{-10}.)

Membrane or organism	Urea	Sucrose
Ox erythrocyte	7·8	–
Beggiatoa mirabilis	1·58	0·14
Chara ceratophylla	0·11	0·0008
Plagiothecium denticulatum	0·0036	0·000008
Dixippus, Malpighian tubule	7·5	0·11

All figures, except for *Dixippus* Malpighian tubule, from Davson & Danielli (1952).

DISCUSSION

The evidence for the proposition that the organic substances used in this investigation enter the tubule by diffusion may be summarized as follows:

(i) The U/P ratio is never greater than 1 (within the limits of experimental error).

(ii) The U/P ratio characteristic of each substance is largely independent of P.

(iii) The effects of rate of flow upon the U/P ratio are in accordance with expectation.

(iv) There is no interference between different substances.

Other possible modes of excretion may be shown to be incompatible with this evidence. Let us suppose, for example, that:

(*a*) The tubule transports in unit time a quantity, X, of substance, proportional to P. This gives a U/P ratio independent of P. The relation between U/P and R is

$$\frac{U}{P}=\frac{X}{R}.$$

If this were true then U/P should exceed 1 at low values of R, e.g. for sucrose (Table 7) at values of $R<0{\cdot}85$, which it obviously does not.

(*b*) The tubule allows the substance to diffuse in very rapidly at some places and reabsorbs it at others; if U/P is to be independent of P it is necessary to suppose that a quantity, X', proportional to P, is reabsorbed in unit time. On this theory an increase in the flow of natural urine would result in an increase in the U/P ratio, which is contrary to observation (Fig. 2).

It is clear that any alternative explanation which accounts for the observations is bound to be somewhat complicated, and in the absence of evidence to the contrary the truth of the proposition may be assumed.

Limiting rates of entry do not seem to have been reached under any of the experimental conditions tested, although these involved abnormally high concentrations and rates of flow. This, and also the absence of interference, suggest that the process is more likely to be one of simple diffusion rather than one of passive transport involving carrier molecules limited in number. On the other hand, the differences in rate of penetration as between one substance and another cannot be reconciled with any simple theory of membrane structure, e.g. the larger molecule of sucrose

(M.W. 342) penetrates more than three times as fast as the smaller molecule of glycine (M.W. 75). Lison (1942) was likewise unable to interpret the excretion of dyes by Malpighian tubules in terms of any well-known physico-chemical property.

In relation to the wider field of the physiology of excretion in insects the most significant observation is that to which reference was made in the Introduction, namely, that metabolically useful substances appear in the urine and can be reabsorbed in the rectum. Elementary text-books adhere to the traditional view in ascribing the excretory function in insects to the Malpighian tubules alone; a more enlightened view recognizes that the rectal glands can enter into the process of excretion by reabsorbing certain constituents of the urine, such as water and salts, and returning them to the haemolymph. The requirement for a brisk circulation through the tubule is obvious when one considers that solid particles, e.g. of uric acid, can only be removed from the tubules by being flushed out with a stream of water; in the rectum, an organ provided with muscles enabling solid contents to be evacuated, it is possible for the water to be almost completely removed. The requirement for a circulation of water and salts is easily understood, but it is not at once obvious that any advantage would accrue from committing metabolically useful substances to this circulation.

It may be permitted at this point to consider the principles of design that are applicable to excretory systems. Granted that a stream of water through the system is provided, either (i) we may secrete unwanted substances into the stream, or (ii) we may let all substances, wanted and unwanted, enter the stream and then reabsorb the wanted substances. A moment's consideration will show that if one function of our proposed excretory system is to remove foreign substances from the body then the second design is the better. We are not in a position to foresee what sorts of foreign substances we may one day wish to eliminate, and the first design requires that specific provision must be made to eliminate each unwanted substance; but on the basis of the second design unwanted substances are automatically eliminated simply by not providing specific mechanisms for their reabsorption.

The filtration-reabsorption process of the vertebrate glomerular nephron conforms to the ideal design. In recognizing the impossibility of filtration in the aglomerular nephron and Malpighian tubule we have perhaps overlooked the possibility of a diffusion-reabsorption process whereby soluble substances of small molecular weight enter the urine by diffusion rather than by being forced through a filter. The filtration-reabsorption process, which is available to vertebrates by virtue of their closed blood system and high arterial pressure, should be very much more efficient than the diffusion-reabsorption process to which insects are condemned by their open blood system and inadequate arterial pressure. In insects one would expect that selective advantage would lie with a tubule in which a rapid rate of urine formation was combined with a high permeability to dissolved substances, not necessarily excluding those that are metabolically useful.

Although the tubule can actively transport substances such as potassium ion and certain dyes, it is not altogether clear how far active transport of organic substances may be a part of the tubule's normal activity. More uric acid can be recovered from

the urine than from the same volume of haemolymph, but in view of the complicated solubility relations of uric acid and its salts one cannot say with certainty that uric acid is transported against an electrochemical gradient. The excretion of dyes has been intensively studied, notably by Lison (1942, and earlier papers), and there is no doubt that many of these, e.g. phenol red, are actively concentrated by factors which Lison estimates as of the order of 10,000. There are striking parallels between the Malpighian tubule and the vertebrate nephron in their behaviour towards dyes; in the kidney tubules of the flounder Puck, Wasserman & Fishman (1952) have measured concentration factors for phenol red as high as 4000. It is not easy to see why natural selection should have operated to provide this one class of substances with such an efficient mechanism of elimination or how this observation can be assimilated to any theory of the normal operation of the excretory system. It may be that the ability to concentrate dyes is an incidental property of some other feature of the excretory mechanism.

The importance which is currently attached to the purely thermodynamic distinction between active transport on the one hand and diffusion (simple or facilitated) on the other, and also to the kinetics of passage through membranes, tends perhaps to distract attention from other features of the process of secretion which are less amenable to interpretation in physico-chemical terms. Biophysicists have confined their attention to the passage of dissolved substances through cell membranes and have shown little interest in cells which liberate their secretions by rupture. Recent electron microscope studies of the Malpighian tubules of the grasshopper by Beams, Tahmisian & Devine (1955) show that the brush border is formed of an array of tubes 3–4 μ in length and less than 1 μ in diameter. Mitochondria are seen in various positions relative to the tubes, and the evidence suggests strongly that they are in process of migrating through the tubes, eventually to be set free into the lumen of the Malpighian tubule. Wigglesworth (1943) describes the excretion of biliverdin by the Malpighian tubules of *Rhodnius* in the form of small discrete masses which assume an elongate form as they pass through the brush border. These observations raise doubts as to the value of physico-chemical models for the interpretation of secretion at the cellular level in Malpighian tubules.

At the level of organ physiology it is now suggested that the role of the Malpighian tubules may be re-defined as follows. The tubule is not concerned in the regulation of the composition of the haemolymph. The tubule is not an organ which selectively removes only specific substances from the haemolymph; it is a means whereby all soluble substances of low molecular weight are indiscriminately removed from the haemolymph, to which they may be selectively returned by the rectal glands. This does not exclude the selective excretion of substances such as uric acid. The whole process of excretion in the insect involves the contributory processes of diffusion, reabsorption and secretion—in the vertebrate, of filtration, reabsorption and secretion. In the vertebrate filtration is exclusive to the glomerulus, reabsorption and secretion taking place in the tubule; in the insect reabsorption takes place mainly in the rectal glands, diffusion and secretion taking place in the tubule.

SUMMARY

1. Contrary to expectation, it is found that metabolically useful substances pass into the urine and are reabsorbed in the rectum.

2. The kinetics of penetration have been investigated for six amino acids, three sugars and urea.

3. The evidence indicates that these substances enter the urine by passive diffusion.

4. The role of the Malpighian tubules in the insect's excretory system is discussed in the light of these findings. It is suggested that the tubule is primarily a means whereby all soluble substances of low molecular weight are removed from the haemolymph and that in this respect it has analogies with the glomerulus as well as with the tubule of the vertebrate nephron.

I am very grateful to Dr K. E. Machin for criticizing the theoretical part of this paper and for valuable suggestions for improving the presentation of the results, most of which I was glad to accept.

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