

## THE FATE OF CALCIUM IN THE DIET OF *RHODNIUS PROLIXUS*: STORAGE IN CONCRETION BODIES IN THE MALPIGHIAN TUBULES

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### Summary

We have investigated the fate of the large amounts of calcium ingested by *Rhodnius prolixus* in its meals of blood.  $^{45}\text{Ca}^{2+}$  injected into the haemolymph or fed to fifth-stage *Rhodnius* reared on rabbits is accumulated at high concentrations in the cells of the upper Malpighian tubules; very little is excreted from the body. This  $^{45}\text{Ca}^{2+}$  accumulation goes on continuously for at least 12 days and the rate of uptake is increased several-fold within 3–4 days of a meal. The extent of calcium accumulation in tubule cells is correlated with the presence of intracellular membrane-bound concretion bodies, which are therefore likely sites of calcium deposition. X-ray diffraction showed that the calcium deposits are non-crystalline. Tubules from rabbit-fed fifth-stage *Rhodnius* contain  $410\text{ mmol l}^{-1}$  calcium; in those from chicken-fed insects the calcium concentration is over  $1\text{ mol l}^{-1}$ ; and in those fed *in vitro* on heparinised low- $\text{K}^+$  sheep blood the calcium concentration is only  $21\text{ mmol l}^{-1}$ . The concentration of calcium in the haemolymph in all these insects was  $8\text{ mmol l}^{-1}$  and its activity determined by an ion-selective electrode was  $2.5\text{ mmol l}^{-1}$ .  $^{45}\text{Ca}^{2+}$  deposited in the tubules is readily exchangeable, but the efflux preferentially passes to the haemolymph side of the tubule epithelium. The ability to sequester calcium in the Malpighian tubules may prevent calcium from interfering with reabsorptive processes in the rectum.

### Introduction

Young (non-adult) stages of *Rhodnius prolixus* take very large meals of blood of

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about 10–12 times their body weight (Buxton, 1930). Since the mammalian or avian blood on which they usually feed contains at least  $1.5 \text{ mmol l}^{-1}$  calcium (Altman and Dittmer, 1974), fed *Rhodnius* seem likely to face a dietary excess of calcium considerably greater than that faced by most other insects and, indeed, most other animals. This paper examines the question of how *Rhodnius* deals with the excess calcium. It turns out that the largest part is deposited in the cells of the upper Malpighian tubules, some is retained in the haemolymph and relatively little is eliminated from the body. Conceivably, the calcium deposits may act as a store and be drawn upon when calcium is in short supply, but their normal function is more likely to be to immobilise excess calcium and so prevent it from interfering with such vital functions as water conservation.

### Materials and methods

The insects used were *Rhodnius prolixus* Stål (Hemiptera) kept in laboratory culture. They came from three different stocks held (1) in the Department of Zoology, Cambridge, UK, (2) at Instituto Venezolano de Investigaciones Científicas (IVIC), Caracas, Venezuela, and (3) at the Centro de Investigaciones Biomédicas, Universidad de Carabobo, (Núcleo Aragua) at Maracay, Venezuela. Experiments were performed with fourth- and fifth-stage (juvenile) insects from the Cambridge stock, except where mentioned.

Malpighian tubules were dissected from insects under saline using a binocular microscope and isolated in drops of saline under liquid paraffin as described earlier (Maddrell *et al.* 1988). Most experiments used the upper parts of the Malpighian tubules only, but for some procedures entire tubules, i.e. the complete length from the tip to the point of attachment to the hindgut (Wigglesworth, 1931), were taken. To stimulate the tubules to secrete,  $10^{-5} \text{ mol l}^{-1}$  5-hydroxytryptamine (5-HT) was included in the saline (Maddrell *et al.* 1971). The standard saline used had the following composition ( $\text{mmol l}^{-1}$ ): NaCl, 145; KCl, 8.6;  $\text{CaCl}_2$ , 2.0;  $\text{MgCl}_2$ , 8.5; Hepes, 5.0; pH adjusted to 7.0 with NaOH. When needed, we omitted the  $\text{CaCl}_2$  to produce a calcium-free saline. Solutions containing  $^{45}\text{Ca}^{2+}$  and  $[^{14}\text{C}]\text{sucrose}$  were obtained from Amersham International, UK. To measure the permeability of the tubule wall to  $^{45}\text{Ca}^{2+}$  and  $[^{14}\text{C}]\text{sucrose}$ , tubules were bathed in saline containing one of these tracers at about  $20\,000 \text{ cts min}^{-1} \mu\text{l}^{-1}$ . Drops of secreted fluid were collected at intervals, their diameters measured and their radioactive content determined in a liquid scintillation counter. The permeability of the wall was then determined from the formula (Ramsay, 1958):

$$b = [(S/M)a] / (1 - S/M), \quad (1)$$

where  $b$  is the permeability in  $\text{nl min}^{-1} \text{ mm}^{-2}$ ,  $a$  is the rate of fluid secretion per  $\text{mm}^2$  of tubule surface ( $7 \text{ mm}^2$  for a fifth-stage tubule) and  $S/M$  is the ratio of the concentration of tracer in secreted fluid ( $S$ ) to that in the medium ( $M$ ).

Insects were fed artificially on saline containing  $^{45}\text{Ca}^{2+}$ , with  $2 \text{ mmol l}^{-1}$  ATP as

a phagostimulant (Friend and Smith, 1975), through a stretched Parafilm membrane with the saline warmed to 38°C in a water bath. The meal size was assessed by weighing the insects before and after feeding.

To inject  $^{45}\text{Ca}^{2+}$  in saline into the haemolymph of an insect, one of its metathoracic legs was cut half way along the femur. A 10  $\mu\text{l}$  Hamilton syringe containing  $^{45}\text{Ca}^{2+}$  in saline was pushed into the cut end of the leg and waxed in position with a beeswax-resin mixture applied with an electrically heated wire loop. After the requisite amount of fluid had been injected, the leg was held tightly at its base with fine forceps while the needle was withdrawn and the cut end of the leg sealed with wax. Haemolymph samples were taken later by cutting a metathoracic and/or prothoracic leg and collecting the haemolymph that emerged in a drawn-out Pasteur pipette while gently squeezing the insect. The haemolymph was blown out under liquid paraffin to measure its volume from the diameter of the spherical drop. The cut end of the leg was then resealed with wax.

$^{45}\text{Ca}^{2+}$  accumulated in Malpighian tubules was routinely determined after washing them for at least 10 min in tracer-free 5-HT-containing saline, so as to remove  $^{45}\text{Ca}^{2+}$  from the surface of the tubule and from the lumen. Each tubule was then osmotically disrupted in a 10  $\mu\text{l}$  drop of distilled water. The tubule plus drop was then transferred to liquid scintillant (Aquasol) in a vial for counting.

X-ray diffraction was performed in the Molecular Structure Laboratory at IVIC using a rotating anode X-ray generator.

For electron microscopy, tubules were immersed overnight at 4°C in a fixative solution consisting of 2.4 % glutaraldehyde in 0.1 mol l<sup>-1</sup> phosphate buffer, pH 7.4, plus 6 % sucrose. Following washing in buffer solution the tissues were post-fixed in 0.5 % aqueous osmium tetroxide solution for 30 min. Specimens were then briefly washed in buffer followed by a further three washings in distilled water, *en bloc* stained with 2 % aqueous uranyl acetate for 1 h, dehydrated through an ascending series of ethanols, 5 min each, to 100 % ethanol, 3×20 min, and thence to propylene oxide, 3×20 min. The tubules were finally embedded in Araldite CY212. Ultrathin sections, 50–70 nm thick, were cut on a Reichert Ultracut microtome, mounted on copper grids, stained with uranyl acetate and lead citrate and examined in a Philips EM 420 at 80 kV.

Analyses of calcium in Malpighian tubules or in samples of haemolymph were made using flame photometry after sonicating the tubules or drops of haemolymph in 25  $\mu\text{l}$  of distilled water in small plastic vials. 50  $\mu\text{l}$  of 0.5 mol l<sup>-1</sup> HCl was then added, and the volume made up to 150  $\mu\text{l}$  with distilled water. Calcium measurements were made by diluting each vial's contents for use with an atomic absorption flame spectrophotometer in the Chemistry Department at IVIC. The results are given as mmol of calcium per litre of fluid for samples of haemolymph and as mmol of calcium per litre of volume of the cells for analyses of the calcium content of tubules.

The activity of calcium in small samples of haemolymph (15–20  $\mu\text{l}$ ) was measured using a calcium-selective membrane electrode, built using a membrane obtained from W. Müller, Zurich, Switzerland (Affolter and Sigel, 1979).

All measurements are given as mean  $\pm$  s.e. (*N*). Experiments were performed at room temperature, 22–24°C.

## Results

### *Transport of calcium across the walls of the Malpighian tubules*

Absorption of the products of digestion in insects usually occurs largely by diffusion through the permeable epithelia of the midgut; this process is accelerated by uptake of fluid, which concentrates the solutes in the lumen of the gut (Treherne, 1967). As a result, even unwelcome or toxic materials, such as sulphate ions, magnesium ions and nicotine, from the diet all readily cross into the haemolymph. The influence of such materials is reduced both by the protective role of the perineurium, the epithelial blood–brain barrier of insects (Treherne and Pichon, 1972), and by the ability of the Malpighian tubules to remove those substances from the haemolymph by active transport (Maddrell and Phillips, 1975a; Phillips and Maddrell, 1974; Maddrell and Gardiner, 1976). It was natural, then, first to examine the ability of the Malpighian tubules of *Rhodnius* to excrete calcium ions.

The upper, fluid-secreting parts of the Malpighian tubules of *Rhodnius* have a low permeability to substances that passively cross the wall *via* the paracellular route and do not enter the cells of the tubule (Maddrell, 1980; O'Donnell *et al.* 1984; Whittembury *et al.* 1986). To determine whether calcium ions cross the epithelium other than by passive means, we measured  $^{45}\text{Ca}^{2+}$  levels in the fluid secreted by upper Malpighian tubules isolated from fifth-stage insects and bathed in standard saline containing  $10^{-5}\text{ mol l}^{-1}$  5-HT and  $^{45}\text{Ca}^{2+}$  at  $25000\text{ cts min}^{-1}\mu\text{l}^{-1}$ . Because even slight damage can greatly increase tubule permeability, we first similarly measured the permeability of the tubules to [ $^{14}\text{C}$ ]sucrose; sucrose is known to cross the tubule wall entirely paracellularly (O'Donnell *et al.* 1984; Whittembury *et al.* 1986). The 10 least permeable tubules of 50 tubules tested had a mean permeability to [ $^{14}\text{C}$ ]sucrose of  $0.033 \pm 0.004\text{ nl min}^{-1}\text{ mm}^{-2}$  ( $5.5 \times 10^{-8}\text{ cm s}^{-1}$ ) but a mean permeability to  $^{45}\text{Ca}^{2+}$  of only  $0.020 \pm 0.002\text{ nl min}^{-1}\text{ mm}^{-2}$  ( $3.3 \times 10^{-8}\text{ cm s}^{-1}$ ). This means that fluid secreted by a tubule at, say,  $70\text{ nl min}^{-1}$  contained on average only 0.2% of the concentration of calcium in the bathing fluid. Clearly, calcium does not receive an accelerated passage across the tubule wall. Indeed, the tubules' permeability to [ $^{14}\text{C}$ ]sucrose is consistently 1.6 times as high as the permeability to  $^{45}\text{Ca}^{2+}$  (Fig. 1).

### *Accumulation of calcium in tubule cells*

Although  $^{45}\text{Ca}^{2+}$  only slowly crosses the tubule wall into the lumen, it is, however, unlike sucrose, rapidly accumulated in the tubule cells. For example, four tubules from two fifth-stage insects taken 7 days after moulting were exposed to [ $^{14}\text{C}$ ]sucrose solution for 2 h then washed twice for 5-min in tracer-free saline. The tubules then contained radioactivity equivalent to only  $1.74 \pm 0.20\text{ nl}$  of the original solution. The other four tubules from the same insects exposed instead to

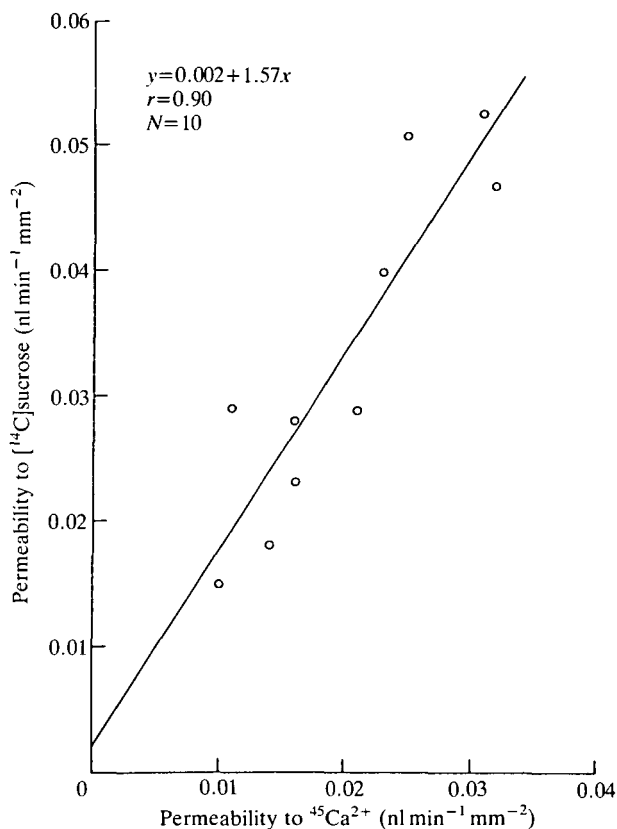


Fig. 1. The relationship between the permeability of isolated Malpighian tubules (from fifth-stage insects) to  $[^{14}\text{C}]\text{sucrose}$  and to  $^{45}\text{Ca}^{2+}$ . The calculated regression line and its equation are shown.

$^{45}\text{Ca}^{2+}$ -containing solution for 2 h contained radioactivity equivalent to  $51.0 \pm 3.6$  nl of the original bathing saline, even after two 5-min washes in tracer-free saline. As described below, tubules exposed for longer periods accumulate proportionately more calcium.

#### *Calcium does not accumulate in the cells of the lower tubule*

Properties of *Rhodnius* Malpighian tubules are often different in the upper, fluid-secreting part of the tubule and in the lower part of the tubule, which is concerned with active transport of uric acid and reabsorption of KCl (O'Donnell *et al.* 1983; Maddrell and Phillips, 1975b). Active transport of nicotine and other alkaloids, however, goes on equally in both upper and lower segments of the tubule (Maddrell and Gardiner, 1976). We investigated whether  $^{45}\text{Ca}^{2+}$  is accumulated in the lower tubule as it is in the upper tubule. We exposed entire Malpighian tubules (i.e. upper and lower regions) from fifth-stage insects taken 10 days after moulting to saline containing  $^{45}\text{Ca}^{2+}$  and 5-HT for periods of up to 7 h. The tubules were then washed for 20 min in non-radioactive saline with 5-HT. The

lower tubules were cut away from the upper parts and the separate pieces counted. The results showed that the lower tubules contained very few counts, equivalent on average to  $0.6 \pm 0.1$  nl ( $N=15$ ) of bathing fluid, while the upper tubules contained radioactivity equivalent to, on average,  $9.9 \pm 1.2$  nl ( $N=4$ ) after 25 min,  $21.5 \pm 2.3$  nl ( $N=4$ ) after 60 min,  $34.4 \pm 1.9$  nl ( $N=4$ ) after 140 min and  $76.2 \pm 3.9$  nl ( $N=4$ ) after 420 min. Even more dramatic differences came from experiments with insects injected with  $^{45}\text{Ca}^{2+}$ . Tubules taken 8–12 days later contained, on average,  $^{45}\text{Ca}^{2+}$  equivalent to  $5563 \pm 594$  nl ( $N=15$ ) of the haemolymph in the cells of the upper tubule but  $^{45}\text{Ca}^{2+}$  equivalent to only  $1.2 \pm 0.3$  nl ( $N=8$ ) of the haemolymph in the cells of the lower tubule, more than 4000 times less.

Evidently, significant accumulation of  $^{45}\text{Ca}^{2+}$  is entirely confined to the upper Malpighian tubules. Their content of  $^{45}\text{Ca}^{2+}$  appears to increase steadily with time. All further experiments were carried out using the upper tubules.

*Progressive accumulation of  $^{45}\text{Ca}^{2+}$  by the upper tubules*

A total of 87 tubules from fifth-stage insects taken 10 days after moulting were incubated in  $^{45}\text{Ca}^{2+}$ -containing saline for periods of up to 400 min and their  $^{45}\text{Ca}^{2+}$  content then determined. The results are shown as the lower line in Fig. 2. They show a continuous accumulation of  $^{45}\text{Ca}^{2+}$ , though at a steadily declining rate. Preliminary experiments had suggested that tubules taken from fed insects accumulated  $^{45}\text{Ca}^{2+}$  more rapidly; many transport properties of the Malpighian tubules of *Rhodnius* are stimulated in the days after a meal (Maddrell and

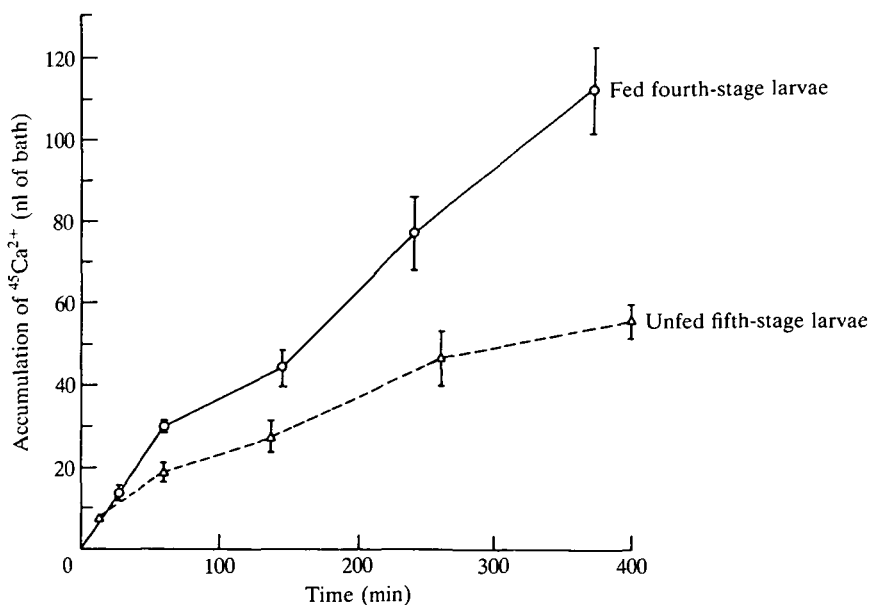


Fig. 2. The amounts of  $^{45}\text{Ca}^{2+}$  accumulated with time *in vitro* by Malpighian tubules isolated either from unfed fifth-stage insects ( $\Delta$ ) or from fourth-stage insects fed 7 days previously ( $\circ$ ). The vertical lines attached to the points show the extent of  $\pm$ s.e. Each point is the mean of 12 determinations.

Gardiner, 1975; O'Donnell *et al.* 1983). So we repeated the experiments with fourth-stage insects fed 7 days previously (i.e. they were taken 2–3 days before moulting). Although these tubules are smaller than those from fifth-stage insects (Maddrell *et al.* 1988), they accumulate  $^{45}\text{Ca}^{2+}$  nearly twice as fast (upper line in Fig. 2).

To test accumulation beyond 7 h, recently fed fifth-instar insects were injected with  $3 \times 10^6 \text{ cts min}^{-1} \text{ } ^{45}\text{Ca}^{2+}$  in  $5 \mu\text{l}$  of saline through a metathoracic leg. After the required period, samples of haemolymph were taken, and the upper tubules were removed and washed for 20 min in tracer-free 5-HT-containing saline. The  $^{45}\text{Ca}^{2+}$  contents of the haemolymph samples and tubules were then determined. The results are given in Fig. 3 and show that  $^{45}\text{Ca}^{2+}$  is taken up at a more or less uniform rate for at least 12 days. At the end of this time, each tubule contained  $^{45}\text{Ca}^{2+}$  equivalent to that found in 6300 nl of haemolymph and more than half of the injected  $^{45}\text{Ca}^{2+}$  had been sequestered in the cells of the upper tubules.

The total volume of the cells (excluding the extracellular spaces) in a single upper tubule was determined to be 100 nl by measurement of the overall tubule dimensions under a binocular microscope fitted with an ocular micrometer and from measurements made on a series of electron micrographs of tubules cut in cross section (courtesy of Dr Helen Skaer). The concentration of calcium in *Rhodnius* haemolymph measured by flame photometry was  $8.13 \pm 0.20 \text{ mmol l}^{-1}$  of fluid ( $N=8$ ). It follows that, 12 days after injection of  $^{45}\text{Ca}^{2+}$ , the calcium concentration in the cells is at least 60 times higher than that in the haemolymph, that is close to  $500 \text{ mmol l}^{-1}$  of cells. As a direct test of this, we measured by flame

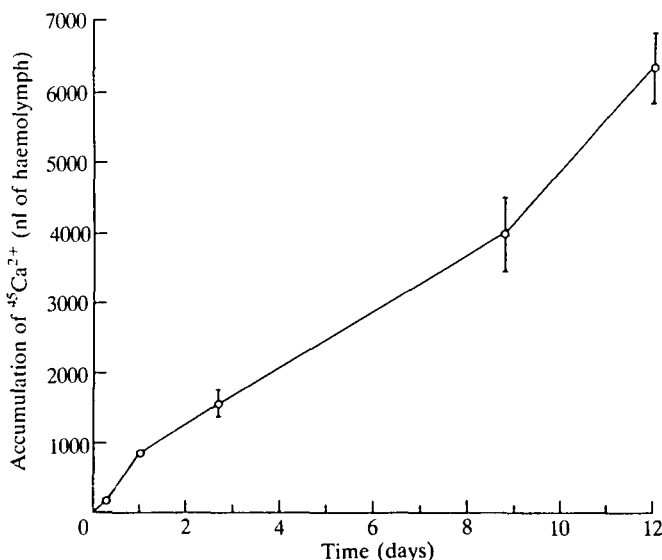


Fig. 3. The amounts of  $^{45}\text{Ca}^{2+}$  accumulated from the haemolymph with time by Malpighian tubules of fifth-stage insects. The insects were fed 1–6 days before injection of  $^{45}\text{Ca}^{2+}$  into the haemolymph. The vertical lines attached to the points show the extent of  $\pm \text{s.e.}$  Each point is the mean of 10 determinations.

photometry the calcium content of 30 tubules from 11 unfed insects. They contained an average of  $410 \pm 53 \text{ mmol l}^{-1}$  of cells ( $N=11$ ).

*Increase in the rate of calcium accumulation after feeding*

To investigate how feeding affects the ability of tubules to accumulate calcium, we exposed tubules from fed and control (unfed) fifth-stage insects for 90 min to saline containing  $^{45}\text{Ca}^{2+}$  and 5-HT. The tubules were washed for 20 min in tracer-free 5-HT-containing saline, before their radioactive content was determined. The results are shown in Fig. 4. By the second day after feeding, the rate of  $^{45}\text{Ca}^{2+}$  accumulation was more than three times higher than in the unfed controls; a significant difference persisted over at least the next 20 days (data for the period beyond 9 days are not shown).

*Retention of calcium in fed fifth-stage insects*

We have seen that calcium levels in upper Malpighian tubules may reach  $0.4 \text{ mol l}^{-1}$ . Thus, it is possible that the greater part of the calcium in the diet may be retained in the tubule cells. On reaching the fifth stage, a *Rhodnius* has in its lifetime consumed a maximum of  $300 \mu\text{l}$  of blood (Gardiner and Maddrell, 1972). Since mammalian blood contains  $1.5 \text{ mmol l}^{-1} \text{ Ca}^{2+}$ , this represents, at most,

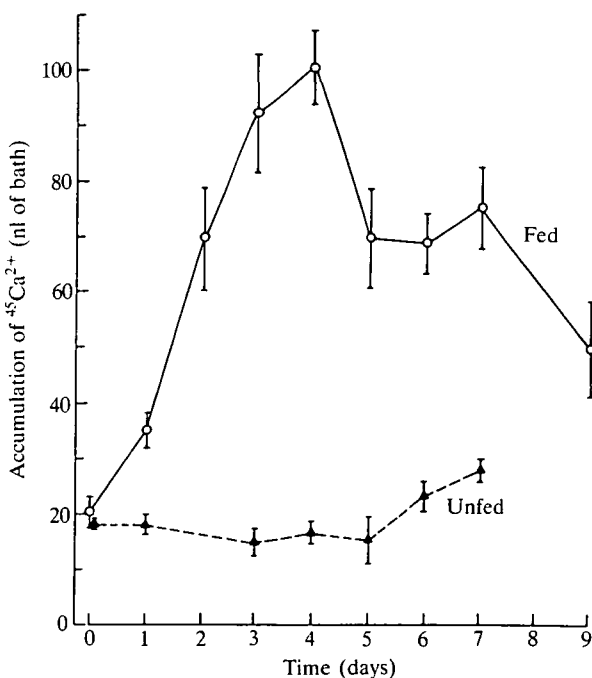


Fig. 4.  $^{45}\text{Ca}^{2+}$  taken up in 90 min *in vitro* by Malpighian tubules from fifth-stage insects fed on day 0 and taken at increasing times afterwards (○;  $N=10$ ) and from control unfed fifth-stage insects (▲;  $N=8$ ). The vertical lines attached to the points show the extent of  $\pm \text{s.e.}$



450 nmol of calcium. The haemolymph volume of a fifth-stage *Rhodnius* is about 15  $\mu\text{l}$  (Maddrell and Gardiner, 1980); at 8  $\text{mmol l}^{-1}$  its calcium content is thus 120 nmol. Four upper tubules, each of 100 nl and with 0.4  $\text{mol l}^{-1}$  calcium in them together contain at least 160 nmol. So, tubules and haemolymph may together hold at least 60 % of the lifetime calcium load. The figure may be considerably higher than this as very few insects succeed in taking a full meal at every possible opportunity.

To test the possibility that calcium is retained in the body and not eliminated, we measured  $^{45}\text{Ca}^{2+}$  elimination from five fifth-stage insects fed 2 days previously and injected through a leg with  $3.3 \times 10^6 \text{ cts min}^{-1} \text{ } ^{45}\text{Ca}^{2+}$  in 5  $\mu\text{l}$  of saline. Each insect was kept in an individual glass vial on a small piece of filter paper. Excreted material was absorbed by the paper and was removed in a daily check and its  $^{45}\text{Ca}^{2+}$  content measured. The results (Fig. 5) showed that  $^{45}\text{Ca}^{2+}$  is steadily eliminated, but at a very slow rate. By 15 days after injection, only  $0.54 \pm 0.09 \%$  ( $N=5$ ) of the injected  $^{45}\text{Ca}^{2+}$  had been excreted.

As a further test, we fed fourth-stage insects on saline containing  $10^5 \text{ cts min}^{-1} \mu\text{l}^{-1} \text{ } ^{45}\text{Ca}^{2+}$  until they had ingested about 15  $\mu\text{l}$ , when they were allowed to feed to completion on rabbit blood (a further 100  $\mu\text{l}$  of fluid). The elimination of  $^{45}\text{Ca}^{2+}$  was followed, this time for a period of 21 days (Fig. 6). The average amount eliminated in this time was  $73\,404 \pm 14\,921 \text{ cts min}^{-1}$  ( $N=7$ ), about 4 % of that ingested.

There is thus a noticeably higher rate of elimination of  $^{45}\text{Ca}^{2+}$  ingested by fourth-stage insects than from fed fifth-stage insects injected with  $^{45}\text{Ca}^{2+}$ . It is instructive to consider why this might be so. The fifth-stage insects were each injected with  $3.3 \times 10^6 \text{ cts min}^{-1} \text{ } ^{45}\text{Ca}^{2+}$ , while the fourth-stage insects were each fed a meal containing about  $1.5 \times 10^6 \text{ cts min}^{-1} \text{ } ^{45}\text{Ca}^{2+}$ ; it follows that both had

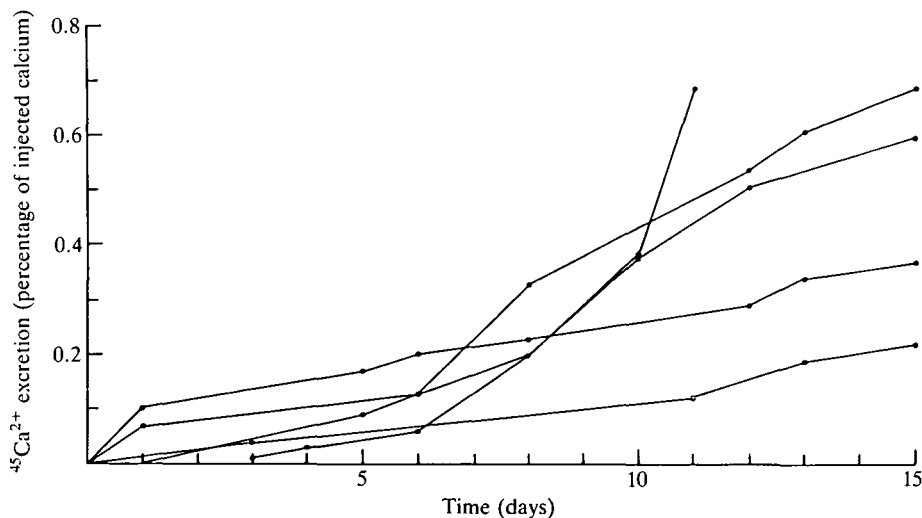


Fig. 5. Cumulative excretion of  $^{45}\text{Ca}^{2+}$  by individual fifth-stage insects fed 2 days before being injected with  $^{45}\text{Ca}^{2+}$  on day 0.

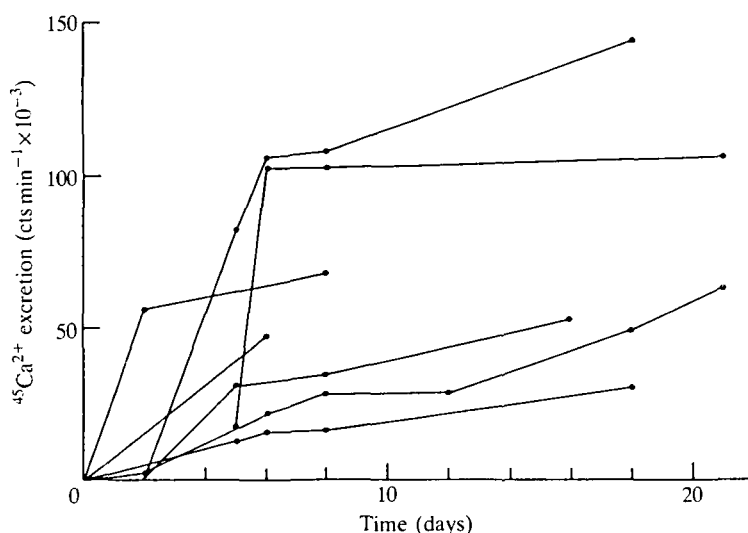


Fig. 6. Cumulative excretion of  $^{45}\text{Ca}^{2+}$  by individual fourth-stage insects fed  $^{45}\text{Ca}^{2+}$  in saline and on blood on day 0. Several insects produced no  $^{45}\text{Ca}^{2+}$ -containing drops after an initial series, so the lines describing their  $^{45}\text{Ca}^{2+}$  elimination do not extend beyond the time at which the last drop was collected.

$^{45}\text{Ca}^{2+}$  at an overall specific activity of about  $5000 \text{ cts min}^{-1} \text{ nmol}^{-1}$  calcium. However,  $^{45}\text{Ca}^{2+}$  injected directly into the haemolymph of fifth-stage insects would initially be present there at a specific activity some five times higher than this. In addition, the Malpighian tubules of these insects would be capable of uptake of  $^{45}\text{Ca}^{2+}$  at a rate about four times higher than at the time of feeding 2 days earlier (Fig. 4). Because of these two factors,  $^{45}\text{Ca}^{2+}$  uptake into the Malpighian tubules of this group of insects would initially be very rapid, leaving less available to be eliminated from the body. In contrast,  $^{45}\text{Ca}^{2+}$  ingested by fourth-stage insects would mix with calcium in both meal and haemolymph before reaching the tubules and would initially be taken up only slowly by them, allowing a more rapid loss of  $^{45}\text{Ca}^{2+}$  by excretion.

Both sets of results agree in showing that much the greater part of the calcium in the diet is retained in the body.

#### *Release of $^{45}\text{Ca}^{2+}$ from upper tubule cells*

The question naturally arises as to what might be the function of the calcium deposits in the Malpighian tubules. We shall see below that changes in diet can greatly affect the quantity of calcium in the tubules. The deposited calcium might, therefore, act as a store through which the haemolymph level can be maintained.

To investigate possible unloading of  $^{45}\text{Ca}^{2+}$  from upper Malpighian tubules, we took tubules from fifth-instar insects injected with  $^{45}\text{Ca}^{2+}$  9 days earlier and, after dissection (when they are immersed in saline for 5 min), they were washed for 10 min in standard saline containing 5-HT, and then were each put in a separate

100  $\mu$ l drop of fresh 5-HT-containing saline. The secreted fluid was collected at 20 min intervals for 80 min, the tubule was removed and the numbers of counts remaining in it, in the bathing drop and appearing in the four secreted drops were measured. The results showed that of an average of  $230\,000 \pm 10\,200$  cts  $\text{min}^{-1}$  ( $N=8$ ) initially present in each tubule,  $17.5 \pm 1.1\%$  was lost from the tubule in 80 min,  $15.7 \pm 0.9\%$  to the bathing drop but only  $1.6 \pm 0.1\%$  to the secreted fluid. The percentages of counts lost in the secreted fluid in the four successive collection periods were, respectively,  $0.47 \pm 0.05\%$ ,  $0.41 \pm 0.03\%$ ,  $0.38 \pm 0.03\%$  and  $0.37 \pm 0.03\%$ . It is clear that counts are continuously lost to the secreted fluid, but that many more counts are lost through the basal cell membrane than through the luminal one.

$^{45}\text{Ca}^{2+}$  injected into the animal for periods shorter than 9 days, and therefore in contact with the tubule walls for periods shorter than 9 days, is lost more rapidly. Up to 48 h following injection,  $24.9 \pm 1.0\%$  ( $N=48$ ) of the intracellular  $^{45}\text{Ca}^{2+}$  is lost (to both bath and secreted fluid) in 1 h; at 72 h after injection,  $18.3 \pm 1.1\%$  ( $N=8$ ) is lost in 1 h and, as we have seen, by 9 days after injection,  $17.5\%$  is lost in 80 min, equivalent to perhaps  $13\%$  in 1 h. In a different set of experiments,  $^{45}\text{Ca}^{2+}$  was also incorporated into the tubules (by injection of  $^{45}\text{Ca}^{2+}$  into the haemolymph) and the percentage of the injected  $^{45}\text{Ca}^{2+}$  appearing in the secreted fluid was measured. 24 h after injection,  $1.39 \pm 0.11\%$  ( $N=16$ ) was lost into the secreted fluid in 20 min; after 52–76 h of loading,  $0.36 \pm 0.02\%$  ( $N=7$ ) was lost in 20 min; and 12 days after injection, only  $0.18 \pm 0.04\%$  ( $N=10$ ) was lost to the secreted fluid in 20 min. Both sets of experiments show that  $^{45}\text{Ca}^{2+}$  in the tubule walls can be unloaded, that unloading is preferentially to the haemolymph and they also suggest that  $^{45}\text{Ca}^{2+}$  becomes somewhat less accessible to the bathing saline with longer times of contact between haemolymph and tubule.

#### *The site of intracellular calcium accumulation*

The upper Malpighian tubules in *Rhodnius*, unlike the lower regions, contain so-called concretion bodies (Wigglesworth and Salpeter, 1962), (sometimes known as spherical bodies, concretions, granular bodies, dense bodies or mineralized granules), well-known in a range of invertebrates (Brown, 1982) and which, in other insects, are thought to contain metals such as zinc, iron and calcium (Sohal, 1974). They are thus promising candidates for the intracellular deposits of calcium in *Rhodnius*. Confirmation that this is so comes from two sources.

*Rhodnius prolixus* in the IVIC stock are routinely fed through a thin membrane on heparinised sheep blood from a stock of sheep whose red cells contain an unusually low concentration of potassium:  $20\text{ mmol l}^{-1}$  in whole blood compared with  $60\text{ mmol l}^{-1}$  present in rabbit whole blood. On dissection of fifth-stage IVIC insects, it is at once clear that the upper tubules in these insects are almost completely transparent, unlike the greyish-white opaque appearance of this part of the tubule in insects from the Cambridge stock. Under the electron microscope, the tubules from these two stocks appear very different. The IVIC insects still have

numerous concretion bodies, but all of them are very small compared with the array of large concretion bodies in the Cambridge insects (Fig. 7). The calcium content of tubules from these insects, determined by flame photometry, was only  $21 \pm 3 \text{ mmol l}^{-1}$  ( $N=11$ ). By a fortunate chance, a few insects were available from another Venezuelan stock of *Rhodnius* (from Maracay). This stock of insects is fed on chicken blood [which is richer in calcium,  $4.5 \text{ mmol l}^{-1}$  in laying hens (Altman and Dittmer, 1974)] and have cells in the upper tubule that are densely white. Here the density of concretion bodies is several times higher than in the Cambridge stock (Fig. 7). In two tubules the average calcium content was  $1190 \text{ mmol l}^{-1}$ .

We injected several IVIC and Cambridge fifth-stage insects with  $^{45}\text{Ca}^{2+}$  in saline as before. After 12 h, four upper tubules from a Cambridge insect contained an average of  $40\,200 \pm 660 \text{ cts min}^{-1} \text{ tubule}^{-1}$ , while four upper tubules from an insect from the IVIC stock had accumulated only  $9 \pm 3 \text{ cts min}^{-1} \text{ tubule}^{-1}$ , more than 4000 times less. By 6 days, upper tubules from two Cambridge insects contained  $142\,300 \pm 9500$  and  $102\,200 \pm 19\,900 \text{ cts min}^{-1} \text{ tubule}^{-1}$ , while upper tubules from two IVIC insects contained  $2050 \pm 340$  and  $66 \pm 18 \text{ cts min}^{-1} \text{ tubule}^{-1}$ , again very many times less.

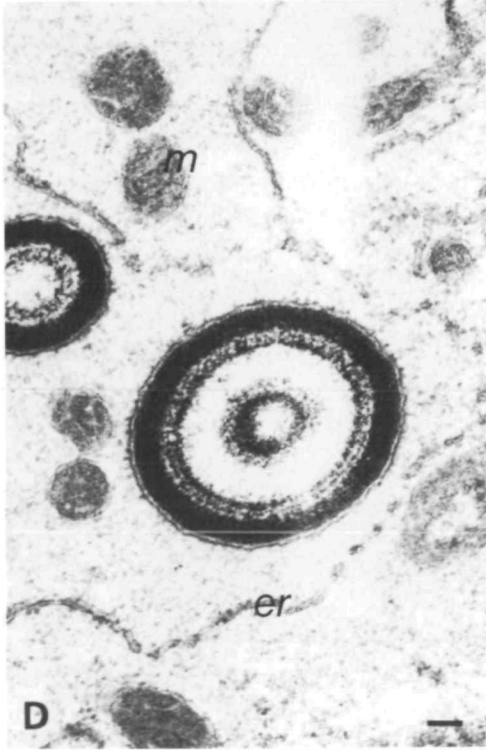
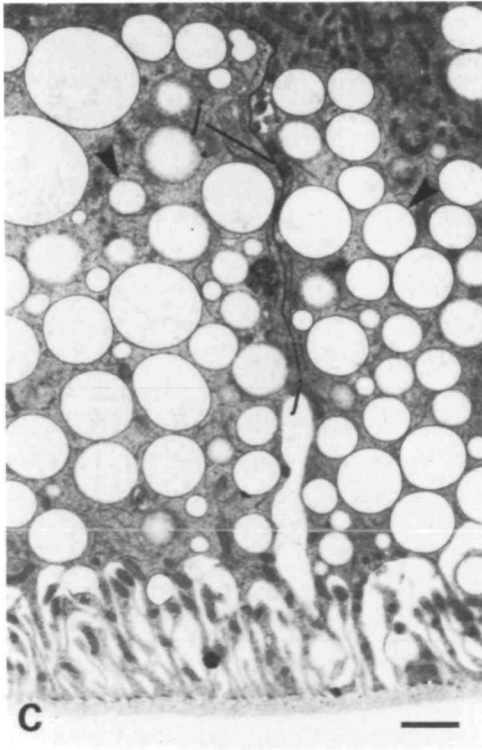
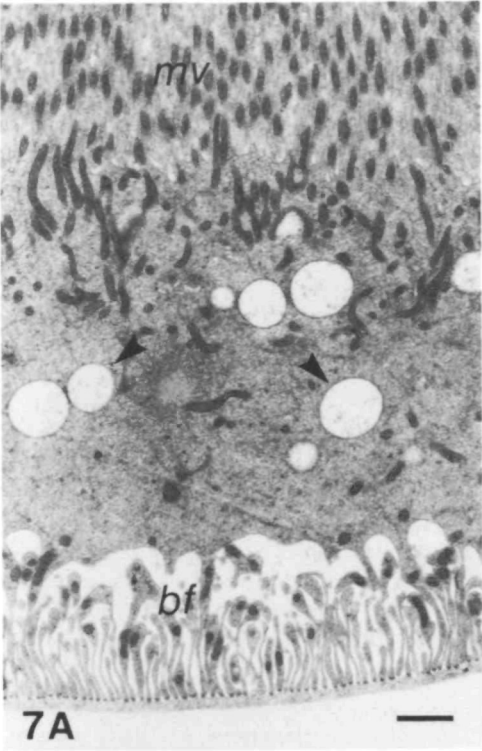
There is evidently a strong correlation between the presence of a large number of sizeable concretion bodies and the calcium content of the tubules or the number of counts of  $^{45}\text{Ca}^{2+}$  taken up by the tubules from the haemolymph. We believe that this is strong evidence that the concretion bodies are the sites of calcium accumulation.

#### *The nature of calcium deposits in the Malpighian tubules*

Since calcium is present at such high concentration in the Malpighian tubule cells, the question arises as to the form in which it is deposited. We collected together 80 of the most opaque Malpighian tubules dissected from eight fifth-stage and twelve adult insects reared on rabbits. The tubules were mounted in the slot of an aluminium sample holder and were allowed to dry. They were then examined by X-ray diffraction to find out whether significant amounts of the calcium deposits were crystalline. Fig. 8 shows the results of the scan together with a scan of a crystalline calcium salt [hydroxyapatite,  $3\text{Ca}_3(\text{PO}_4)_2 \cdot \text{Ca}(\text{OH})_2$ ]. Clearly, no

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Fig. 7. Electron micrographs of longitudinal sections through the upper Malpighian tubule. (A) Tubule from Cambridge stock showing a moderate density of large concretion bodies (arrowheads), maximum diameter  $1.0 \mu\text{m}$ . Note the loss of electron-dense material from within the bodies owing to extraction during the processing procedure. (B) Tubule from IVIC insect showing moderate density of small concretion bodies (arrowheads), maximum diameter  $0.3 \mu\text{m}$ . (C) Tubule from Maracay stock showing a very high density of concretion bodies (arrowheads), some of them attaining a diameter of  $2.0 \mu\text{m}$ . (D) High-power micrograph of membrane-bound concretion body from Cambridge stock showing annular ring type deposition of electron-dense material. *bf*, basal folds; *bm*, basement membrane; *er*, endoplasmic reticulum; *j*, junctional region between two cells; *m*, mitochondria; *mv*, microvilli. Scale bars: A, B, C,  $1.0 \mu\text{m}$ ; D,  $0.1 \mu\text{m}$ .



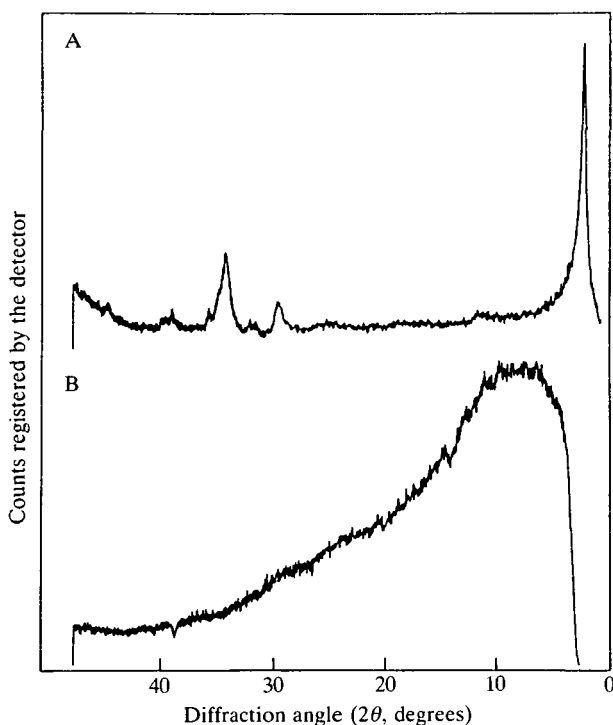


Fig. 8. X-ray diffraction scans of a sample of hydroxyapatite (A) and of 80 Malpighian tubules (B). Several distinct diffraction intensities are clearly visible in A. In B, there are no such peaks, only a scattering curve is seen; the increased numbers of counts at low angles derive from the large amount of organic material present.

reflections arise from crystalline material in the tubules. Calcium must be deposited in an amorphous form in them.

*The effects of a change of diet on the density of concretion bodies and on the calcium content of tubules*

We fed *in vitro* heparinised low- $K^+$  sheep blood to 20 fifth-stage insects previously fed *in vivo* on rabbit blood (batch A) and allowed 20 fifth-stage insects that had been reared *in vitro* on heparinised low- $K^+$  sheep blood to feed on rabbit blood (batch B). At daily intervals thereafter, we examined the tubules from insects from each group. Within 48 h, tubules from insects in batch B were significantly more opaque than tubules from control insects reared on heparinised blood alone. By 8 days the tubules appeared as opaque as those from insects reared on rabbit blood since hatching. By 13 days, the tubules had reached an average calcium concentration of  $239 \pm 27 \text{ mmol l}^{-1}$  ( $N=4$ ) compared with a value of  $410 \pm 53 \text{ mmol l}^{-1}$  ( $N=11$ ) obtained in the animals fed on rabbit blood *in vivo*. Tubules from insects in batch A did not appear any less opaque for the first 8 days after feeding. Then they lost opacity; at least some of the tubules, taken in the

period 8–12 days after feeding with heparinised blood, were not much less transparent than those from insects reared on heparinised blood. By 13 days, the average calcium content of 16 tubules from four insects in batch A had declined from the  $410 \text{ mmol l}^{-1}$  of control insects to  $202 \pm 61 \text{ mmol l}^{-1}$ .

*Uptake of  $^{45}\text{Ca}^{2+}$  from the diet in insects fed heparinised low- $\text{K}^+$  sheep blood*

To test the idea that insects fed heparinised low- $\text{K}^+$  sheep blood might not be able to take up calcium from the diet we fed fifth-stage insects about  $40 \mu\text{l}$  each of  $^{45}\text{Ca}^{2+}$  in saline at  $135\,000 \text{ cts min}^{-1} \mu\text{l}^{-1}$ . Four insects (batch 1) previously reared on heparinised blood were then immediately allowed to feed to completion on heparinised low- $\text{K}^+$  sheep blood (a further  $300 \mu\text{l}$ ). Three further insects reared on rabbit blood *in vivo* (batch 2) were fed to completion on heparinised low- $\text{K}^+$  sheep blood immediately after ingesting  $^{45}\text{Ca}^{2+}$  in saline. Finally, three insects reared on rabbit blood were fed to completion on rabbit blood straight after taking  $^{45}\text{Ca}^{2+}$  in saline (batch 3). The concentrations of calcium in these different bloods are the same, so there were no consequent differences in specific activity of  $^{45}\text{Ca}^{2+}$ .

We took haemolymph samples from the insects on days 3, 4 and 5 after feeding. The concentration of  $^{45}\text{Ca}^{2+}$  in the haemolymph declined in all the insects by about 25 % during this period. The concentrations of  $^{45}\text{Ca}^{2+}$  on day 5 were  $57\,909 \pm 13\,850 \text{ cts min}^{-1} \mu\text{l}^{-1}$  (batch 1),  $78\,180 \pm 1890 \text{ cts min}^{-1} \mu\text{l}^{-1}$  (batch 2) and  $64\,870 \pm 20\,380 \text{ cts min}^{-1} \mu\text{l}^{-1}$  (batch 3). These results indicate that the insects fed heparinised blood (batches 1 and 2) had absorbed as much  $^{45}\text{Ca}^{2+}$  as those fed on rabbit blood (batch 3). The variability in the figures was probably due to the fact that all animals did not ingest exactly the same amount of  $^{45}\text{Ca}^{2+}$  in the saline ingested. To normalize for this, we removed the intact midgut containing the blood meal from each insect on day 5 and disrupted it by sonication in  $3 \text{ cm}^3$  of  $0.3 \text{ mol l}^{-1}$  KOH and determined its content of  $^{45}\text{Ca}^{2+}$  from a  $30 \mu\text{l}$  sample. We could then compare the  $^{45}\text{Ca}^{2+}$  concentration in the haemolymph with the  $^{45}\text{Ca}^{2+}$  still present in the blood meal. Each microlitre of haemolymph from insects in batches 1, 2 and 3 contained  $1.87 \pm 0.15 \%$ ,  $2.11 \pm 0.30 \%$  and  $1.07 \pm 0.20 \%$ , respectively, of the  $^{45}\text{Ca}^{2+}$  still in the gut. On this criterion, insects fed heparinised low- $\text{K}^+$  sheep blood (batches 1 and 2) had significantly higher levels of  $^{45}\text{Ca}^{2+}$  in the haemolymph than had insects fed on rabbit blood (batch 3). Therefore, there is no evidence of any suppression in  $^{45}\text{Ca}^{2+}$  absorption from heparinised blood, indeed the figures suggest that more is absorbed, not less.

We also took all four upper Malpighian tubules from each insect of the three groups and counted their  $^{45}\text{Ca}^{2+}$  contents without washing them further than occurred during the 5 min dissection period under saline. We found that the tubules from the insects fed heparinised low- $\text{K}^+$  sheep blood had taken up considerable amounts of  $^{45}\text{Ca}^{2+}$ , equivalent to that in  $927 \pm 98 \text{ nl}$  of haemolymph (batch 1) and  $1175 \pm 153 \text{ nl}$  of haemolymph (batch 2). However, tubules from insects fed on rabbit blood (batch 3) had taken up significantly more  $^{45}\text{Ca}^{2+}$ , equivalent to  $2625 \pm 665 \text{ nl}$  of haemolymph.

To estimate the total  $^{45}\text{Ca}^{2+}$  absorbed by each insect, we added the  $^{45}\text{Ca}^{2+}$

found in the tubules to that in the haemolymph, assuming from the quantities of haemolymph obtained in bleeding the insects that the haemolymph volume was  $25\ \mu\text{l}$  (unfed fifth-stage insects have  $15\ \mu\text{l}$  of haemolymph). By comparing this total with the sum of that in the gut and the amount absorbed, we could estimate the  $^{45}\text{Ca}^{2+}$  absorbed from the gut as a fraction of the  $^{45}\text{Ca}^{2+}$  ingested. Our results showed that the three groups of insects did not differ in the degree to which  $^{45}\text{Ca}^{2+}$  was absorbed; insects in batch 1 absorbed  $34.7 \pm 1.7\%$  of the  $^{45}\text{Ca}^{2+}$  in the meal, while those in batches 2 and 3 absorbed  $34.8 \pm 7.7\%$  and  $38.2 \pm 3.8\%$  of the  $^{45}\text{Ca}^{2+}$  in the meal, respectively.

In these experiments, much more  $^{45}\text{Ca}^{2+}$  was apparently taken up into the tubules of insects fed heparinised blood than in our earlier experiments (see above). We believe that removing tubules straight into scintillation fluid, after exposing them only to the dissection fluid, without first washing them for 20 min in 5-HT-containing saline may well explain the difference.

#### *Regionalisation of $^{45}\text{Ca}^{2+}$ uptake in upper tubules*

Particularly in insects from the Maracay stock, so opaque are the upper tubules that it is possible to see regional differences in them. The lowermost portion (nearest the transparent lower tubule) appeared decidedly more opaque than did the uppermost, tip end of the upper tubule. We removed four tubules from a fifth-stage Cambridge insect and bathed them for 135 min in a  $^{45}\text{Ca}^{2+}$ -containing saline with 5-HT. The tubules were then washed for 10 min in saline containing 5-HT alone. Each upper tubule was cut into upper and lower halves and the  $^{45}\text{Ca}^{2+}$  content of each was measured. The uppermost portions contained an average of  $2063 \pm 123\ \text{cts min}^{-1}$  but the lower halves contained on average  $4315 \pm 373\ \text{cts min}^{-1}$  each, more than twice as much. As before, there is a correlation between the density of concretion bodies, estimated from the turbidity (opacity) of the tubule segment, and the ability to take up  $^{45}\text{Ca}^{2+}$ .

#### *Calcium activity in the haemolymph*

We measured the activity of calcium in three samples of haemolymph. The first two were volumes of  $20\text{--}30\ \mu\text{l}$  each taken from five fifth-instar insects from the Cambridge stock. The third was  $15\ \mu\text{l}$  taken from 13 fourth-instar insects from the IVIC stock fed on heparinised low- $\text{K}^+$  sheep blood. The activities of calcium in the first two samples were  $2.75$  and  $3.39\ \text{mmol l}^{-1}$ , respectively, and that in the third was  $2.24\ \text{mmol l}^{-1}$ . These figures can be compared with total haemolymph calcium concentrations of  $8.5 \pm 1.2\ \text{mmol l}^{-1}$  (rabbit-fed insects) and  $8.0 \pm 1.8\ \text{mmol l}^{-1}$  (insects fed on heparinised low- $\text{K}^+$  sheep blood).

### **Discussion**

Our findings provide evidence that most of the calcium in the blood consumed by *Rhodnius* is not eliminated from the body but is deposited at very high concentration in an amorphous form in concretion bodies in the cells of the upper



Malpighian tubules. The average concentration of calcium in tubule cells from rabbit-fed *Rhodnius* was  $410 \text{ mmol l}^{-1}$ . From the relatively trivial amounts of calcium measured in cells with reduced concretion bodies, we can assume that calcium is mostly confined to the concretion bodies. Since the bodies occupy only a fraction of the cell volume, the calcium concentration in them must be much higher than in the cell as a whole, perhaps of the order of  $5\text{--}10 \text{ mol l}^{-1}$ . Since the calcium content of Malpighian tubules can be depleted, for example by feeding *Rhodnius* on heparinised low- $\text{K}^+$  sheep blood, it is conceivable that calcium can be withdrawn from the deposits in the Malpighian tubules to maintain haemolymph levels when the insect feeds on blood deficient in calcium. However, this is most unlikely to be the normal function of the deposits, as all bloods likely to be ingested by *Rhodnius* contain significant amounts of calcium. It is more probable that the concretion bodies are the means whereby calcium is effectively removed from the metabolic pool – so-called deposit excretion (Maddrell, 1971). Calcium ions are relatively small, so that, during rectal concentration of urine by reabsorption of water (Ramsay, 1952), they (and other small solutes) would tend to cross passively back into the haemolymph by diffusion through the rectal wall and so be excreted only relatively ineffectually (Maddrell, 1980). More important still, high concentrations of calcium ions in the rectum might well interfere with the ability of the rectal cells to take up water. Water recovery is so vital for most insects (Edney, 1977; Phillips *et al.* 1986; Maddrell, 1980, 1987) that preventing interference by metal ions, such as calcium, may well be the major role of the ability of concretion bodies to sequester such potentially interfering substances. The need for them in insects would be greater than in other animals because the concentrating action of the rectum is more pronounced than that in the homologous parts of the excretory system in other animals (Phillips and Dockrill, 1968). One might argue that free calcium levels in the rectum would be lowered by binding to macromolecules in the faecal material. However, calcium binding sites on macromolecules entering the rectum from upstream parts of the alimentary canal are likely already to be saturated with non-absorbed calcium. So, any calcium entering the urine would tend to remain in the ionic form as the rectal contents are concentrated; no macromolecules accompany calcium in urine formation as they are retained by the filtering action of the Malpighian tubule wall (Coast, 1960).

Under normal circumstances, when the insect takes full meals in each instar, the amount of calcium deposited in the tubules increases steadily. The opacity of the tubules also increases at each succeeding instar. The number of concretion bodies in the tubules probably increases; their size certainly does. It seems likely that calcium is deposited in insoluble form in the concretion bodies so that the earliest deposited calcium lies in the centre. Our data show that  $^{45}\text{Ca}^{2+}$  deposited over periods of the order of 10–12 days is released less rapidly than  $^{45}\text{Ca}^{2+}$  incorporated for shorter periods. In electron micrographs where their contents have been preserved, the concretion bodies appear as layered structures suggestive of a series of phases of deposition, occurring perhaps after every meal when calcium uptake rates are higher. The post-feeding increase in calcium uptake is very reminiscent,

especially in its timing, of the increases in transport of para-aminohippuric acid and of uric acid that occur after a meal (Maddrell and Gardiner, 1975; O'Donnell *et al.* 1983).

Insects from the Maracay stock whose tubules had very high densities of concretion bodies had been routinely fed on chicken blood. At least in laying hens, the blood calcium concentration is more than twice as high as in rabbits (Altman and Dittmer, 1974). Since hens are in lay for at least 80 % of the year, it is very likely that, by maturity, these *Rhodnius* had ingested twice as much calcium as insects fed on rabbit blood. If so, and if only the usual amounts of calcium are excreted, contained in the haemolymph and used elsewhere in the body, then more than twice as much is left to be deposited in the Malpighian tubules. This may be the explanation of the observation that the density of the concretion bodies in the upper tubules of fifth-stage Maracay insects was so much higher than in the Cambridge insects. Two tubules from the Maracay stock had an average calcium content of  $1190 \text{ mmol l}^{-1}$ , nearly three times the average of tubules from rabbit-fed insects.

We have found that  $^{45}\text{Ca}^{2+}$  recently incorporated into the concretion bodies is steadily released into saline *in vitro* at a rate of about 25 % per hour. A meal of heparinised sheep blood also promotes calcium release from the tubules. Because cytosolic levels of free calcium are so low (about  $10^{-7} \text{ mol l}^{-1}$ , Campbell, 1973), lower than those in saturated solutions of such sparingly soluble salts of calcium as carbonate, sulphate, oxalate and phosphate, energy would have to be continuously expended in maintaining the calcium content of the concretion bodies if it is present there as any of these salts. So, it is not surprising to find that  $^{45}\text{Ca}^{2+}$  steadily passes into the bathing solution from tubules *in vitro*; presumably this represents the steady-state efflux from the concretion bodies. The effects of heparinised low- $\text{K}^{+}$  sheep blood could take effect at the level of the calcium concentrating mechanism of the concretion bodies. This could explain why tubules from insects fed heparinised low- $\text{K}^{+}$  sheep blood accumulate significant amounts of  $^{45}\text{Ca}^{2+}$  but nearly all this is lost if the tubules are washed for 20 min in tracer-free saline. If the concretion bodies are to prevent calcium from reaching the rectum in significant amounts, it is of course important that any calcium released from them passes to the haemolymph rather than to the lumen of the tubule. In support of this, we have found that about 10 times as much  $^{45}\text{Ca}^{2+}$  passes to the haemolymph side in unit time as enters the lumen *in vitro*. This is in spite of the observation that the area of cell membrane facing the lumen is at least twice that in direct contact with the haemolymph (Maddrell, 1980). Presumably, membrane-sited calcium-exporting pumps are predominantly found on the haemolymph-facing cell membrane. Of course, if calcium levels in the urine are to be kept low, the tubule walls should be relatively impermeable to calcium. We have found that tubules are consistently 60 % more permeable to  $^{14}\text{C}$ sucrose than to  $^{45}\text{Ca}^{2+}$ , although calcium ions are smaller than sucrose molecules. Sucrose molecules have a radius of 0.45 nm while calcium ions have a hydrodynamic radius of 0.31 nm and a hydrated radius of 0.41 nm. We found earlier (Skaer *et al.* 1987) that the

intercellular junctions are more permeable to anions than to cations, so it is not surprising to find a low transepithelial permeability for  $^{45}\text{Ca}^{2+}$ .

One can try to calculate the loss of  $^{45}\text{Ca}^{2+}$  through the tubules as a fraction of that taken up into the cells. In recently fed fifth-stage insects, the amount of  $^{45}\text{Ca}^{2+}$  taken up in 1 h from the haemolymph by one upper tubule is equal to that contained in 35 nl of haemolymph (based on the uptake rate over the first 24 h, Fig. 3). Assuming that the rate of secretion by each of the tubules is  $0.5 \text{ nl min}^{-1}$  and that the permeability of the tubule to  $^{45}\text{Ca}^{2+}$  is  $0.02 \text{ nl min}^{-1} \text{ mm}^{-2}$ , it can be calculated, using Ramsay's formula (equation 1), that the  $^{45}\text{Ca}^{2+}$  loss through each tubule in 1 h is equal to that contained in about 8 nl of haemolymph, equivalent to 22 % of the amount taken up by the cells. However, not all the calcium in the haemolymph is free; its activity is about  $3 \text{ mmol l}^{-1}$  and its concentration  $8 \text{ mmol l}^{-1}$ . So  $^{45}\text{Ca}^{2+}$  loss passively through the tubule wall in 1 h will only be equivalent to that contained in 3 nl of haemolymph, i.e. calcium is taken up and deposited in the tubules 12 times as fast as it is lost into the primary urine. No doubt this is a major part of the explanation of how *Rhodnius* retains in its body most of the calcium from its meals of blood.

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