ACTIVE TRANSPORT OF POTASSIUM BY THE MALPIGHIAN TUBULES OF INSECTS

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INTRODUCTION

The study of excretion in Malpighian tubules by the direct method of collecting the fluid from them and analyzing it has had to await the development of methods capable of handling the relatively small volumes obtainable. A beginning has been made with the blood-sucking bug *Rhodnius* (Ramsay, 1952) and with the larva of the mosquito *Aedes* (Ramsay, 1950, 1951, 1953).

In *Rhodnius* the haemolymph usually contains very much more sodium than potassium. In the fluid collected from the tubules (which will be called 'urine' in this paper), as compared with the haemolymph, the concentration of potassium is greater and that of sodium is less. Since the blood on which the insect feeds contains more potassium and less sodium than the haemolymph and since the excretory mechanism is presumably adapted to maintain the normal ionic ratio in the haemolymph, this greater concentration of potassium in the urine is entirely in accordance with expectation.

The picture in *Aedes* is somewhat different. As in *Rhodnius* the haemolymph contains very much more sodium than potassium and the urine always contains more potassium and less sodium than the haemolymph. Both these ions are reabsorbed into the haemolymph from the urine in the rectum. Although the larva of *Aedes aegypti* is a fresh-water animal, it can survive in solutions of pure NaCl or KCl, of concentration approaching 1%. Under these conditions the cation present in the external medium enters the haemolymph through the anal gills and leaves by the Malpighian tubules; reabsorption from the rectum is not complete, so that the cation entering the body is eliminated via the anus. It is surprising, however, to find that when the animal is kept in a solution of pure NaCl, a condition in which there is a substantial flux of sodium through the body, the concentration of potassium in the urine is always greater than in the haemolymph and the concentration of sodium is always less. Further, when the animal is kept in a solution of pure KCl, under which condition it may be supposed that the excretory mechanism is actively eliminating potassium, there is still considerable reabsorption of potassium from the rectum. It follows that under all conditions there is a circulation of potassium within the body, from the haemolymph into the tubule and from the urine back into the haemolymph via the rectum or the mid-gut.

In the case of *Rhodnius* it is reasonable to interpret the high concentration of potassium in the urine as adaptive to the animal's immediate excretory problem;

but in *Aedes* we cannot regard it as adaptive since it persists under conditions in which the intake of potassium is zero. If it is not adaptive is it simply fortuitous or is it in some way connected with the fundamental processes of urine formation in Malpighian tubules?

Clearly the next step must be to extend these observations to other insects and determine the concentrations of sodium and potassium in haemolymph and urine.

A further question will then be posed: are the differences in concentration the result of active transport or can they be accounted for by passive diffusion? When the movements of electrolytes are under consideration it is not sufficient to know the differences in concentration; it is also necessary to know the difference in electrical potential across the wall of the tubules (see Ussing, 1949). The scope of the investigation has therefore been widened to include measurements of potential difference (p.d.) as well as analysis for sodium and potassium.

In approaching the general problem two considerations must be borne in mind. First, the structure of the Malpighian tubules and their anatomical relations with other organs of the body present a great range of variation; it is reasonable to suppose that this anatomical diversification is not unreflected in their physiological activities. One particularly striking disposition is known as 'cryptonephridism' (Poll, 1934). The distal (alternatively, upper or blindly-ending) portions of the tubules are closely applied to the wall of the rectum and enclosed with it in a 'perinephric' membrane which separates the tubules from the haemolymph. Cryptonephridism is widespread in Coleoptera, and is also found among the larvae of Lepidoptera where it seems to have been independently evolved (Poll, 1938). In relation to a possible circulation of potassium this arrangement is suggestive. Secondly, there is wide variation in the normal sodium/potassium ratio of the haemolymph from one species of insect to another. Boné (1944) has shown that these variations are correlated with feeding habits, carnivorous insects having high ratios, of the order of 20, and herbivorous insects having low ratios, of the order of 0·5 or less. It may be that these variations are directly related to the sodium/potassium ratio of the food, but it is also possible that the normal ratios are maintained by the activity of the excretory system.

A survey of insect excretion, such as has been contemplated above, must clearly be planned to bring these known anatomical and physiological relations within its scope. On the other hand, choice of material is limited by technical considerations and by availability. In the work which this paper describes eight species of insect have been investigated. This is a modest figure; but, as is brought out in the Discussion, a substantial range of conditions has been covered.

MATERIAL AND METHODS

The following insects have been used in this investigation:

Locusta migratoria migratorioides R. & L., adult (Orthoptera, Acridiidae).
Dixippus morosus (al. *Carausius morosus*), adult (Orthoptera, Phasmidae).
Pieris brassicae L., larva (Lepidoptera, Pieridae).

Dytiscus marginalis L., adult (Coleoptera, Dytiscidae).
Tenebrio molitor L., larva (Coleoptera, Tenebrionidae).
Tabanid larva (species not identified) (Diptera, Tabanidae).
Aedes aegypti L., larva (Diptera, Culicidae).
Rhodnius prolixus Stål., adult (Hemiptera, Reduviidae).

A brief description of the excretory system in each of these insects is given in Appendix I, with notes on the operations carried out.

In the previous work on *Aedes* the urine was collected by means of a cannula inserted through the anus into the intestine as described in an earlier paper (Ramsay, 1951). The urine of *Rhodnius* was collected directly from the tubules by piercing the wall with a pipette, but the operative technique used at that time was difficult and unsatisfactory. Means have now been devised which make the operation relatively easy, and these are described in Appendix II.

The analysis by flame photometry is described in detail by Ramsay, Brown & Falloon (1953). It is necessary, however, to add a word about the accuracy of the method. The full potential accuracy is only realized when it is possible to anticipate the order of concentration which is to be measured, so that the apparatus can be previously calibrated over a relatively narrow range. When the order of concentration cannot be anticipated, as in the present work, it is necessary to adjust the apparatus so that it will deal with the extreme limits of concentration which it is reasonable to expect, and this entails some loss of accuracy. This loss of accuracy is difficult to estimate exactly. The figures in Table 1 are qualified by 'estimated error', and this represents the error which in the writer's opinion may effect the analysis in question. Greater accuracy could of course have been achieved by repeating the experiments after the order of concentration had been established; but this would have taken time and the results as they stand are deemed adequate to support the conclusions which are to be drawn from this survey.

The operative technique used for the collection of urine is readily adaptable to the measurement of p.d. across the wall of the tubule. A pipette electrode is inserted into the tubule and an indifferent electrode is dipped into the haemolymph (see also Appendix II).

RESULTS

The concentrations of sodium and potassium in haemolymph and urine are given in Table 1, from which it can be seen that in every case the concentration of potassium in the urine is substantially greater than in the haemolymph. The difference in concentration is more marked in the aquatic insects (*Dytiscus*, *Aedes*, Tabanid) and in *Rhodnius* than in the other terrestrial insects. The concentration of sodium in the urine is generally less than in the haemolymph, but the difference is not so marked as in the case of potassium.

The results of the p.d. measurements are summarized in Table 2, but before they are further considered it is necessary to describe certain observations which, although merely incidental to the measurements, reflect upon them. The normal procedure was (1) to measure the asymmetry potential with both electrodes in the haemolymph, (2) to penetrate the tubule and to thrust the pipette in until the p.d.

reached a constant value, (3) to withdraw the pipette and measure the asymmetry potential again. As an alternative to withdrawing the pipette it was sometimes convenient to thrust it out through the wall of the tubule. While this second penetration, from within outwards, was being made it was often possible to measure a stable

Table 1. *Analysis of haemolymph and urine*

(Concentrations in m.equiv./l.)

	Haemolymph, C_2		Urine, C_1		$\frac{C_1}{C_2}$ (Na)	$\frac{C_1}{C_2}$ (K)
	Na	K	Na	K		
Locusta (1)	75±10	20±3	31±5	167±10	0·41	8·3
(2)	74±10	15±3	66±5	79±10	0·89	5·3
			41±5	146±10	0·55	9·7
(3)	102±10	22±3	128±10	73±10	1·25	3·3
			95±10	95±10	0·93	4·3
					Av. 0·81	6·2
Dixippus (1)	14±3	16±3	13±3	160±10	0·93	10·0
			6±3	186±10	0·43	11·6
			3±3	145±10	0·21	9·1
			7±3	118±10	0·50	7·4
(2)	14±3	17±3	4±3	144±10	0·29	8·5
			1+3	148±10	0·07	8·7
			6±3	125±10	0·43	7·3
			5±3	128±10	0·36	7·5
					Av. 0·40	8·8
Pieris (1)	9±3	30±3	8±3	165±10	0·89	5·5
(2)	5±3	27±3	3±3	144±10	0·60	5·3
			3±3	191±10	0·60	7·1
					Av. 0·70	6·0
Tenebrio (1)	77±5	32±5	14±3	283±10	0·18	8·8
(2)	54±5	53±5	17±3	217±10	0·31	4·1
					Av. 0·24	6·4
Dytiscus (1)	140±15	5±2	44±5	156±10	03·1	31·1
Tabanid (1)	151±15	5±2	25±5	160±10	0·17	3·20
Rhodnius (1)	158±15	6±2	108±10	79±10	0·68	13·2
			93±10	119±10	0·59	19·8
(2)	158±15	4±2	165±10	62±10	1·04	15·5
			113±10	89±10	0·71	22.2
					Av. 0·75	17·7
*Aedes**						
Dist. water adapted	87	3	24	88	0·28	29·3
NaCl adapted	113	3	71	90	0·63	30·0
KCl adapted	87	6	23	138	0·26	21·0

* Average values (from Ramsay, 1953).

p.d., obviously differing from that measured with the pipette in the lumen. It seems likely that in these cases the pipette had entered the cells of the tubule wall and that the p.d. thus measured was developed across the membranes separating the cytoplasm from the haemolymph. Some examples are given in Table 3 from the insects in which this effect was most clearly seen, namely *Pieris*, *Tenebrio* and *Dytiscus*. In these insects the p.d. lumen/haemolymph is positive and the p.d. cells/haemolymph is negative. In four cases, however, a negative p.d. was measured

when the pipette was judged to be fairly in the lumen, although owing to the opacity of the tubule it was impossible to be quite certain of this. These four measurements are included in Table 2, in brackets, but they have been disregarded in calculating the average figures.

Table 2. *Measurements of p.d. across wall of tubule*

(The figures are millivolts. A positive sign indicates that the interior of the tubule was positive with respect to the haemolymph.)

Insect		Measurements	Av.
Locusta	(1)	+1, −16, −13, −11, −12, −8, −15	−16
	(2)	−19, −17, −21, −25, −25, −22, −21, −18, −12	
Dixippus	(1)	+8, +16, +20, +31	
	(2)	+18, +9, +22, +30	+21
	(3)	+29, +16, +19, +34, +31, +29, +15, +21	
Pieris	(1)	+10, +7, +28, +26, +28	
	(2)	+22, (−35), +36, +42	+28
	(3)	+31, +22, +37, +47	
Tenebrio	(1)	+52, +27, (−10), +58	
	(2)	+30	+45
	(3)	+35, +54, +62	
Dytiscus	(1)	+23, +23, (−43), +27, +17, +21	+22
	(2)	+18, +28, +23, (−47), +23	
Rhodnius	(1)	−47, −45, −20, −32, −28, −57, −32	−35
	(2)	−25, −34, −39, −29, −29	
Aedes			
Dist. water adapted	(1)	+24, +30, +24	+21
	(2)	+8, +29, +18, +15	
NaCl adapted	(1)	+10, +22	+13
	(2)	+7, +10, +16, +13	
KCl adapted	(2)	+12, +15, +23	+16
	(2)	+15, +13, +16	

Table 3

(The figures after the insects serve to identify them with the individuals correspondingly numbered in Table 2. The figures for p.d. are in millivolts. For further explanation see text.)

Insect		P.d. lumen/ haemolymph	P.d. cells/ haemolymph
Pieris	(2)	+42	−23
	(3)	+37	−16
		+31	−4
		+47	−12
Tenebrio	(1)	+58	−16
	(2)	+30	−20
	(3)	+54	−25
Dytiscus	(1)	+23	−45
		+27	−18
		+17	−18
		+21	−10
	(2)	+18	−6
		+28	−7
		+23	−47
		+23	−41

Table 2 shows that in all cases except two, *Locusta* and *Rhodnius*, the interior of the tubule is positive with respect to the haemolymph. There does not appear to be any obvious correlation between the p.d. and the concentrations of sodium and

potassium in haemolymph and urine, and in the case of *Aedes* it is to be noted that the p.d. is not greatly influenced by the medium to which the insect is adapted.

In order to decide whether the differences in concentration can be accounted for by passive diffusion, or whether it is necessary to postulate some process of active transport, it is convenient to express the concentration ratios in terms of p.d., using the relation

$$E=\frac{RT}{nF}\log_e\frac{C_1}{C_2},$$

or, for a monovalent cation,

$$E=-58\log_{10}\frac{C_1}{C_2},$$

where E is the p.d. in millivolts, C_1 and C_2 are the concentrations of the ion in the urine and in the haemolymph respectively, and the negative sign indicates that the interior of the tubule is negative with respect to the haemolymph.

Table 4

(The figures are millivolts. For explanation see text.)

Insect	$p.d._{meas.}$	$p.d._{eq}(Na)$	$p.d._{eq}(K)$	$p.d._{eq}(Na)-p.d._{meas.}$	$p.d._{eq}(K)-p.d._{meas.}$
Locusta	−16	+5	−46	+21	−30
Dixippus	+21	+23	−55	+2	−76
Pieris	+28	+9	−45	−19	−73
Tenebrio	+45	+36	−47	−9	−92
Dytiscus	+22	+29	−86	+7	−108
Tabanid	—	+45	−87	—	—
Rhodnius	−35	+7	−72	+42	−37
Aedes					
Dist. water adapted	+21	+32	−85	+11	−106
NaCl adapted	+13	+12	−86	−1	−99
KCl adapted	+16	+34	−79	+18	−95

Thus, for example, if C_1 is 100 m. equiv/l. and C_2 is 10 m. equiv/l. and if the system is equilibrium then the p.d. should be −58 mV. If the p.d. is measured and is found to be less than 58 mV. negative, or if it is positive, then the concentration difference C_1-C_2 must be brought about by active transport.

In preparing Table 4 the average values of C_1/C_2 in Table 1 have been used to calculate the p.d. corresponding to the condition of equilibrium ($p.d._{eq}$). The average value of the p.d. measured ($p.d._{meas.}$) is obtained from Table 2 and is subtracted algebraically from $p.d._{eq}$, and if the sign of the difference $p.d._{eq}-p.d._{meas.}$ is negative then active transport is to be assumed.

Taking first the case of potassium, it is obvious that for those insects in which $p.d._{meas.}$ is positive the presentation of figures in Table 4 is superfluous; potassium must be actively transported across the wall of the tubule. In the cases of *Locusta* and *Rhodnius*, although the differences are negative, some assurance is needed that the figures are significant in relation to the scatter of the observations. The '*t*'

test for significance has been applied, and in both cases the difference is found to be highly significant ($P \ll 0{\cdot}01$). It is therefore concluded that in all cases in which the necessary measurements have been made there is active transport of potassium; and in the case of the Tabanid active transport is highly probable since $p.d._{eq}$ is -87 mV., the highest value recorded.

In the case of sodium the difference $p.d._{eq} - p.d._{meas.}$ is in general more positive than for potassium. Having regard to the scatter of the observations the only case of a negative difference which merits consideration is that of *Pieris*, -19 mV., and here the concentrations of sodium are so small in relation to the errors of analysis that little significance attaches to this figure. There are insufficient grounds for assuming active transport of sodium; in fact, it is admissible as a working hypothesis that the differences in concentration of sodium are brought about by passive diffusion.

DISCUSSION

The main conclusion which can be drawn from the results just described is that in all the insects studied potassium is actively secreted into the tubule. The question now arises as to how far this conclusion is of general application. Although only eight insects have been investigated: (1) they include representatives of five orders (Orthoptera, Hemiptera, Lepidoptera, Coleoptera, Diptera); (2) they include terrestrial insects (*Locusta*, *Dixippus*, *Pieris*, *Tenebrio*, *Rhodnius*) and aquatic insects (*Dytiscus*, *Aedes*, Tabanid); (3) they include insects having a variety of feeding habits—omnivorous (*Locusta*, *Tenebrio*), carnivorous (*Dytiscus*, Tabanid), herbivorous (*Dixippus*, *Pieris*), detritus feeder (*Aedes*), blood-sucker (*Rhodnius*); (4) they include insects having cryptonephridial systems (*Pieris*, *Tenebrio*).

It therefore seems reasonable to suggest that if future work brings to light other insects which do not actively secrete potassium, these will prove to be the exceptions to a general rule.

In the Introduction the suggestion was made that the active secretion of potassium into the tubule—and its subsequent reabsorption into the haemolymph, which is known to occur in *Rhodnius* and *Aedes*—might be an essential process in the formation of urine. It seems possible that the active secretion of potassium, accompanied by some anion, might produce a high osmotic pressure in the tubule which would cause water to pass inwards through the wall; and that this in its turn would promote a passive diffusion of sodium into the tubule. This hypothesis has the attraction of simplicity, but very little further evidence can be adduced in its support. In the course of this work some measurements were made of the freezing-point depression of haemolymph and urine. It was found that in general the osmotic pressure of the urine did not differ widely from that of the haemolymph, but it could be either greater or less, even in different tubules of the same animal. The possibility of some slight concentration or dilution of the haemolymph during the course of these experiments was not rigorously excluded in all cases, and it was decided not to report these observations in detail in this paper.

It is not to be forgotten that the Malpighian tubules are able to concentrate other substances, e.g. dyes (Lison, 1942 and earlier papers) and that they are able to

eliminate discrete masses of insoluble substances, e.g. biliverdin (Wigglesworth, 1943). When one reflects upon the lack of information concerning the composition not only of the urine of insects, but also of the haemolymph, in which organic substances often predominate, it becomes clear that to put forward any comprehensive theory of urine formation would be premature. What appears to be needed is a more detailed knowledge of the process in at least one insect, and in the immediate future work will be directed towards this end.

SUMMARY

1. The concentrations of sodium and potassium in the haemolymph and in the urine have been measured in eight species of insect.

2. The concentration of potassium in the urine is always greater than in the haemolymph. The concentration of sodium in the urine is generally less than in the haemolymph.

3. In seven of the species the difference of electrical potential across the wall of the tubule has also been measured.

4. In these seven species the results lead to the conclusion that potassium is actively secreted into the tubule. It is very probable that the same is true in the eighth case.

5. It seems likely that the excretion of sodium can be brought about by passive diffusion into the tubule.

I wish to thank Lord Rothschild for advice on the method of measuring p.d. and for the loan of a pH meter; Prof. A. L. Hodgkin for advice and for reading this paper in draft; Miss R. Eccles for practical assistance in the early stages of the p.d. measurements.

APPENDIX I

Locusta migratoria migratorioides R. & L., adult (Orthoptera, Acridiidae). A general account has been given by Schindler (1878). There are about 100 tubules, among which he distinguishes two kinds, yellow and white. I have not observed such differences in this species, all the tubules being brownish red in colour. They are of considerable length and are present as loose coils in this posterior part of the abdomen. The pigment makes it difficult to see the exact position of the point of the pipette after its insertion.

Dixippus morosus (al. *Carausius morosus*), adult (Orthoptera, Phasmidae). The tubules are described by de Sinéty (1901). They are numerous and are of three kinds: (1) 'superior' tubules, opening at the annulus between mid-gut and hind-gut, making a short forward loop and running back to end blindly close to the posterior region of the hind-gut. The 'superior' tubules are of uniform appearance throughout their length and are supplied by small branches of the tracheal system reaching them at various points. They are of relatively large diameter and have transparent walls. (2) 'Inferior' tubules, opening at the annulus in pairs and running directly backwards. Their distal regions are dilated and filled with a milky fluid and they terminate in clumps of cells (cells of Sidorot) embedded in the fat body. Each tube has its own trachea which accompanies it over most of its length. Over their non-dilated regions they present the same appearance as the 'superior' tubules. (3) 'Appendices of the mid-gut.' These appear to be very thin tubules opening separately

into the mid-gut and running back over the hind-gut. The 'superior' and 'inferior' tubules are ideal for operations; no attempt has been made to operate on tubules of the third kind.

Pieris brassicae L., larva (Lepidoptera, Pieridae). The tubules of *Vanessa urticae* are described in detail by Henson (1937), who states that his description also applies to *Pieris brassicae*. There are six tubules arising three on each side from a small pulsatile bladder which is derived from the hind-gut. Thin-walled tubules run forwards over the mid-gut and then turn backwards, their walls becoming thicker, and after some convolutions they pass beneath the muscle layer of the rectum and end in a 'cryptonephridial chamber'. Urine was collected from the thin-walled portions near the bladder. Measurements of p.d. were made on these portions and also on the thick-walled portions. The cryptonephridial region has not been investigated.

Dytiscus marginalis L., adult (Coleoptera, Dytiscidae). Described by Rungius (1911). There are four tubules of considerable length, but their walls are brown and opaque and for this reason it is impossible to collect urine except from a short transparent region near the opening into the gut. Several attempts were made but only one was successful in obtaining sufficient urine for analysis. The p.d. was measured in the transparent region and attempts were also made, 'blind', to insert the pipette electrode into the lumen of the opaque region. There is no cryptonephridism in *Dytiscus*.

Tenebrio molitor L., larva (Coleoptera, Tenebrionidae). A very complete description is given by Poll (1934). There are six tubules. Near their opening into the gut they are transparent, but over most of their course through the body cavity they are brownish and rather opaque. They are gathered together into a common trunk and enter the cryptonephridial region where they spread out over the walls of the rectum. Collections of urine were made from the transparent region and measurements of p.d. were made on the pigmented region.

Tabanid larva (Diptera, Tabanidae). Two of these larvae were brought in by a collector. Preliminary dissection of one of them showed that it was very suitable for operation; the other was used for the collection of urine. No measurement of p.d. was attempted. There are four tubules, briefly described by Stammer (1934), which run freely in the body cavity. Over most of their length their walls contain a brownish pigment which is not so dense as to make operation difficult.

Aedes aegypti L., larva (Diptera, Culicidae). Described by Wigglesworth (1933). There are five short tubules, their blind ends being closely applied to the rectum. They are opaque except just before they open into the gut. As already mentioned, the urine was collected from the intestine. It was not possible to operate on the tubules *in situ*, and p.d. had to be measured on preparations torn open upon a slide under liquid paraffin.

Rhodnius prolixus Stål., adult (Hemiptera, Reduviidae). Very fully described by Wigglesworth (1931). There are four tubules. Each tubule enters the rectum through a specialized region, the ampulla. The proximal portion has transparent walls with a brush border ('bürstensaum') internally and appears to be the site of precipitation of uric acid and reabsorption of water. The distal portion has slightly opaque walls with a honeycomb border ('wabensaum') and it is here that the urine is formed. The tubules are relatively long and convoluted and end blindly near the rectum. Immediately after the animal is fed there is a period of rapid diuresis during which the greater part of the water and salts taken in with the blood-meal is eliminated. In the present experiments urine was collected from the distal portion of the tubule, and the measurements of p.d. were also made on this portion, during the period of diuresis.

APPENDIX II

Briefly, the technical problem of operation is as follows. The wall of the tubule can readily be penetrated by a fine-pointed pipette (5μ diameter) but the finer the orifice the more likely it is to become blocked. If a wide-mouthed (30μ diameter) pipette is used the force required to penetrate is greater; the tubule is stretched and is usually dragged into an unfavourable position. To penetrate with a wide-mouthed pipette it is necessary to hold the tubule firmly and to apply the pipette as close as possible to the place at which the tubule is held. This calls for the use of forceps with very fine points. For this purpose ordinary steel forceps are unsuitable because the points become exceedingly delicate when ground sufficiently thin. This difficulty is overcome by using forceps tipped with tungsten.

The blades of the forceps (Fig. 1*a*) are made from strips of steel about 2·5 × 0·2 × 0·02 in. (old blades of the miniature 6 in. hacksaw are used). They can be spot-welded together at the base, but it is more convenient to bolt them to a small block of brass since this makes possible a final adjustment to bring the points together. A piece of tungsten wire 0·5 in. long and 0·02 in. in diameter is brazed to the tip of each blade (Fig. 1*b*). The tungsten

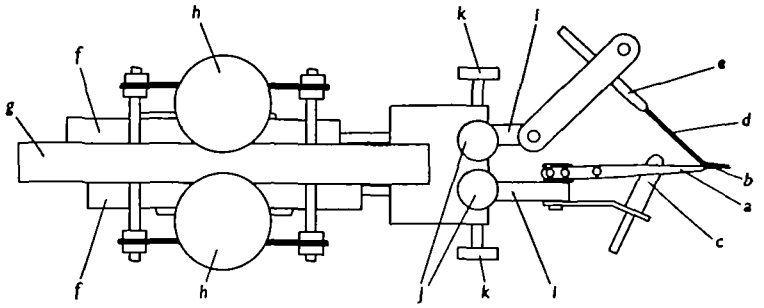

Fig. 1. Micromanipulator assembly carrying pipette and forceps. *a*, blades of forceps; *b*, tungsten tips of forceps; *c*, rubber bulb between blades of forceps; *d*, silica pipette; *e*, wider tube into which pipette is sealed; *f*, bar taking fore and aft movement; *g*, bed upon which bar slides; *h*, adjustment controlling fore and aft movement; *i*, square-section bar spring-mounted on bar *f*; *j*, adjustment controlling lateral movement; *k*, adjustment controlling vertical movement.

wires are then dipped into molten sodium nitrite and allowed to dissolve slowly until they taper to moderately fine points. The points are then ground by hand upon an Arkansas stone, under a medium power binocular. The 'flats' where the points meet are made by grinding on a thin sheet of bakelite covered with the finest carborundum, the sheet being slipped between the points. An adjusting screw is provided which brings the points together in the 'at rest' position. For separating the points a small rubber bulb (fountain pen reservoir) is mounted between the blades and connected to a rubber tube which is held in the operator's mouth (Fig. 1*c*).

The pipette (Fig. 1*d*) is drawn from thin walled silica tubing of about 1 mm. diameter down to an orifice of about 2μ. A jagged end makes penetration easier and can be produced by crushing the point with fine forceps under the binocular. The final orifice may be as much as 30μ in diameter. The terminal portion is then bent over to an angle of about 30°. Prior to use the pipette is sealed into a wider tube (Fig. 1*e*) and the terminal portion is filled with liquid paraffin stained with Sudan Blue.

It is of course necessary to mount both the forceps and the pipette on micromanipulators, but these do not require to have the high precision of instruments used for operations upon cells. The fore and aft movement is of the rack and pinion style, but operates by friction; a steel bar (Fig. 1*f*) of circular cross-section is made to slide back-

wards and forwards along a brass bed (Fig. 1*g*) by rotation of a handwheel (Fig. 1*h*). The steel bar carries a short length of spring steel wire which joins it to a piece of square-section brass bar (Fig. 1*i*). The lateral and vertical movements are brought about by screws (Fig. 1*j*, *k*) which displace the brass bar against the reaction of the spring. It is convenient to build the two manipulators upon the same framework and the combined instruments are carried on a Palmer adjustable stand.

Nearly all the operations described in this paper were carried out on tubules *in situ*. One of the troubles met with is that light from an external source can be reflected from the numerous curved surfaces of partly exposed organs and the glare from these makes it difficult to see below the surface of the haemolymph. This is overcome by illuminating through a glass rod, about 1 mm. in diameter at the end, which dips into the haemolymph and is equivalent to a submerged source of light.

It is also necessary to protect the preparation from desiccation. This might be arranged by working in a moist chamber, but such a chamber would have to be inconveniently large to accommodate the various pieces of apparatus which are used. In any case, a completely saturated atmosphere is difficult to achieve in a chamber which has to admit the operator's hands and allow them sufficient freedom of movement. A neater method is to generate a stream of saturated air and direct it over the preparation. Compressed air is humidified by bubbling through hot water and is then allowed to pass through a length (about 15 ft.) of tubing so that it can take up the temperature of the room. The air stream leaving the tubing is saturated with water vapour and is if anything slightly above room temperature. When tested with a mirror it shows a slight tendency to produce fogging. This, however, is advantageous rather than otherwise since the heat produced by the animal and by the illuminator will tend to keep the preparation at a slightly higher temperature than the air. Experience has in fact shown that on the whole there is a slight increase in the osmotic pressure of the haemolymph in experiments which last about 1 hr.

The insect is pinned out on a small operating table and the Malpighian tubules are exposed. The illuminator is placed in position. A suitable length of tubule is selected and the manipulator is lowered so that the forceps, with their points held apart, descend upon the tubule and are allowed to grasp it. The point of the pipette is laid upon the tubule just beyond the forceps, is lowered so as to indent the tubule and is then thrust forwards. If it is seen to penetrate air pressure is applied to drive out a droplet of liquid paraffin. The pipette is thrust forwards a second time so that its point lies just beyond the droplet which then serves as a seal to prevent the entry of haemolymph through the wound. It is often necessary to apply slight suction to start the flow of urine into the pipette, but once this has begun capillary forces usually become effective and suction is no longer needed. As the urine rises into the pipette the walls of the tubule can be seen to collapse slightly. Much of the urine momentarily present in the tubule is thus collected at once. If the tubule is secreting actively the urine continues to rise in the pipette and as much as 0·1 cu.mm. may be collected in 15 min. under favourable conditions. If secretion is slow there is very little movement after the initial rise and one has to be content with 0·01 cu.mm. or less. At the end of the period of collection the pipette and forceps are raised together (by the adjustment on the Palmer stand) so as to clear the haemolymph. The vertical adjustment of the pipette mounting is moved so as to raise the pipette slightly, but this merely bends it since the point is still inside the tubule. The pipette is drawn quickly backwards and as it emerges from the tubule it flicks clear of the forceps and there is no time for adherent haemolymph to be drawn into it. The pipette is then removed from the manipulator with all possible speed and the sample is blown out into a watch-glass filled with liquid paraffin. If the watch-glass has previously been varnished with bakelite there is no tendency for the droplet to spread over the surface of the glass.

For the measurement of p.d. a silver wire, coated with silver chloride, is inserted into a pipette which is filled with a saline approximating in composition to the urine, and the pipette is thrust into the tubule in the ordinary way. If necessary a droplet of liquid paraffin can be drawn up into the tip of the pipette and later expelled inside the tubule to form a seal to the wound. The other electrode is a glass tube of wider aperture which dips into the haemolymph; this tube contains a saline of approximately the same composition as the haemolymph, set in agar, and contact with it is made through a chloride-coated silver wire as before. The asymmetry potential is first measured with both electrodes dipping into the haemolymph and is compared with the potential measured when the pipette is inserted into the tubule. The potentials are measured with the valve voltmeter which forms part of the Cambridge Instrument Co. pH meter. Since this instrument will only measure potentials negative with respect to earth a backing-off potential of -500 mV. is applied from a potentiometer in series with the lead to the pipette.

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