J. exp. Biol. (1975), 63, 237–248
With 8 figures
Printed in Great Britain

THE REMOVAL OF SULPHATE BY THE EXCRETORY APPARATUS OF THE BLOWFLY *CALLIPHORA VOMITORIA*

BY G. KNOWLES*

Zoology Department, University of Newcastle upon Tyne

(*Received* 23 *January* 1975)

SUMMARY

The excretion of sulphate by the isolated Malpighian tubules of *Calliphora vomitoria* has been investigated. Contrary to expectation, it was found that the isolated tubules are freely permeable to sulphate. The rate of sulphate secretion is comparable to the rates of secretion of both phosphate and chloride. The excretion of sulphate by the intact fly has also been verified.

INTRODUCTION

The isolated Malpighian tubules of insects are freely permeable to some organic solutes (Ramsay, 1958; Knowles, 1975; Maddrell & Gardiner, 1974). Thus, even inulin (molecular weight of 5500) can relatively rapidly enter the tubule lumen of *Calliphora* (Knowles, 1974; Maddrell & Gardiner, 1974). However, it is postulated that *ionic* molecules of roughly similar size cannot penetrate the tubule wall. Hence, Berridge (1969), Maddrell (1969) and Pilcher (1970) found that fluid secretion by isolated Malpighian tubules would cease if the tubules were bathed by a Ringer solution in which sulphate was the only anion present. It was suggested that the large sulphate anion (hydrated molecular diameter 7·5 Å) could not pass rapidly enough across the tubule wall to accompany the secreted cations. Eventually ionic secretion would fail because electrical neutrality has to be maintained and the cessation of ion secretion would probably result in the breakdown of standing osmotic gradients and stop fluid formation. However, experiments by Berridge (1969) on the isolated tubules of *Calliphora* showed that the presence of sulphate at lower concentrations, which were used for the osmotic balancing of phosphate or chloride Ringer solutions, did not prevent fluid secretion. Similar results were also obtained by Pilcher (1970).

The question thus arises, do sulphate ions enter the tubule lumen when other permeating ions are present (e.g. phosphate or chloride), which may seem possible in view of the relatively high inulin permeability, or is the tubule impermeable to sulphate as other results suggest (Berridge, 1969; Maddrell, 1969; Pilcher, 1970)? The permeation of charged molecules can often not be predicted on size alone, and as Maddrell (1971) has written, 'it is the size of decrease in free energy of an ion as

* Present address: Department of Medical Biochemistry, Stopford Building, Oxford Road, Manchester M13 9PT.

it becomes associated with a membrane which determines the permeabilities of the membrane to the ion'. However, if the permeating ion traverses the membrane through pore channels then the hydrated ion size is, of course, important.

The presence of some mechanism facilitating sulphate entry cannot be dismissed outright, since previous work has shown that several sulphate transporting mechanisms exist in other systems. For instance, Berglund & Forster (1958) have shown that the aglomerular kidneys of the marine teleost fish *Lophius* will excrete sulphate. In addition, Webb (1940) has stated that the antennal gland of *Carcinus* secretes sulphate. Both *Lophius* and *Carcinus* are marine animals where the level of environmental sulphate is high, and this could result in an excess of body sulphate which is excreted. Berglund & Lotspeich (1956*a*, *b*) present evidence to suggest that dog kidneys can reabsorb sulphate ions from the nephron tubules.

MATERIALS AND METHODS

The penetration of sulphate through the walls of the Malpighian tubules from *Calliphora* was investigated by using radioactive $^{35}SO_4$. The isotope was supplied by the Radiochemical Centre, Amersham.

Malpighian tubules were dissected from young, adult, male flies and were set up as isolated tubule preparations. Preparations were bathed by Ringer solutions with a varied sulphate concentration (0·5–50·0 mM) to which $^{35}SO_4$ had been added. Osmotic adjustments to balance differing sulphate concentrations were achieved by adding varying amounts of NaCl. Glucose at a concentration of 10 mM was added to the bathing media as an energy source for the isolated tubules. The tubules were allowed to secrete fluid for set periods, samples were then taken and their radioactivities counted on an Isotopes Developments Ltd end-window anti-coincidence counter. Providing samples from each experiment are counted within a limited period, not usually longer than 12 h, then it can be assumed that the extent of the radioactive decay of ^{35}S in each sample is negligible. Results are initially expressed as the radioactivity in the tubule fluid sample (cpm/nl of sample) divided by the radioactivity in the bathing medium sample (cpm/nl of sample). This expression is the *TF/M* radioactivity ratio. Since the results from the various experiments are expressed as ratios, then the effects of radioactive decay on the count rate for readings taken at different times during an experiment is, in any case, eliminated.

The potential differences across the tubule wall were measured with an E.I.L. Vibron Electrometer. Ag/AgCl in saturated KCl electrodes were used. The arrangement was similar to that used by Pilcher (1970).

The pattern of sulphate excretion by the intact fly was also investigated. A male fly was taken and restrained on a waxed slide with its ventral side uppermost. A known volume (*ca.* 4·7 μl) of 50 mM $^{35}SO_4$ Ringer solution was injected through the mesopleuron. This volume of Ringer solution, which is approximately one-third of the haemolymph volume, induced a diuresis in the fly. Urine samples were collected at regular intervals. Haemolymph samples were taken less frequently and not more than three times from any one fly to prevent too great a loss of haemolymph. The radioactivities of the samples were counted as previously described for the isolated tubule experiments.

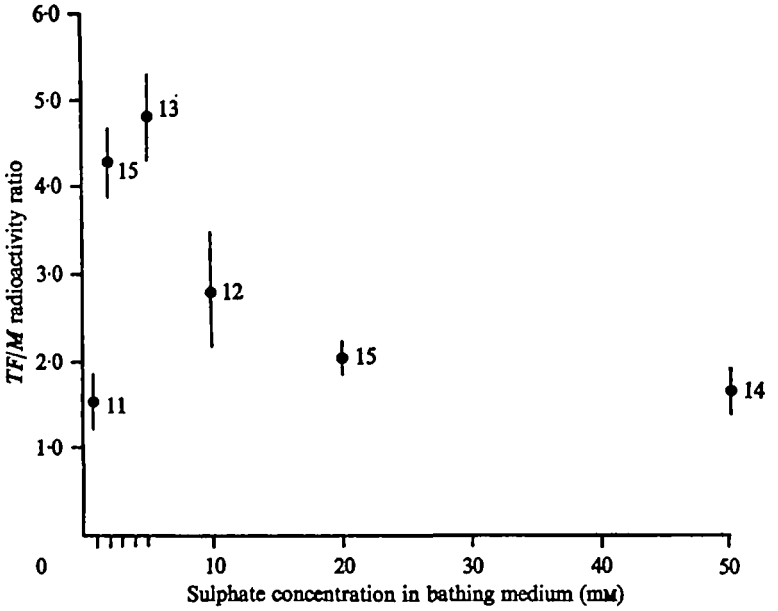

Fig. 1. The effect of the bathing medium sulphate concentration upon the *TF/M* radioactivity ratio from isolated Malpighian tubules. All error bars are given as the mean ± S.E. The numbers adjacent to the means represent the total number of observations from which that mean was calculated.

Some experiments required the separation of ionic sulphate from samples. This was achieved by adding aliquots of 2 μl of 200 mM $BaCl_2$ to each 1 μl of sample, thus precipitating $BaSO_4$. The precipitates were spun down in capillary tubes placed in a haematocrit centrifuge. The organic content, if any, of this precipitate was tested by washing the precipitate three times with distilled water, placing it on platinum foil and then heating to 200 °C in a muffle furnace. Control procedures were undertaken whereby an organic substance was included with the precipitate. In such cases brown spots occurred when the sample was heated to 200 °C.

If labelled sulphate becomes incorporated into proteins or mucopolysaccharides, then absolute ethanol should precipitate the radioactivity (Ray & Trudinger, 1970). This was tested by adding 2 μl of absolute ethanol to each 1 μl of haemolymph or urine. The precipitate was removed and the radioactivity of the supernatant counted.

RESULTS

(1) *The TF/M radioactivity ratio of isolated tubules*

The *TF/M* ratios for several bathing medium sulphate concentrations are at all times above unity and greatest at a medium concentration of about 6 mM (Fig. 1). These results alone seriously question the theory hitherto proposed, that the Malpighian tubules are impermeable to sulphate.

Although the chemical identity of the radioactive molecules in the tubule fluid samples themselves has not been verified, injection of $^{35}SO_4$ into intact animals and subsequent collection of urine and haemolymph samples suggests that the

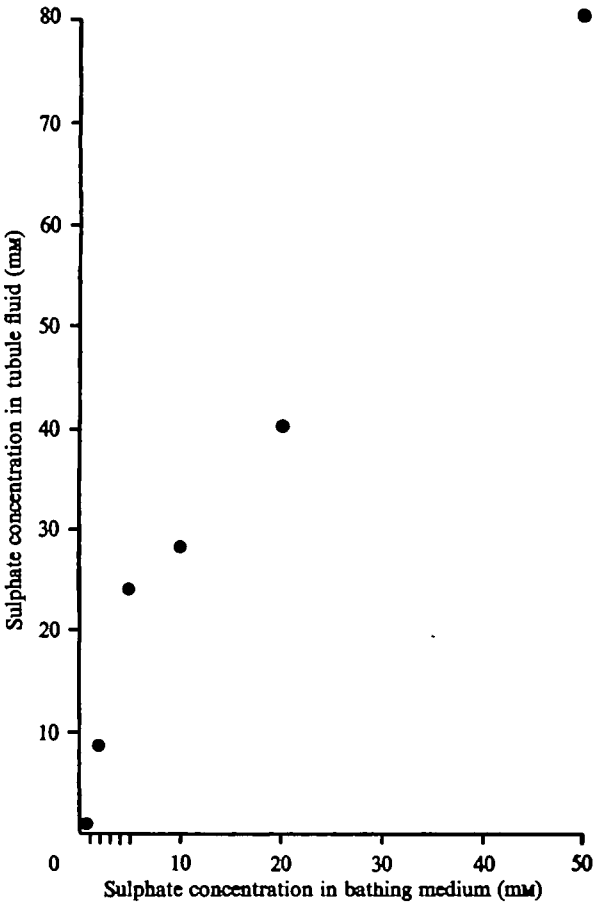

Fig. 2. The calculated tubule fluid sulphate concentration in relation to the bathing medium sulphate concentration.

radioactivity is present as inorganic sulphate. If the radioactivity in the tubule fluid is derived from labelled ionic sulphate, then the *TF/M* ratios can be used to calculate tubule fluid sulphate concentrations (Fig. 2).

The rates of tubule fluid production at several bathing medium sulphate concentrations are shown in Table 1. From these data the rates of sulphate ion secretion have been calculated (Fig. 3). At low bathing medium concentrations the rate of ion secretion rises sharply, and then, at an external concentration of 5 mM, the rate of increase of ion secretion is reduced. Thus, between the bathing medium concentrations of 0·5–5·0 mM the rate of ion secretion increases from 1·42 to 58 p-mol/min/tubule, i.e. approximately 40-fold.

(2) *The potential difference across the tubule wall*

The driving force for the rapid transfer of sulphate may be derived from potential differences across the tubule wall. To establish whether this is so, the potential difference (p.d.) across the tubule wall was measured. The p.d. was measured at three bathing medium sulphate concentrations; 0·5, 5·0 and 50·0 mM (Fig. 4). The

Table 1. *The rates of tubule fluid production of isolated Malpighian tubules when bathed by media of varying sulphate concentrations*

Bathing medium SO_4^{2-} conc. (mM)	Rate of tubule fluid production (nl/min)	S.E.	*n*
0·5	1·82	0·35	11
2·0	1·93	0·27	15
5·0	2·42	0·51	13
10·0	2·71	0·46	12
20·0	2·45	0·38	15
50·0	2·29	0·19	14

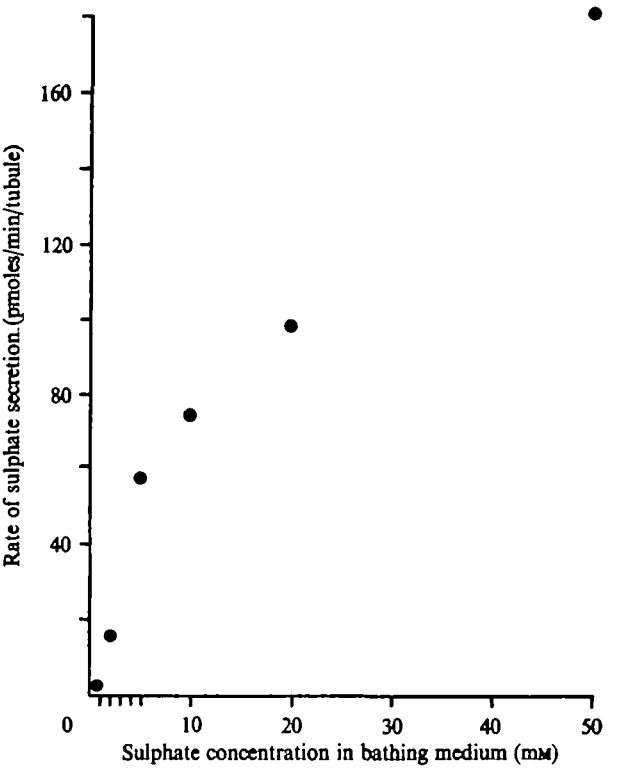

Fig. 3. The calculated rate of sulphate secretion (p-moles/min/tubule) by isolated tubules in relation to their bathing medium sulphate concentration.

sulphate equilibrium potentials calculated from the *TF/M* ratios of Fig. 1 are also shown in Fig. 4. It is apparent that steady membrane potentials were not achieved, but over a 2 h period there was a gradual decline from approximately 15 mV lumen positive to 5 mV lumen positive. There were no discernible differences between the potential profiles from the three sulphate concentrations. Maddrell (1971) quotes some unpublished results of Berridge that, in *Calliphora*, the tubule lumen is positive with respect to the bathing medium. These results are in agreement with this. At bathing medium concentrations of 0·5 and 50·0 mM sulphate the membrane potentials may initially exceed the equilibrium potentials and the potential difference might

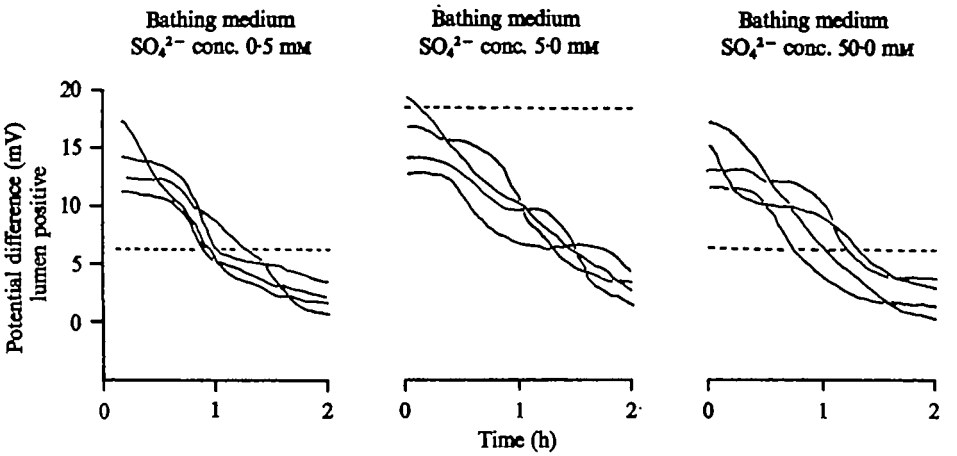

Fig. 4. The potential differences across the wall of the isolated tubules at three different bathing medium sulphate concentrations. The sulphate equilibrium potentials have been calculated from the *TF/M* radioactivity ratios from Fig. 1 and are denoted by dotted lines.

be thought to be responsible for the sulphate transport. However, at a medium sulphate concentration of 5·0 mM the membrane potential appeared to be insufficient to account for any sulphate transport, and this despite the fact that a 40-fold increase in sulphate secretion had occurred when the bathing medium sulphate concentration was increased from 0·5 to 5·0 mM. The potential difference profiles at these two bathing medium sulphate concentrations were not sufficiently different to account for this large increase in ion secretion. It is therefore doubtful if the observed potential difference is a significant factor in determining the high rate of sulphate transport.

(3) *The excretion of sulphate by the intact fly*

It is important to verify that the transport of sulphate as demonstrated by the isolated Malpighian tubule also operates in the intact fly. Therefore, labelled sulphate was injected into flies and the excretion of isotope with respect to time was followed.

Assuming for the moment that the radioactivity is in the sulphate ion (the evidence for this is to be given shortly), then the sulphate concentrations of the haemolymph and urine can be calculated (Fig. 5). It is clear that much sulphate is excreted. The high concentration of sulphate in the urine could obviously be caused by the secretory activity of the tubules. It is also possible that water reabsorption from the tubule fluid or secretion of sulphate by the alimentary canal might elevate the sulphate concentration in the urine. It is known, for example, that if a non-metabolizable monosaccharide is injected into the fly, considerable amounts of the radioactivity are excreted at high concentration, indicating that water reabsorption may be occurring even during a diuresis (Knowles, 1974).

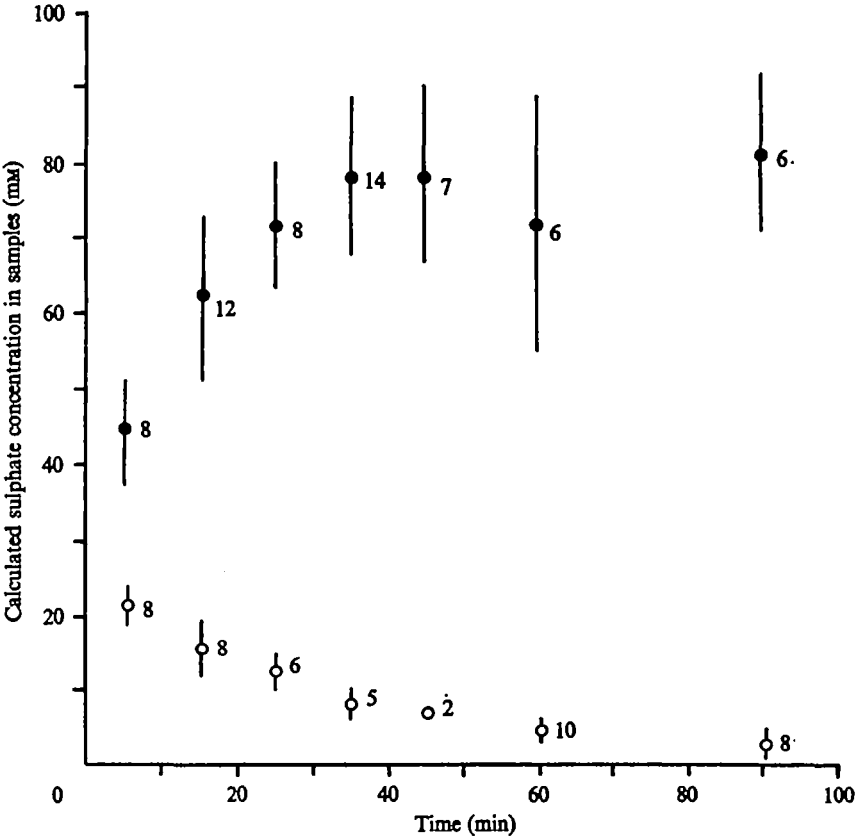

Fig. 5. The calculated sulphate concentrations of the haemolymph (O–O) and urine samples (●–●) from the intact fly in diuresis, after injection of $^{35}SO_4^{2-}$.

Table 2. *Radioactivity of the supernatant after treating* 1 *µl of haemolymph* and urine samples* with* 2 *µl of* 200 mM-$BaCl_2$

	Urine			Haemolymph		
	cpm	S.E.	*n*	cpm	S.E.	*n*
With $BaCl_2$	175	26	5	206	32	5
No $BaCl_2$	8967	621	5	1621	98	5

* Samples taken 45 min after injection of labelled sulphate.

(4) *The chemical identity of isotope in the haemolymph and urine from intact flies*

The radioactivity detected in either the haemolymph or urine samples could have been conjugated to sulphated mucopolysaccharides. Several workers have shown that there are specialized cells (mucocytes) within Malpighian tubules which are thought to secrete mucopolysaccharides (Martoja, 1959; Taylor, 1971). The stellate cells from the Malpighian tubules of *Calliphora* bear resemblances to mucocytes (Berridge & Oschman, 1969).

The separation of ionic sulphate was achieved by the addition of $BaCl_2$ and the

Table 3. *Observations on the organic content of body fluids after using $BaCl_2$ as a precipitating reagent*

Solution spotted		Colour of spots
Haemolymph + $BaCl_2$	(ppt not separated)	Black
Urine + $BaCl_2$	(ppt not separated)	Black
Haemolymph + $BaCl_2$	(ppt washed and separated)	White
Urine + $BaCl_2$	(ppt washed and separated)	White
$BaCl_2$ + Na_2SO_4	(ppt washed and separated)	White

Table 4. *Radioactivity of the supernatant after treating 1 µl of haemolymph and urine samples with 2 µl of absolute ethanol*

	Urine			Haemolymph		
	cpm	S.E.	*n*	cpm	S.E.	*n*
With ethanol	7650	726	5	1520	208	5
No ethanol	9995	810	5	1719	262	5

ubsequent removal of the precipitate, presumably $BaSO_4$. $BaCl_2$ was found to precipitate 98% and 87% of the radioactivity in the urine and haemolymph samples respectively (Table 2). If the washed precipitate is heated to 200 °C then charring would indicate some organic content of the precipitate. Control procedures were undertaken and the observations are given in Table 3. These observations are indicative of an inorganic precipitate and suggest that the radioactivities of the haemolymph and urine are in the sulphate molecule. The addition of ethanol, which precipitates macromolecules, does not precipitate appreciable radioactivity (Table 4).

Although the evidence indicates that the radioactivities of the haemolymph and urine samples are in the sulphate ion, it is still possible that for the tubule fluid samples radioactivity is in molecules other than ionic sulphate. Thus, as haemolymph sulphate passes through the tubule wall it may become conjugated to mucopolysaccharides, only to be released from its macromolecular binding as it is carried along the hindgut. Nevertheless, as previously shown, 98% of the radioactivity would have to be released as inorganic sulphate. It would seem more likely that it remains as inorganic sulphate during its passage through the excretory system.

DISCUSSION

Berridge (1969) investigated the role of anions in maintaining fluid flow by the isolated Malpighian tubules from female *Calliphora*. Approximate comparisons can be made between the rate of excretion of sulphate ions, derived from the present investigation, and the rate of excretion of both phosphate and chloride derived from Berridge's (1969) results.

In solution, phosphate exists partly as polyvalent ions, and since the pH of the tubule fluid was not measured, the abundance of each valent type cannot be determined. Therefore, the phosphate concentrations have been expressed as mg atoms of phosphorus/l. The tubule fluid concentrations of phosphate and sulphate anions have been calculated and are compared in Fig. 6 where it is apparent that these two anions are secreted by the isolated tubules at comparable rates.

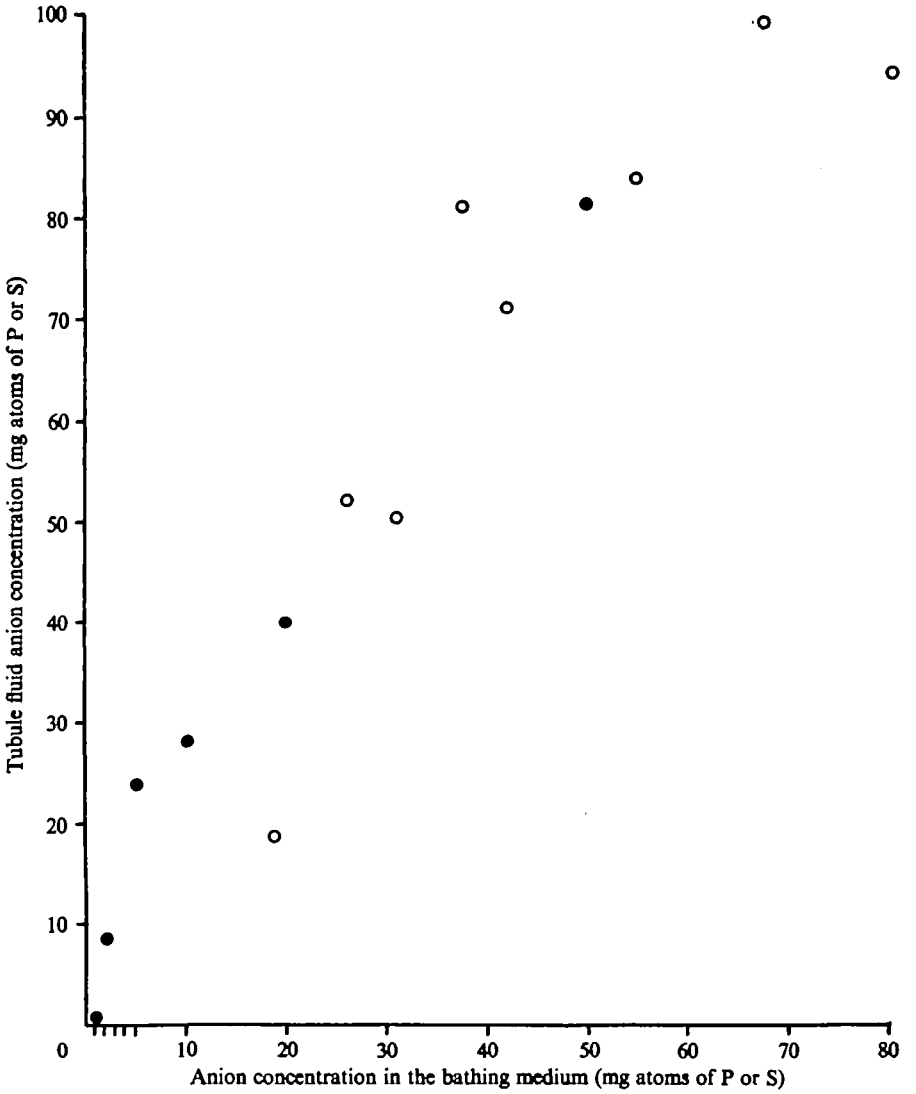

Fig. 6. A comparison between the tubule fluid concentrations of sulphate (mg atoms of sulphur) and phosphate (mg atoms of phosphorus) in relation to their bathing medium concentrations. ●–●, Sulphate; O–O, phosphate. Phosphate data taken from Berridge (1969).

If the rate of fluid secretion is taken into account, the similarity between phosphate and sulphate becomes less clear. Berridge's (1969) investigation of the effects of anions on fluid secretion was performed on tubules isolated from female flies which generally secrete fluid several times faster than the tubules from male flies. Thus, taking the observed rates of fluid secretion into account the rates of anion secretion have been calculated and are shown in Fig. 7. Because the rates of fluid secretion were generally much higher for the phosphate experiments, it appears that phosphate is secreted faster than sulphate.

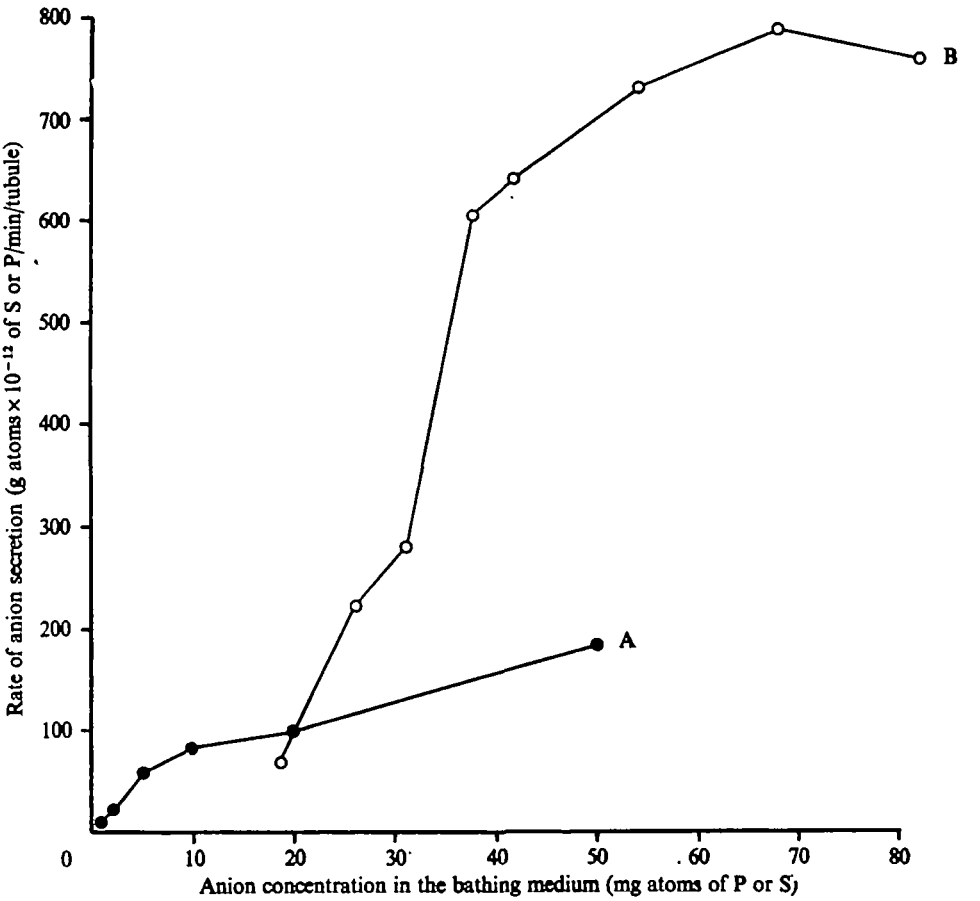

Fig. 7. A comparison between the rates of sulphate and phosphate secretion. (A) ●–●, Sulphate secretion (g atoms × 10^{-12} of S/min/tubule). (B) O–O, Phosphate secretion (g atoms × 10^{-12} of P/min/tubule). Phosphate data taken from Berridge (1969).

Similarly, the excretion of sulphate and chloride can be compared. Berridge (1969) used either sulphate or phosphate to balance the osmolarity of varying chloride concentrations. In this comparison both sets of chloride data have been presented, but the data when chloride is balanced with phosphate approximates to a comparable situation which occurred when in the present investigation the sulphate permeability was being investigated. This is because in the present experiments excess phosphate ($\sim 4{\cdot}7\ \mu g\ PO_4/\mu l$) was present. When the absolute rates of ion secretion are calculated from the available data the secretion of sulphate exceeds that of chloride at low medium concentrations, but at a medium concentration of 100 m-equiv/l they are similar (Fig. 8).

The preceding comparisons between the transport of sulphate with either phosphate or chloride suggests that sulphate can be transported remarkably quickly. However, it must be emphasized that comparisons have been made between results from experiments of different design, and this imposes limitations upon any interpretation. The comparison of anion secretion rates should not be accepted without caution.

The permeability of Malpighian tubules to sulphate has now to be reconciled

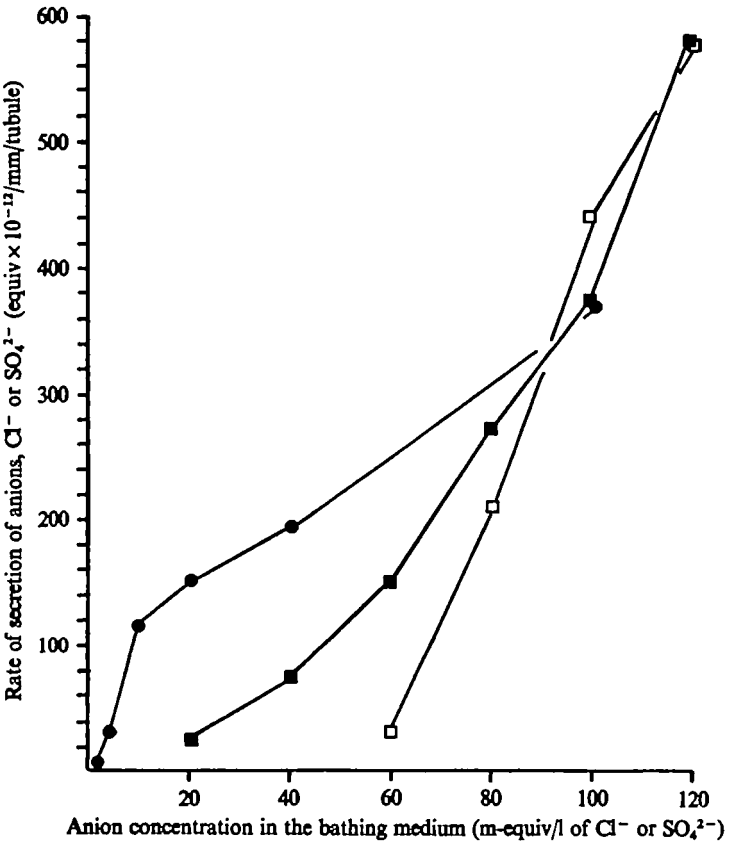

Fig. 8. A comparison between the rates of secretion of sulphate (equiv × 10^{-18}/min/tubule) and chloride (equiv × 10^{-18}/min/tubule) in relation to their bathing medium concentrations. ●–●, Sulphate; ■–■, chloride with phosphate ions to balance the osmolarity; □–□, chloride with sulphate ions to balance the osmolarity. Chloride data taken from Berridge (1969).

with Berridge's (1969) results, which suggested that the tubules were impermeable to sulphate. The criterion for sulphate impermeability was the inability of sulphate ions to support fluid secretion. It is possible that the transported sulphate ion is not utilized in the establishment of standing osmotic gradients, which may be responsible for fluid secretion. Indeed, the site of sulphate transport in the tubule cells may be entirely different from other ion transport sites.

The ability of the intact fly to excrete sulphate has been verified, but it is difficult to assess the role of sulphate excretion in a terrestrial insect. It is possible that the metabolic turnover of molecules containing sulphur acts as a source of haemolymph sulphate. Thus, sulphur atoms of sulphated polysaccharides and amino acids could be removed, oxidized to sulphate and then excreted. However, in insects, which are generally short-lived, the bodily build up of sulphur in the body, in whatever form, may not be so great as to require its elimination from the insect and storage excretion may suffice.

My thanks are due to Professor J. Shaw, under whose generous supervision this work was undertaken, and also to the S.R.C. for their financial assistance.

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Note added to proof.

It has recently come to the author's attention that the Malpighian tubules of *C. erythrocephala* conjugate β-ecdysone with sulphate (Price & Russell, 1975). Gilbert (1974, p. 372) also reports that the metabolities of insect juvenile hormone can combine with sulphate to form conjugates which are then excreted. Thus the ability of the Malpighian tubules to secrete sulphate may be linked to the metabolism of these hormones.