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THE PASSIVE PERMEABILITY OF INSECT MALPIGHIAN TUBULES TO ORGANIC SOLUTES

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INTRODUCTION

The excretory system of insects is essentially a secretion/reabsorption system; the Malpighian tubules secrete a fluid which is passed to the hind gut where selective reabsorption occurs (Ramsay, 1958; Maddrell, 1971). As Ramsay (1958) discovered, Malpighian tubules are highly permeable to such organic solutes as amino acids and sugars. These useful small molecules are reabsorbed in the hind gut, particularly in the rectum (Ramsay, 1958), where the uptake of amino acids has recently been shown to be an active process (Balshin & Phillips, 1971). As Ramsay (1958) was the first to realize, this apparently pointless committal of useful substances to the excretory flow and their later reabsorption is but one aspect of what from the insects point of view is a particularly sane arrangement. New toxic molecules or elevated concentrations of other useless or toxic molecules are *automatically* excreted simply by there being no mechanism for their reabsorption.

To repeat, the reabsorption of useful molecules from the excretory fluid goes on mainly in the hind gut. The hind gut of insects is lined with cuticle and, in the locust, this cuticular lining of the rectum has a rather low permeability to organic solutes as small as disaccharides (Phillips & Dockrill, 1968). Yet the Malpighian tubules of the stick insect are highly permeable to disaccharides; for example, the fluid they secrete contains sucrose at 45% of the concentration that it occurs in the bathing fluid (Ramsay, 1958). Were the locust to operate in a similar fashion it would seem that the excretory system would rapidly drain the haemolymph of substances of the order of the size of trehalose, because such molecules would cross the walls of the Malpighian tubules but would be too large to cross the cuticular lining of the rectum and so could not be reabsorbed there.

As a first step to investigate this type of paradox this paper describes experiments to determine the passive permeability of a range of Malpighian tubules to a range of organic solutes of varying size. To anticipate the results it appears that Malpighian tubules have a considerable permeability even to molecules as large as inulin. The consequences of this finding are discussed on p. 648.

MATERIALS

(1) *Insects*

Malpighian tubules of the following insects were examined: *Calliphora erythrocephala* (Diptera) adult insects; *Schistocerca gregaria* (Orthoptera) adult and larval

insects; *Rhodnius prolixus* (Hemiptera) 5th-stage larvae; *Triatoma phyllosoma* (Hemiptera) 5th-stage larvae; *Manduca sexta* (Lepidoptera) newly emerged adults.

All the insects were taken from laboratory cultures maintained in the Department of Zoology, Cambridge. *Triatoma phyllosoma* is a very large blood-sucking insect closely related to *Rhodnius* and included for comparison with *Rhodnius*.

(2) *Malpighian tubule preparations*

Preparations of isolated Malpighian tubules from the above species were made as described elsewhere (*Calliphora erythrocephala* (Berridge, 1966); *Schistocerca gregaria* (Maddrell & Klunsuwan, 1973); *Rhodnius prolixus* (Maddrell, 1969); *Manduca sexta* and *Triatoma phyllosoma* as for *Rhodnius*). Basically tubules are dissected from the insect and placed in an appropriate Ringer's solution under liquid paraffin. Secreted fluid is collected at the cut end of the tubule held away from the bathing solution on a glass rod stuck into the wax base of the experimental chamber. Secretion by Malpighian tubules of *Rhodnius* and *Triatoma* was stimulated with solutions containing 5-hydroxy-tryptamine (Maddrell, Pilcher & Gardiner, 1971); the other tubules secrete without the need for an added stimulant. Only the upper, secretory parts of the Malpighian tubules of *Rhodnius* and *Triatoma* were used.

(3) *Organic test substances*

The following substances were used in this investigation: urea, benzoic acid, L-valine, L-aspartic acid, L-glutamic acid, L-glutamine, D-xylose, L-lysine, L-3-phenylalanine, L-fucose, uric acid, L-tyrosine, maltose, sucrose, lactose, maltotriose and inulin. These compounds, labelled either with carbon-14 or tritium, were obtained from the Radiochemical Centre, Amersham.

EXPERIMENTAL METHOD

In a typical experiment a solution containing the test substance at a suitable concentration was made up, and individual Malpighian tubules or sets of tubules were set up in it. After sufficient fluid had been secreted to flush out the fluid initially in the lumen of the tubules further drops of secreted fluid were collected for assay of their contained radioactivity, and the rate at which the fluid was secreted was measured. After the volume of such drops had been noted (from measurements of their diameter (Maddrell, 1969)) they were absorbed on to the tips of small triangular pieces of Whatman no. 3 chromatography paper which were carefully dried and then transferred for counting to glass vials containing the scintillation mixture (0·3 % PPO in xylene). Alternatively, the drops of fluid were transferred by fine pipette straight into scintillation vials containing Bray's fluid. Samples were counted using an Intertechnique ABAC SL40 scintillation counter. As a check that a particular test substance was not metabolized in crossing the walls of the Malpighian tubules, samples of the secreted fluid and bathing fluid were subjected to paper chromatography in three different solvent systems and scanned radiometrically with a Berthold Scanner Model LB 2722. Of the substances used, very nearly all were found not to be metabolized to a measurable extent by Malpighian tubules. The concentration of a test substance in the secreted fluid was expressed as a fraction of that in the bathing medium, henceforth called the S/M ratio.

Table 1. *The measured surface areas and rates of fluid secretion of the Malpighian tubules used*

Species	Average surface area of Malpighian tubules (mm^2)	Average fluid rate of secretion ($nl.min^{-1}$)	Value of a ($nl.min^{-1}.mm^{-2}$)
Calliphora erythrocephala	4	13	3·3
Schistocerca gregaria	4	2	0·5
Manduca sexta	18	18	1·0
Rhodnius prolixus	7	36	5·1
Triatoma phyllosoma	17	60	3·5

As Ramsay (1958) pointed out, for a substance to be regarded as crossing the wall of the tubule passively, the following criteria must be fulfilled: (1) the S/M ratio should not exceed 1; (2) the S/M ratio should be largely independent of M; (3) the S/M ratio should be affected by the rate of fluid secretion according to the formula

$$S/M = b/(a+b),$$

where a is the rate of fluid secretion per unit area of wall and b is a constant, a measure of the permeability of the tubule wall to the particular substance.

Values for a were calculated from measurements of the dimensions and the secretory rates of several Malpighian tubules from each species of insect used. The measurements taken as typical are set out in Table 1.

Ramsay himself was not able extensively to use the third criterion in his work on Malpighian tubules isolated from *Carausius* because of the difficulty of altering the rate of secretion of fluid. He was able, however, to perfuse tubules at differing rates and in the one experiment he carried out, using urea as the test substance, he was able to conclude that this substance did cross the tubule wall in a passive fashion.

Using *Rhodnius* tubules which can be made to secrete at a variety of rates as they slow down following stimulation with 5-hydroxytryptamine, we have been able to use the third criterion routinely. An improvement in the analysis of the results was to plot M/S against the rate of secretion. As can be seen by rearranging the formula given above

$$M/S = (a+b)/b = a/b+1,$$

so such a plot for a substance passively appearing in the secreted fluid will be a straight line with slope $1/b$ and an intercept on the y axis of 1. In the examples we show of this type of analysis, straight lines have been fitted to the points by linear regression.

RESULTS

Permeability of Malpighian tubules of Rhodnius

The fullest set of tests was carried out using 5HT-stimulated tubules of *Rhodnius*. The permeabilities of the wall of this tubule to the various test substances are set out in Table 2, together with their molecular weights and the results of tests relevant to the manner in which the substances enter the tubule lumen. In Fig. 1 results for four test substances are plotted to show the dependence of the M/S ratio on the rate of fluid

Table 2. *Permeability of* Rhodnius *tubules to the various organic substances*

Substance	M.W.	*b* permeability ($nl.mm^{-2}.min^{-1}$)	Criteria of passive transport: $S/M \neq 1$	Criteria of passive transport: S/M independent of M	Criteria of passive transport: $\frac{S}{M} = \frac{b}{a+b}$	Concs tested (mM)	Substance not metabolized	No. of tubules used
Urea	62	26·2	Yes	Yes	Yes	0·015, 0·12	Yes	12
Benzoic acid	124	NA	Yes	NT	No	0·12	NT	12
L-Valine	125	0·38	Yes	Yes	Yes	0·0018, 0·007, 0·022, 0·033, 0·12	Yes	20
L-Aspartate	133	NA	Yes	NT	No	0·007	No	10
L-Glutamate	147	0·62	Yes	NT	Yes	0·007	Yes	12
L-Glutamine	148	NA	Yes	NT	No	0·21	No	10
D-Xylose	150	2·8	Yes	Yes	Yes	0·35, 0·68, 0·79, 2·67	Yes	16
L-Lysine	161	0·10	Yes	Yes	Yes	0·007	Yes	10
L-3-Phenylalanine	165	0·29	Yes	Yes	Yes	0·042, 0·32, 0·36, 0·48, 0·70	Yes	16
L-Fucose	168	0·60	Yes	Yes	Yes	0·069, 0·084, 0·1, 0·15	Yes	20
Uric acid	170	0·36	Yes	Yes	Yes	0·004, 0·039, 0·040, 0·049	Yes	20
L-Tyrosine	181	0·50	Yes	NT	Yes	0·007	Yes	10
Sucrose	360	0·39	Yes	Yes	Yes	0·11, 0·12, 0·24, 1·07, 1·25	Yes	10
Maltose	360	0·053	Yes	Yes	NT	0·031, 0·51, 0·58, 0·75	Yes	14
Lactose	360	0·14	Yes	Yes	Yes	0·076, 0·25, 0·33	NT	13
Maltotriose	504	0·053	Yes	Yes	NT	0·066, 0·21, 0·32	NT	11
Inulin (hydroxymethyl)	5200	0·039	Yes	Yes	Yes	0·27, 0·42, 0·53	Yes	20
Inulin (carboxylic acid)	5200	0·060	Yes	Yes	Yes	0·077, 0·084, 0·79, 0·87, 1·16	Yes	20

NA = Not applicable. NT = Not tested.

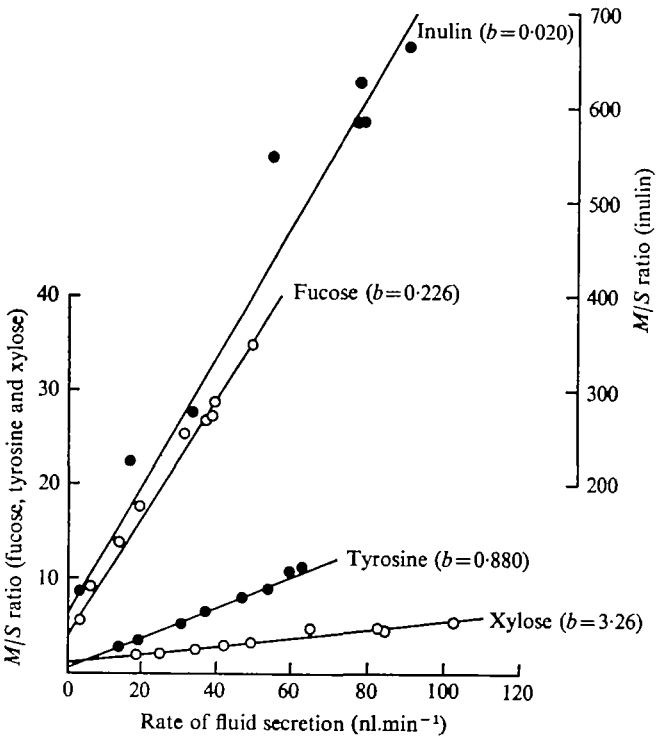

Fig. 1. The relationship between M/S ratio and rate of fluid secretion for isolated Malpighian tubules of *Rhodnius*.

secretion; the linear relationships are what one would expect for substances crossing the tubule wall passively. Fig. 2 shows how the S/M ratio depends on the molecular weight of test substances. Not unexpectedly, the larger compounds penetrate the tubule wall more slowly, but even they penetrate the wall surprisingly easily so that inulin, for example, reaches a concentration close to 1 % of that of the bathing solution in a tubule secreting at 36 nl.min^{-1}. There is also an apparent tendency for the more highly charged molecules to penetrate more slowly than the more electrically neutral compounds. Specific examples are L-valine and D-xylose which appear at 7 and 35 % respectively in fluid secreted at 36 nl.min^{-1} and even more striking, maltose and sucrose which appear in such fluid at about 1 and 7 % respectively, though their molecular weights are the same. As a result, of course, the line drawn through the points in Fig. 2 should be taken only as an indication of the way in which molecules of a particular size might behave. Clearly other factors such as the charge on the molecule have a large effect on the rate of penetration of any particular substance.

Permeability of Malpighian tubules of other insects

The results of exactly similar experiments using different Malpighian tubules are presented in Table 3 and Fig. 3. In Table 3 have been included, for comparison, figures for the Malpighian tubules of *Carausius morosus* calculated from those of Ramsay (1958) and Ramsay & Riegel (1961). Again, what is striking is the permeability of the tubule walls even to compounds as large as inulin. In individual cases

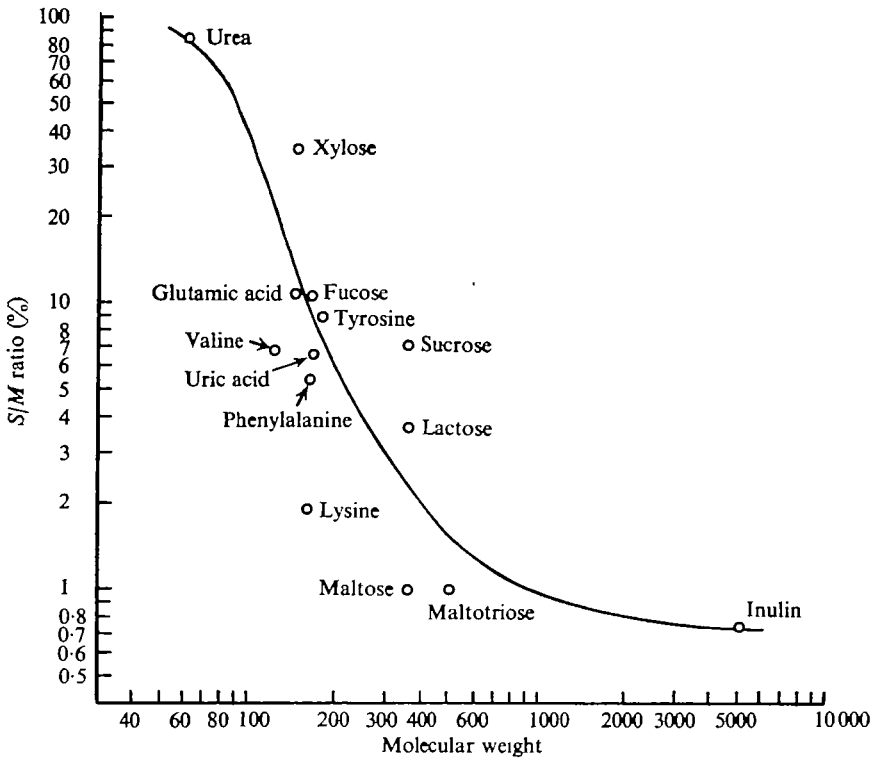

Fig. 2. The relationship between S/M ratio and the molecular weight of organic substances for isolated Malpighian tubules of *Rhodnius*, secreting fluid at 36 nl.min^{-1}.

where tubules secrete fluid particularly slowly, this high permeability results in the concentration of inulin in the secreted fluid reaching nearly 50% of its concentration in the bathing fluid.

As before, the essentially passive nature of the movements tested is shown by the facts that the concentration of test substances was never higher in the secreted fluid than it was in the bathing fluid and that, where it was measured, the S/M ratio was not affected by changes in M.

Apart from the tubules of *Calliphora*, which have distinctly higher permeabilities to the substances tested, the Malpighian tubules of the different insects studied are closely similar in their permeability properties.

Comparison of in vitro *experiments with* in vivo *findings*

It is a point of some concern to establish whether the experiments so far described can be used in an extrapolation to the situation *in vivo*. To investigate this point we injected non-metabolized tracer substances into insects and followed the subsequent course of their excretion. For these experiments we chose inulin and L-fucose as markers and injected them into adult *Schistocerca* and fed 5th-stage larvae of *Rhodnius*, respectively. While inulin is not metabolized by the locust, it does become sequestered in the fat body and gonads (Loughton & Tobe, 1969). We therefore followed the concentration of inulin in the haemolymph of locusts over a period of up to 10 days

Table 3. *Permeability of the* Malpighian *tubules of various insects to organic substances*

Insect	Substance	*b* permeability ($nl.mm^{-2} min^{-1}$)	$S/M > 1$	Concentrations tested (mM)	No. of tubules tested
Calliphora erythrocephala	D-Xylose	19·7	Yes	0·79	8
	Maltose	0·35	Yes	0·58	5
	Maltotriose	0·30	Yes	0·41	9
	Inulin (carboxylic acid)	0·13	Yes	0·133	6
	Inulin (hydroxymethyl)	0·12	Yes	0·25	20
Schistocerca gregaria	D-Xylose	0·27	Yes	0·68	22
	Maltose	0·040	Yes	0·55	9
	Maltotriose	0·034	Yes	0·65	14
	Inulin (carboxylic acid)	0·022	Yes	0·23	10
Triatoma phyllosoma	L-Valine	1·01	Yes	0·007	7
	L-3-Phenylalanine	0·55	Yes	0·713	5
	Maltose	0·104	Yes	0·55; 0·58; 0·41	9
	Inulin (hydroxymethyl)	0·030	Yes	0·87	12
Manduca sexta	D-Xylose	2·7	Yes	0·52	17
	Maltotriose	0·076	Yes	0·44	8
	Inulin (carboxylic acid)	0·018	Yes	0·40	30
*Carausius morosus**	Urea	45·0	Yes	Wide range	25
	Glucose	2·46	Yes	Wide range	33
	L-Valine	0·15	Yes	Wide range	32
	Sucrose	0·34	Yes	Wide range	31
	Inulin	0·019	Yes	0·38	5

* Figures for *Carausius* from Ramsay (1958) and Ramsay & Riegel (1961).

after injection. The results are set out in Fig. 4 from which it is clear that to a first approximation the inulin concentration declines exponentially.

The faeces collected from eight injected locusts was found to contain 4·5% of the inulin injected 5 days previously. From Fig. 4, the haemolymph at this time contains 61% of the inulin injected initially. On the bold assumption that the fat body and gonads sequester inulin from the haemolymph at a rate proportional to its concentration therein, one can calculate from these figures the time constant for the removal of inulin by the Malpighian tubules as 126370 min. Using the inulin dilution method of Loughton & Tobe (1969), we measured the haemolymph volume of ten adult locusts and found this to be 608 ± 41 μl (mean ± S.E.M.). The Malpighian tubules of adult locusts secrete fluid into the hindgut at a rate of 8 $\mu l.h^{-1}$ (Phillips, 1964). From these figures one can calculate that the *in vivo* U/P ratio for inulin is 0·036 and that, from the formula $U/P = b/(a+b)$, the permeability of the Malpighian tubules to inulin, b, is 0·019 $nl.mm^{-2}.min^{-1}$ which is in excellent agreement with the figure of 0·022 $nl.mm^{-2}.min^{-1}$ found in our *in vitro* experiments (Table 3).

As a further test of the relevance of our *in vitro* results to the *in vivo* situation, we injected radioactive L-fucose into the haemolymph of recently fed *Rhodnius* and followed its appearance in the urine for about 30 min before taking a haemolymph

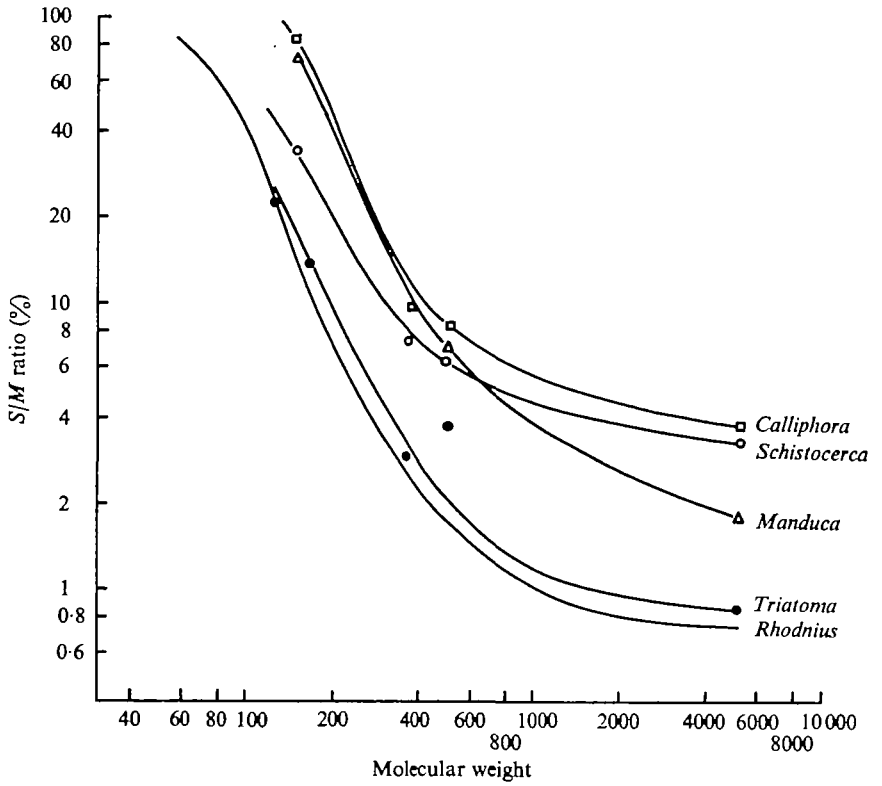

Fig. 3. The relationship between S/M ratio and the molecular weight of organic substances for isolated Malpighian tubules of various insects, secreting fluid at their characteristic rates (Table 1). The linedrawn for *Rhodnius* is that from Fig. 2 included here for comparison.

sample. This gave simultaneous measurements of the concentration of L-fucose in the haemolymph and urine. Using the formula $U/P = b/(a+b)$ as before, the results for five insects gave a value of b for L-fucose of 0·66 $nl.mm^{-2}.min^{-1}$ which is in good agreement with the figure of 0·60 $nl.mm^{-2}.min^{-1}$ from our *in vitro* experiments (Table 2).

CONCLUSIONS AND DISCUSSION

What emerges from this survey of insect Malpighian tubules is that they are permeable to a wide variety of organic substances. This of course makes good sense in an excretory organ, where as pointed out earlier, automatic excretion of toxic molecules is ensured by a relatively unselective committal of materials to the primary excretory fluid followed by a selective reabsorption of useful substances. Thus passive excretion of relatively large molecules requires that the Malpighian tubule be permeable to them; from results of the present investigation it appears that this is the case.

What is at first sight surprising is that if one compares the permeability of the locust Malpighian tubules with that of the cuticular lining of its rectum (Phillips & Dockrill, 1968), they appear to be poorly matched. For example, from the present results one would expect the fluid leaving the Malpighian tubules to contain disaccharides at

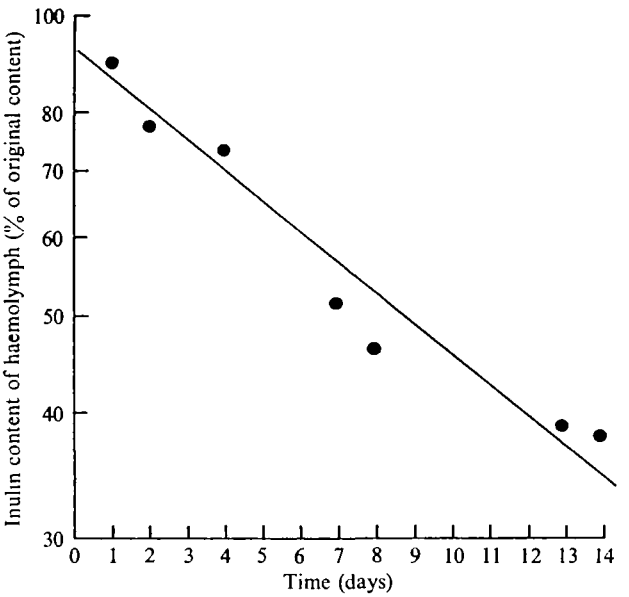

Fig. 4. The decline in the content of radioactive inulin in the haemolymph of *Schistocerca gregaria* with time. The line drawn through the points is the linear regression calculated by the least squares method.

concentrations of about 5–10% of the levels occurring in the haemolymph. Yet the rectal cuticle has a very low permeability to substances as large as this. If such materials are not to be lost, then it follows that they must be reabsorbed elsewhere. It is entirely possible that the hind gut anterior to the rectum (through which the primary excretory fluid must in any case pass *en route* to the rectum) is lined with more permeable cuticle and that reabsorption of useful molecules other than the very smallest can go on here. As has been emphasized before (Maddrell, 1971), the study of the anterior hind gut (often called the ileum and/or the colon) seems to be very worthwhile.

With the benefit of hind sight it is possible to make sense of the apparently greater permeability of Malpighian tubules compared with the rectal wall. Absorption of water in the rectum must lead of course to a great concentration of substances dissolved in the rectal fluid. Were the rectal wall to be permeable to materials intended for excretion there would be a strong tendency for them to re-enter the haemolymph passively, which would be counter-productive. In addition, as Phillips & Dockrill (1968) have pointed out, the rectal cells, on whose controlling activity the insect vitally depends, would be bathed by high concentrations of substances some of which are toxic. Again the impermeability of the rectal cuticle makes sense.

From the point of view of compounds that an insect might wish to circulate in its haemolymph, the permeability of the Malpighian tubules forces a choice on the insect. If a substance of low molecular weight is to be circulated, then the insect must be prepared either continually to reabsorb the substance from the excretory fluid or countenance its steady loss from the haemolymph. Alternatively, materials must be circulated in a form in which they are attached to some large carrier substance, the complex then being sufficiently large to escape excretion. In this connexion it is worth

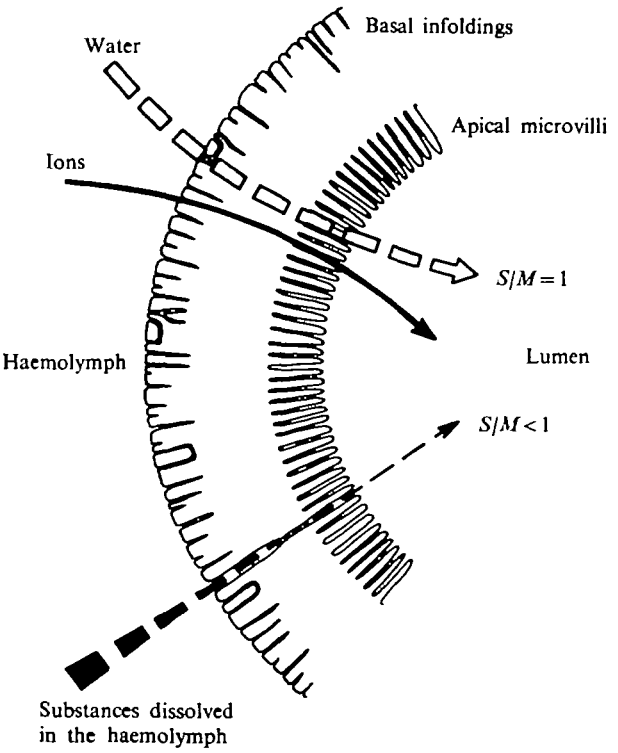

Fig. 5. Schematic section of the wall of a Malpighian tubule to show the routes of transport of ions and water and diffusion of organic substances through the cell wall.

noting that lipids, which many insects use as the fuel for flight, are mainly transported as diglyceride-protein conjugates and it is possible that unesterified fatty acids are also bound to protein (Gilbert, 1967). In addition, it is possible that insect juvenile hormone is circulated as a hormone-protein complex (Pratt, 1972).

So large are some of the substances to which Malpighian tubules are permeable that one can scarcely doubt that they must cross the tubule wall in the lateral spaces between the cells. These spaces are long, thin and narrow and are 'closed' by septate desmosomes and 'tight' junctions. However, such an arrangement may be permeable as has been emphasized by Frömter & Diamond (1972). Fluid secretion by Malpighian tubules is thought to be achieved by an osmotic coupling of water fluxes to a primary active transport of ions (Maddrell, 1971). To be efficient, such a mechanism demands that ion transport occurs across a membrane which is osmotically relatively tight. If the intercellular route is leaky, then it follows that ion and water movements probably use an intracellular route. One envisages then that Malpighian tubules produce an iso-osmotic flow of fluid through the cells and that the excretorily useful permeability is achieved by a parallel leaky intercellular route (Fig. 5). The continuous fluid flow into the lumen ensures that a concentration gradient always exists between the haemolymph and the primary excretory fluid and this in turn ensures the passive appearance in this fluid of substances able to diffuse across the tubule wall. Just how important this function is emerges clearly from the fact that fluid is secreted by the Malpighian

tubules far faster than it is allowed to leave the insect. A similar disparity arises in mammalian kidneys (Smith, 1951) for exactly the same reason.

Although Malpighian tubules are permeable structures this is not to deny the existence in them of specific active transport systems for materials which the insect has a constant need to excrete. As will be described in a later paper (Maddrell *et al.* 1974) at least two such mechanisms exist in insect Malpighian tubules.

SUMMARY

1. The permeability of Malpighian tubules of five insect species to a range of organic solutes has been measured by both *in vitro* and *in vivo* techniques.

2. Nearly all the substances tested were found, in *in vitro* experiments, to penetrate the walls of Malpighian tubules in a manner which, on several criteria, was judged to be passive.

3. The walls of Malpighian tubules are more permeable to small molecules than to large ones; but even inulin (MW 5200) penetrates fast enough to reach concentrations which, in tubules secreting fluid slowly, can be as high in the secreted fluid as 50% of its concentration in the bathing fluid.

4. Inulin injected into *Schistocerca* and L-fucose injected into *Rhodnius* appeared in the excreta at rates which could be accurately predicted from the *in vitro* behaviour of Malpighian tubules of these insects.

5. Taken with the fact that the cuticular lining of the rectum of insects is thought to be not very permeable, the high permeability of insect Malpighian tubules means that the reabsorption of useful compounds of the order of size of disaccharides must to some extent occur before excretory material reaches the rectum.

6. It is suggested that the circulation of lipids and hormones in the form of complexes with proteins is a device to prevent their rapid loss through the excretory system.

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