PHYSIOLOGICAL MECHANISMS OF URINE FORMATION IN *LOCUSTA MIGRATORIA MIGRATORIOIDES* (R&F) AND *ZONOCERUS VARIEGATUS* (L).

by

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ABSTRACT

The functioning of the Malpighian tubules of Locusta migratoria migratorioides (R.& F.) and Zonocerus variegatus (L.) has been studied using an in vitro preparation.

The effect of corpora cardiaca extracts on the rate of secretion of urine by the Malpighian tubules was tested. These extracts of storage lobes contain a diuretic hormone. Saline extracts of storage lobes were unstable and more than 75% of the activity was lost within 3 hours at room temperature. The diuretic activity was stable in methanol extracts. Locust tubules were stimulated by diuretic hormones from Rhodnius, Glossina and Periplaneta.

Several compounds mimicked the action of diuretic hormone: cyclic-AMP, dibutyryl cyclic-AMP, adrenalin, histamine, ecdysterone, cholesterol, aldosterone, phlorizin and phloretin produced marked increases in urine secretion. No effect was obtained with 5-HT or ouabain. Known inhibitors of some stimulants, such as phentolamine and ethacrynic acid, inhibited the effects of adrenalin and aldosterone respectively, but failed to inhibit the action of either diuretic hormone or cyclic-AMP.

The ability of the tubules to handle toxic chemicals was investigated. The transport of ouabain was investigated in Zonocerus which is predisposed to feeding on toxic plants and compared with Locusta tubules. It was found that Zonocerus tubules excreted ouabain more efficiently than those of Locusta. Moreover, the presence of ouabain in the diet of Zonocerus induced, after a few days, a higher rate of ouabain

secretion to a point where the concentrations in the secreted fluid reached levels higher than that found in the bathing medium. ^{3}H-phlorizin was found to be transported actively by the tubules of Locusta.

The effect of phlorizin and phloretin on the permeability of the tubules to glucose was tested. The glucose permeability was found to increase in the presence of phlorizin, phloretin as well as ouabain. This effect was investigated further using different sugars and Na^{+}.

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1. INTRODUCTION

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1. INTRODUCTION

Insects do not possess a closed blood circulatory system and therefore the initial force responsible for the formation of a primary urine filtrate present in most animals is absent in insects. However, in many ways the Malpighian tubules of insects have analogies with the glomerulus of the mammalian kidney. They supply the hindgut with an ultrafiltrate of the haemolymph by specific active transport systems without the aid of a high blood pressure. This produces a continuous flow of fluid containing the smaller sized constituents of the haemolymph including metabolically useful substances such as sugars and amino acids. The secreted fluid is iso-osmotic with the haemolymph but as it moves down the tubule and when it reaches the rectum reabsorption of water and other useful substances occurs. Thus despite the fact that an anatomical equivalent to the counter-current system in vertebrates is absent in insects they are capable of producing a hyper-osmotic urine. The insects have therefore evolved an alternative method to perform the necessary function of all excretory organs, that is, filtration-reabsorption-secretion (Ramsay, 1961).

1.1. STRUCTURAL FEATURES OF MALPIGHIAN TUBULES

1.1.1. Cellular differences.

The Malpighian tubule is a thin-walled tube formed from an epithelial layer one cell thick. The cells of the wall may be one type only (primary cells) or of two cell types (Berridge & Oschman, 1969). In the two cell type tubules one type is predominant over the other (stellate) cell. Typically the primary cells have the following features (Fig. 1.1.1.):

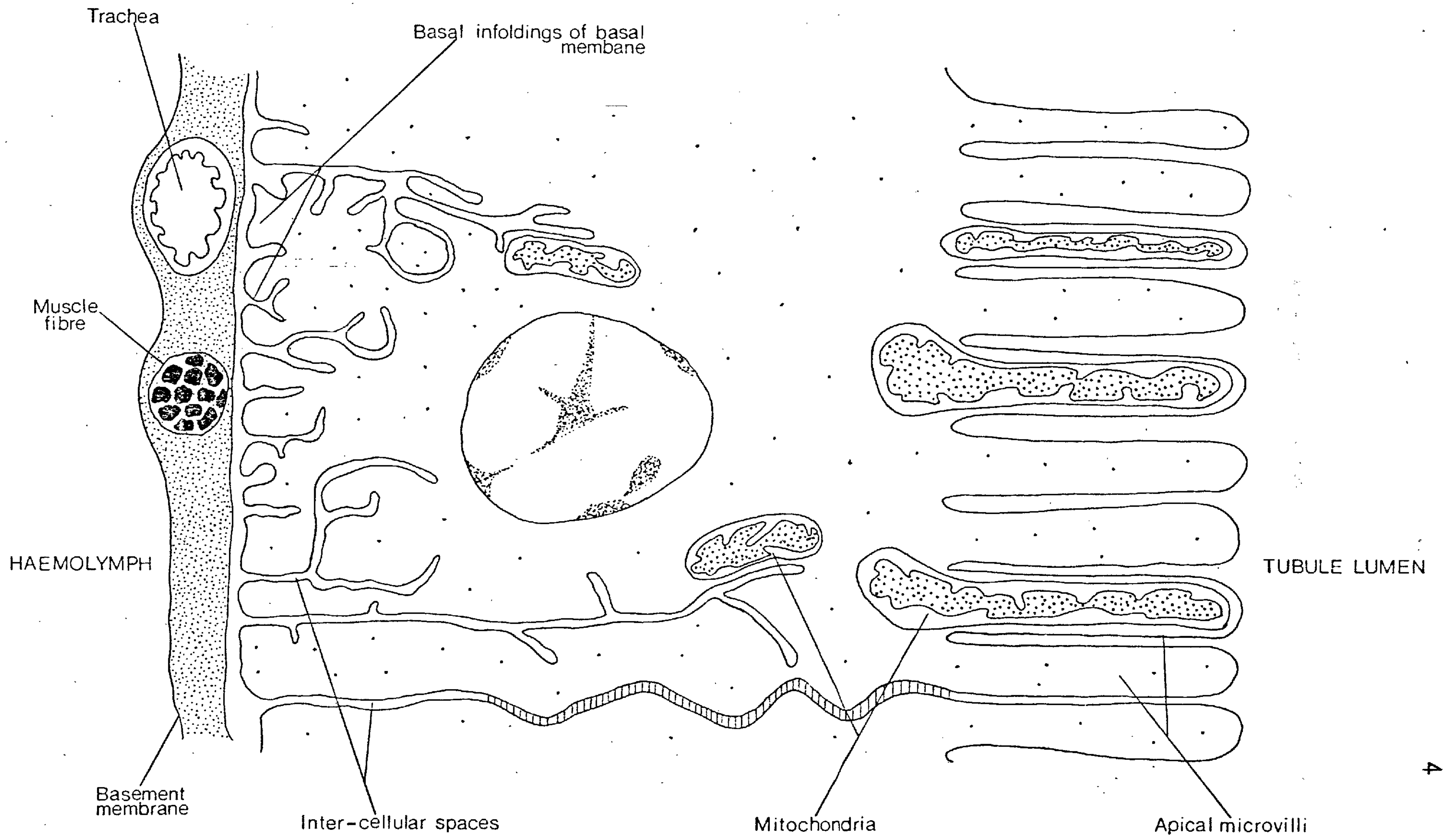
(a) A basement membrane along the haemolymph (basal) side of the cells.

(b) The basal cell membrane forms deep infoldings. The basal infolds form clear channels extending from the basement membrane towards the apical surface. Berridge & Oschman (1969) found in *Calliphora* tubules that the width of the channels varies with the type of fixation used and with the secretion rate. The cytoplasmic compartments between the basal infolds contain mitochondria and rough endoplasmic reticulum. This region also contains dense membrane-bound structures resembling microbodies.

(c) The basal region is followed by an area with a few basal infoldings, the nucleus, endoplasmic reticulum, Golgi bodies and various autophagic vacuoles and clear vacuoles.

(d) The lumen (apical) side forms long closely packed

Fig. 1.1. Diagrammatic representation of a cross section of a typical primary tubule cell. (After Berridge & Oschman, 1972).


Trachea
Basal infoldings of basal membane
Muscle fibre
HAEMOLYMPH
Basement membrane
Inter-cellular spaces
Mitochondria
Apical microvilli
TUBULE LUMEN

microvilli, closely associated with mitochondria. The microvilli provide about a 40-fold increase in surface area when compared to a flat membrane. The cytoplasmic surface of the microvilli and the basal infoldings is coated with vesicles in different stages of communication with the exterior. Larger smooth vacuoles are associated with the apical surface.

The typical features of the stellate cells are:

(a) A basal region composed of surface infoldings forming a network of interconnecting channels but they do not extend up to the apical surface as in the primary cells.

(b) The central region is similar to that of the primary cells but does not contain lipid droplets or clear vacuoles.

(c) The apical microvilli are shorter and are not associated with mitochondria. These microvilli also lack the coating found in the primary cells.

(d) Processes of the cell radiate from the nuclear region extending between the primary cells or over them facing the blood. Junctions between the primary cells occurs very infrequently.

Carausius tubules similarly contain two types of cells (Taylor, 1971). The primary cells closely resemble that described for Calliphora. A function of mucus production was proposed for the other cell type. Joyner (1970) also reports of two cell types in the tubules of Schistocerca.

1.1.2. Fine structure related to functional aspects.

It can be argued that a variation in the physiological condition of the insect (nature of food, availability of water, composition of waste products, age etc.) can result in a corresponding variation in the structure of the Malpighian tubules so that tubules vary according to the insect's habitat. This may be true but at the fine structural level basic similarities do exist which can be correlated to basic common physiological processes.

The present findings on the functional significance of the various components of the cells of the Malpighian tubules reveal a common plan. The basement membrane appears to act as a physiological sieve providing access to certain sized molecules, any larger molecules are denied entry. Thus it is important in the primary determination of the composition of the secreted tubular fluid. Berridge & Oschman (1969) suggest that the basement membrane in *Calliphora* tubules do not concentrate proteins. Locke & Collins (1967) showed that the basement membrane of the fat body in *Calpodes* allowed blood proteins and injected peroxidase (MW 40,000) to penetrate freely, but that of the Malpighian tubule did not. This property of the basement membrane would be a desirable factor if a standing-gradient osmotic flow is proposed (see Section 1.2.2.3. & 1.3.5.) as the mechanism responsible for water transport. This would effectively prevent clogging of the basal infoldings

by proteins swept in by the fluid stream (Taylor, 1971). However, this feature is not found in all Malpighian tubules. Uptake of colloids, proteins and horseradish peroxidase from the haemolymph has been demonstrated for tubules of *Gryllus* (Berkaloff, 1960), *Drosophila* (Wessing, 1965) and the dragonfly *Libellula* (Kessel, 1970). Taylor (1971) suggests that a probable function of the basement membrane in *Carausius* is to protect the tubules against distortion by intraluminal pressures created both by muscular activity and trans-tubular transport.

It is possible that the vesicles coating the basal and apical surfaces are involved in the entry or exit of substances into or out of the cells. It is also possible that water and/or solute could enter the cells inside vesicles by cytopempsis (Moore & Ruska, 1957). This was suggested by Wessing (1962) and Wigglesworth *et al.* (1962) as the transport mechanism of the Malpighian tubules. Wessing (1965) and Berkaloff (1960) respectively working on *Drosophila* and *Gryllus* demonstrated the incorporation of metal-labelled colloids and proteins from the haemolymph into vacuoles in the Malpighian tubule cells and their subsequent liberation into the lumen. Kessel (1970) showed that injected horseradish peroxidase is first incorporated into vesicles coating the basal infoldings and its subsequent incorporation into larger vacuoles. The transport must in some way

involve selective binding along the surface of the cell which produces the pinocytotic vesicles. This may be due to a positive filamentous coat (Marshall, 1965) which acts as a cationic exchanger binding ions which are subsequently taken into the cell.

Riegel (1966) showed the presence of membrane-limited formed bodies within the lumen and between the microvilli of excretory systems of several animals. It is possible that these structures are mere artifacts of fixation especially due to the fact that they are found in the lumen outside the cells. But micropuncture samples taken from living *in situ* excretory organs still showed the presence of these formed bodies in the urine of *Rhodnius* after a blood meal. However, it can still be argued that the formed bodies are merely products of ageing cells being replaced. Riegel (1966) suggests that these may be sites of accumulation and release into the lumen of substances such as albumen and peroxidase or other substances unable to enter by any other method. It thus seems likely that the formed bodies do play a role in excretion but their exact function is clearly an area open to further research.

Both the apical and basal surfaces of the Malpighian tubules possess long narrow channels formed by the infoldings of the membrane. The distribution of mitochondria is such that it provides close association

with the basal infoldings and apical microvilli suggesting that energy-requiring processes occur in these regions. Beams *et al.* (1965) report of evidence that the mitochondria could also provide a passageway for the actively trasnsported substances. Taylor (1971) expresses the possibility of this role taken over by the endoplasmic reticular system. More conclusive evidence is required however, if the elevation of these organelles to the status of transporting systems is to be made. The penetration of mitochondria into the microvilli, (Taylor, 1971) which is common in Malpighian tubules, does suggest that they are intimately involved in urine formation.

1.2. SOME THEORETICAL CONSIDERATIONS OF TRANSPORT SYSTEMS

The excretory system usually provides for the exchange of substances between the body and its environment but ultimately it is the properties of the membrane surrounding individual cells of these epithelial tissues which regulate the passage of substances. The chemical content of living cells differs from its environment. This is kept so by the limiting membrane of the cell. In order to support cell life substrates have to be supplied through this membrane and metabolic waste products removed. In addition, cell membranes are permeable to water and thus intracellular ionic concentrations need to be controlled to maintain cell volume.

1.2.1. Passive membrane transport.

By definition passive transport is the transport of compounds in which the energy for their transport is a function of the concentration gradient and requires no special link with metabolism. The permeant molecules, which traverse the membrane either through the lipid bilayer or through protein-lined channels or pores in the bilayer, come to equilibrium when the concentration inside and outside the cells are equal. In this way passive transport has a feature in common with Fickian diffusion. Briefly, Fick's Law states that flow of matter is proportional to the concentration gradient (Eq. 1.2.1.1.):

$$dm/dt \propto dc/dx \quad \text{.....(1.2.1.1.)}$$

or

$$dm/dt = -DAdc/dx \quad \text{.....(1.2.1.2.)}$$

where dm/dt is the flow of matter (mass, moles per unit time), D is the diffusion coefficient (small, cm^2/s), A is the area, dc/dx is the concentration gradient (c measured in units consistant with m). Biological flows are often expressed as fluxes. The flux is the rate of flow of matter per unit area (Eq. 1.2.1.3.):

$$J = 1/Adm/dt \quad \text{......(1.2.1.3.)}$$

where J is the flux. One often speaks loosely about concentration gradients when all that can be measured are concentration differences. The above equation

1.2.1.3. for flux in the direction from the outside to the inside of the cell becomes:

$$J = P(c_o - c_i) \quad \ldots\ldots(1.2.1.4.)$$

where P is the permeability coefficient, c_o is the concentration outside and c_i is the concentration inside.

Ramsay (1958) put forward three criteria for regarding a transport system as passive transport:

(a) The concentration ratio i.e. the concentration of a test substance in the secreted fluid i.e. (inside the cell) expressed as a function of that in the bathing medium (outside the cell) should not exceed 1 (Eq. 1.2.1.5.):

$$S/M \leqq 1 \quad \ldots\ldots\ldots\ldots(1.2.1.5.)$$

where S/M is the concentration ratio.

(b) The concentration ratio should be largely independent of the concentration of the substance in the bathing medium.

(c) The concentration ratio should be affected by the rate of fluid secretion according to the relationship in Eq. 1.2.1.6.:

$$S/M = P/(a+P) \quad \ldots\ldots\ldots(1.2.1.6.)$$

where P is the permeability coefficient and a is the rate of fluid secretion per unit area (flux). Eq. 1.2.1.6. can be rearranged to form Eq. 1.2.1.7.:

$$M/S = a/P+1 \quad \ldots\ldots\ldots\ldots(1.2.1.7.)$$

Eq. 1.2.1.7. expresses a straight line relationship of slope 1/P and intercept of 1 at the y-axis (Maddrell & Gardiner, 1974).

However, the transport kinetics of the majority of compounds passing through cell membranes do not comply with simple diffusion and usually involve interactions with specific carrier systems.

1.2.2. Facilitated diffusion and active transport.

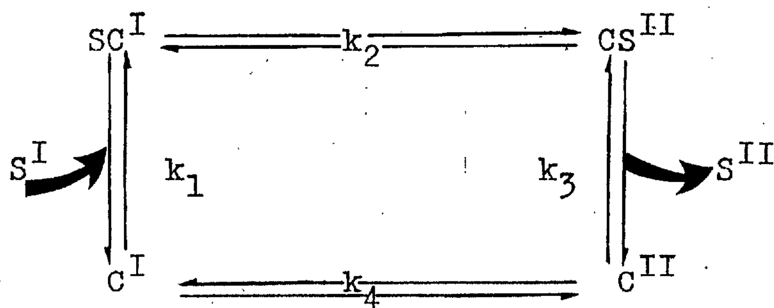
As our knowledge of passive transport is greater than that of active transport, the definition of active transport tends to be of a negative character. A substance can be regarded as actively transported only if the transfer of the substance cannot be accounted for by the action of purely physical forces. In other words, active transport mechanisms are linked with metabolism. Transport involving carriers and therefore agreeing with saturation kinetics can be passive e.g. facilitated diffusion or exchange diffusion. The distinction between active transport and facilitated or exchange diffusion must therefore be made.

1.2.2.1. The distinction between facilitated diffusion and active transport.

Passive carrier mechanisms consist of a reversible reaction between a substrate S and a membrane constituent C, the carrier, to form a complex CS which can cross the membrane and release the substrate at the other side.

SUBSTRATE I	MEMBRANE CARRIER	SUBSTRATE II

The carrier can cross the membrane in two forms, loaded or unloaded:



k_1 and k_3 are the dissociation constants:

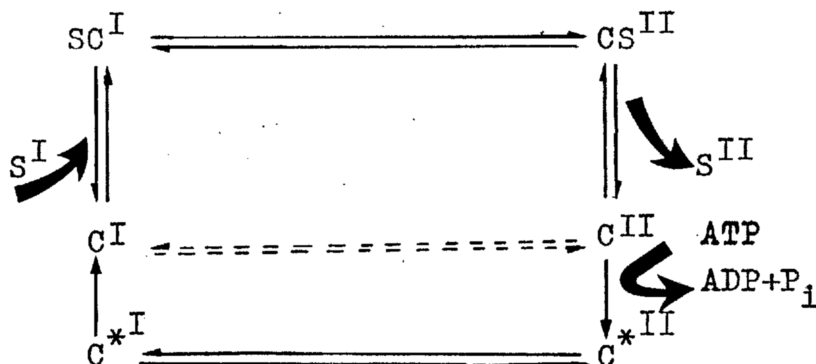
$$k_1 = \frac{[S^I][C^I]}{[SC^I]} \quad ; \quad k_3 = \frac{[S^{II}][C^{II}]}{[SC^{II}]}$$

and k_2 and k_4 are the distribution constants:

$$k_2 = \frac{[SC^I]}{[CS^{II}]} \quad ; \quad k_4 = \frac{[C^I]}{[C^{II}]}$$

loaded unloaded

The carrier site itself can be a metabolic intermediate so that the substrate carrier complex is directly coupled with metabolism: i.e. active transport



A new form of carrier thus appears as a result of a reaction with ATP and there are two alternative cyclic sequences for the carriage of S because there are two different ways in which the unloaded carrier can return to its starting point. For this system to have a biological advantage it is postulated that the movement of C is much slower than CS complex or C^*.

Stein (1967) proposed the following criteria for identifying facilitated diffusion systems:

(a) The transport operates on an existing electro-chemical gradient leading to its disappearance and no input of free energy is required.

(b) The optical enantiomorph will have a different rate of penetration if it is due to facilitated diffusion. (It would have the same rate of penetration if it is to be accounted for by simple diffusion).

(c) The rate of penetration reaches a saturation level and therefore Fick's Law is not obeyed, i.e. competition between identical molecules occurs.

(d) The rate of penetration may be reduced by inhibition by the presence of structural analogues, i.e. competition between opposed or similar molecules occurs.

(e) The rate of penetration can be reduced by inhibitor chemicals which may also be active as enzyme poisons.

(f) The rate of penetration of net transfer of the permeant differs from that measured as unidirectional flux by isotopically labelled permeant.

(g) The transport of the permeant may have a link to the movement of a structurally analogous molecule in the opposite direction i.e. counter-transport. Co-transport, a variant of (g) may also occur whereby the transport of the permeant is linked to the transport of another molecule in the same direction.

Facilitated transfer systems can be expressed by Eq. 1.2.2.1.1.:

$$J = \frac{SV_{max}}{K_m + S} \quad .....(1.2.2.1.1.)$$

where V_{max} is the maximum flux that the cell can demonstrate toward the permeant, a constant depending on the number of cells present, cell type and class of permeant. K_m is the Michaelis constant characterizing the affinity of the permeant to the cell membrane.

1.2.2.2. Active transport of Na^+ - the Na^+ pump.

One of the great mysteries of nearly all living cells is how they maintain a relatively high K^+ and low Na^+ content while they are surrounded by the reverse concentration ratio of the two cations. Not only is this important for regulating the Na^+ and K^+ content of cells, but it plays a particular role in several physiological functions including excitation and renal secretory and reabsorptive processes. In addition

water movements are intimately coupled.

The small ions such as K^+ and Cl^- tend to distribute in a way that compensates for the large negative charge produced by the larger protein molecules inside the cell. Thus K^+ will predominate the inside of the cell whilst Cl^- will predominate the outside thus forming the Gibbs-Donnan equilibrium. However, there are permeant ions that do not distribute as expected. Ions to which the membrane is permeable tend to diffuse down their concentration gradient, thus K^+ will tend to diffuse from the inside to the outside of the cell and Cl^- from outside to the inside of the cell. Because of the opposing need to keep positive ions and negative ions on either side of the membrane, few ions have to pass before a potential difference is set up large enough to oppose the movements due to concentration gradients. That is, as K^+ ions are positively charged, the inside of the cell, being negative relative to the outside will attract any K^+ ions that diffuse down their concentration gradient. Thus the membrane potential can be determined by the balance of the opposing forces:

(a) the force $RT \ln \frac{[K_o^+]}{[K_i^+]}$

which is the tendency of K^+ ions to diffuse out of the cell, down their concentration gradient, where

R is the gas constant, T is the absolute temperature and ln $[K_o^+/K_i^+]$ is the rate of diffusion of K^+ expressed logarithmically.

(b) the force zFE_{K^+}

which is the tendency of K^+ ions to be pulled back into the cell by the buildup of negative charge where z is the valency of the ion, F is the Faraday number and E_{K^+} is the membrane potential for K^+.

At equilibrium these two forces make up the Nerst equation (Eq. 1.2.2.2.1.):

$$zFE_{K^+} = - RT \ln [K_o^+/K_i^+] \quad \ldots (1.2.2.2.1.).$$

i.e. $$E_{K^+} = -RT/zF \ln [K_o^+/K_i^+] \quad \ldots (1.2.2.2.2.).$$

If K^+ was the only ion involved in producing this potential difference than the expected membrane potential should be equivalent to the membrane potential for K^+, i.e. E_{K^+}. However, in most cells the membrane potential is less negative than E_{K^+}, E_{K^+} being some 10-15 mV more negative. Thus K^+ is subjected to a force equivalent to 10-15 mV which will tend to drive it out of the cell and result in a steady leakage of K^+ down its concentration gradient until no membrane potential exists. Since this does not occur there must be some mechanism which will maintain a high intracellular K^+ concentration. This is maintained by an active sodium

pump. This active extrusion of Na^+ in exchange for K^+ is the main energy requiring function of most cells, in fact, almost one third of a cell's energy production is linked to this pump (Whittam, 1962).

Na-K-ATPase is responsible in making ATP available to this type of transport. There is now much evidence available that Na-K-ATPase can operate either in an electrogenic mode in which Na^+ ions are extruded on their own in such a way that the pump hyperpolarizes the membrane or in a coupled mode in which there is an electrically neutral exchange of one Na^+ for one K^+ ion. The active transport process may therefore involve Na^+ ions alone, moving outwards, or K^+ ions moving inwards as well. Na-K-ATPase is inhibited by cardiac glycosides by their action directly on the carrier mechanism (see review by Glynn, 1975).

Whittam (1962) using red cell ghosts showed that replacing the Na^+ ions in the medium with either choline or K^+ ions did not affect ATPase activity, however there was a 50% fall when K^+ was omitted from the medium. A maximum stimulation was obtained when 10 mM K^+ was present in the medium. On the other hand an increase in the Na^+ concentration in the red cell ghosts stimulated ATPase whereas changes in the intracellular K^+ concentrations had no effect.

The synergic stimulation of ATPase by internal Na^+ and external K^+ was counteracted by ouabain.

The action of ouabain on Malpighian tubules is discussed in Chapter 4 and 6. This system has been shown by several workers (see review by Jungreis, 1977) not to be involved in the mechanism of fluid secretion by Malpighian tubules where active K^+ transport is the major force of fluid flow (see Section 1.3.1.; reviews by Maddrell, 1971 and Edney, 1977, Chapter 6). However, it should be pointed out that although it may not function in the process of fluid secretion by the Malpighian tubules, Na-K-ATPase is present in microsomal fractions of not only the tubules but several other areas of the insect's gut (Peacock *et al*., 1972; Anstee & Bell, 1975; Peacock *et al*., 1976; and Tolman & Steel, 1976). A ouabain-sensitive ATPase has also been suggested through morphological evidence in *Drosophila* tubules (Weber-von-Grotthuss *et al*., 1974). Its possible importance in the reabsorptive function of the tubules is discussed in Chapter 6. Na-K-ATPase is also still an important enzyme in other insect tissues, notably the excitable tissues (Treherne, 1966).

1.2.2.3. Transport of water.

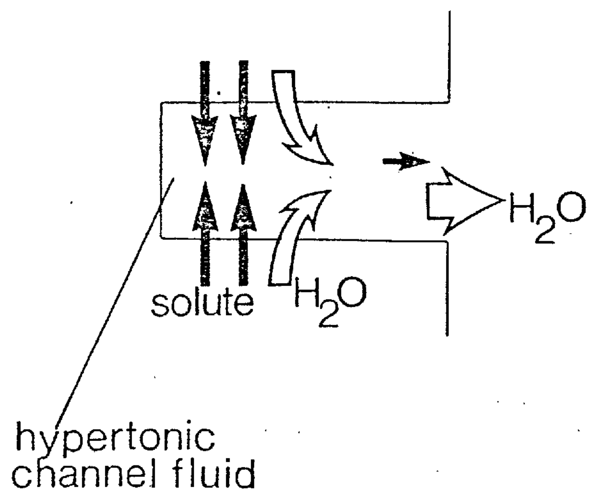
Most cases of water transport have been shown to be closely coupled to the movement of solute. Procedures used to inhibit solute movement have been shown to inhibit water transport as well (Parsons,1967). In most cases water is transported from isotonic bathing solutions but there exists cases, particularly the insect rectum, where water movement is against a gradient of water activity (Beament, 1964; Phillips, 1968, 1970; review by Keynes, 1972). Uptake of water occurs independent of the movement of other substances and also when the potential difference is reversed which thus eliminate the possibility of electro-osmosis. Beament (1964) showed that absorption of water through the cuticle is brought about by a temporary reorganization of the lipid molecules of the cuticle involving a reorientation of the polar lipid layer forming an 'inverted' monolayer which acts as a semi-permeable membrane.

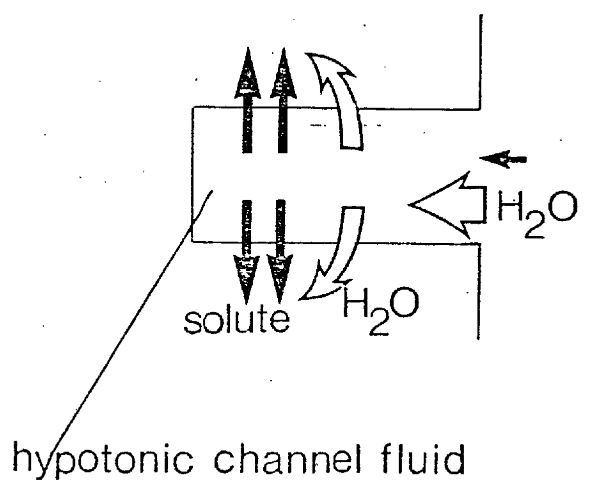
The double membrane theory proposed by Curran (1960) to explain coupled water and solute transport proposes three compartments separated by two membranes. Solute is actively transported from the first compartment across a membrane to a second compartment where the osmotic pressure is raised. This results in a passive flow of water and causes an increase in the hydrostatic pressure in the second compartment. This causes a flow to the

third compartment through a second membrane. Thus water transport is due to the active transport of solutes in a multicompartmental system in which these compartments are arranged serially and separated by membranes with differing permeability characteristics. Patlack *et al*. (1963) have shown that by varying various physiological parameters one can produce a fluid which can be either hyper-, iso-, or hypo-osmotic to the medium.

Diamond & Bossert (1968) have extended the above model by emphasizing the advantage in having the second compartment as a long narrow intercellular channel. In this way a standing gradient is produced by the active solute transport into the blind endings of the channel. They distinguished between backward and forward channels where forward channels transport water towards the open end and backward channels transport water away from the open end (fig. 1.2.2.3.1.). They pointed out that where the solute is transported *out* of the channel, as in the backward channels, the channel becomes hypotonic and thus there is solute exhaustion which imposes a limited value on the rate of water transport. This could be increased either by the presence of a larger quantity of these channels or a higher permeability to water.

Fig. 1.2.2.3.1. A comparison of forward and backward channels (after Diamond & Bossert, 1968). For explanation see text.


FORWARD
CHANNEL
H_2O
solute
H_2O
hypertonic
channel fluid


BACKWARD
CHANNEL
H_2O
solute
H_2O
hypotonic channel fluid

However, the measured passive permeabilities of many epithelia to water reveal lower values than the theoretical value required for isotonicity, even in the forward channels. Diamond & Tormy (1966) argue that if an active transport of solute is concentrated at the closed end of the channel and the channels are long and narrow a sweeping effect would be produced. This leads to the important point of the presence of tight junctions in these epithelia which would prevent the passive leakage of ions down their concentration gradients (Frömter & Diamond, 1972). Tight junctions would thus maintain steeper solute gradients and provide for 'active' water transport, whereas'leaky' junctions would be related to isotonic water transport. However, tight junctions have not been widely observed (Satir & Gilula, 1973). Moreover, Mills & Dibona (1978) have shown that solute active transporting sites (Na^+ pump sites) are associated with the entire length of the intercellular space and are not concentrated at the apical ends. Uniform distribution has also been observed by many other workers (Farquhar & Palade, 1964; Stirling, 1972). It is thus clear that leaky junctions and a uniform distribution of solute pumps are the main objections brought about by experimental investigations.

Hill (1975a) has critically examined the standing gradient theory and has pointed out that very high

values for the osmotic permeabilities of the cell membrane of many systems are required for isotonic water transport, which have not been observed. He argues that even if the solute pumping region is confined to the 10% of the channel length, the osmotic permeability would have to be 3-4 times greater than that measured. He proposes (1975b) an electro-osmotic theory for fluid transfer whereby water movements are coupled to ionic currents. Thus the electric potential of a cell sets up ionic movements to which water movements are 'frictionally' attached.

Sackin & Boulpaep (1975) have postulated two alternative models for Necturus proximal tubules. The first model, the continuous model, is an extension of the Diamond & Bossert model whereby the boundary conditions are altered, the boundary being the capillary wall rather than the end of the channel. In this way the solute pumps can be uniformly distributed along the channel and the tight junction region is permeable to solute and water and treated as an effective membrane with specific permeability coefficients. By extending the boundary area to the capillary wall at which isotonicity is achieved soluted mass balance is satisfied. The second model, the compartment model, extends the original Curran double membrane model but consists of 5 compartments: (1) the lumen; (2) the cell; (3) the interspace; (4) the peritubular space; (5) the capillary. Both electrical and chemical driving forces for Na^+ and Cl^- ions, and hydraulic and osmotic forces for water are considered.

1.3. MALPIGHIAN TUBULE FUNCTION

1.3.1. Active potassium transport.

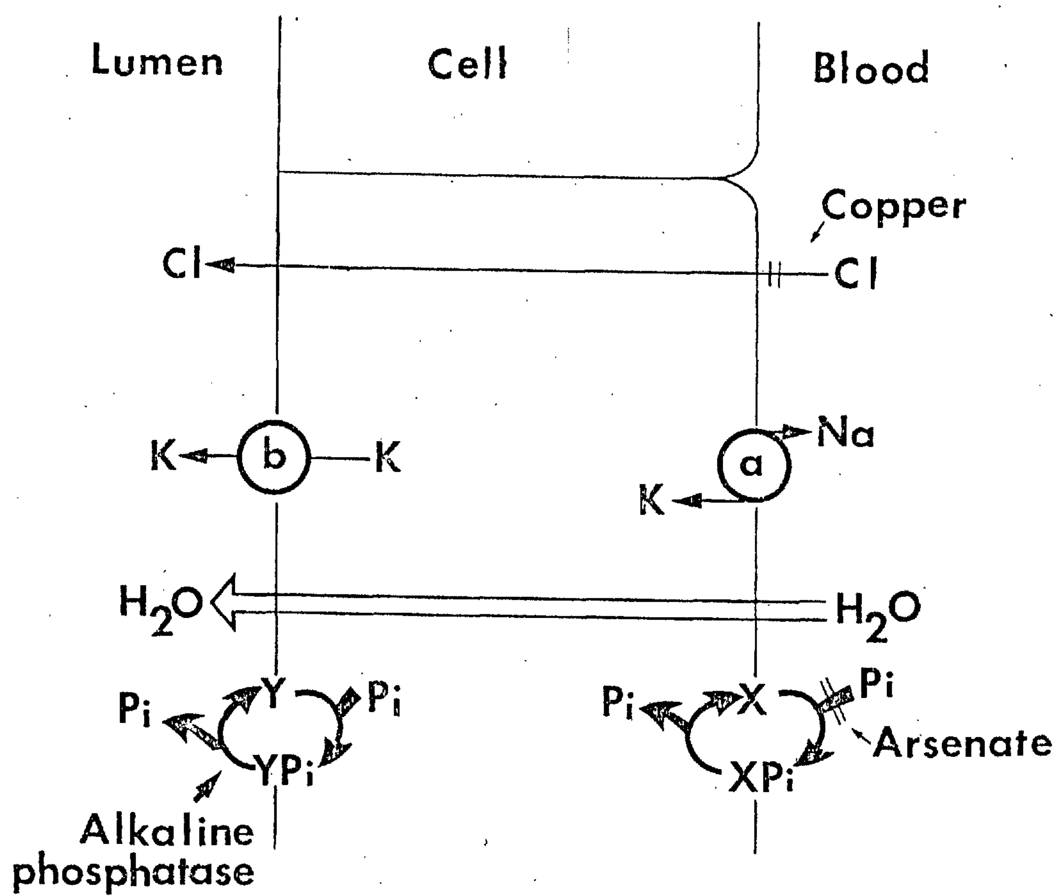
The insect gut, especially that of a herbivore, encloses a potentially hostile environment as the contents reflect the high K^+ concentration and low Na^+ concentration of the plant food injested. The concentrations of these ions are reversed in most animal cells, high cellular K^+ but low extracelluar K^+, particularly in nerve and muscle cells where the function is based on the resting membrane potential. Thus K^+ must be concentrated on the inside whilst Na^+ is concentrated on the outside. If the insect haemolymph equilibrates with the gut's ionic environment the concentration of K^+ in the blood would disrupt the resting membrane potential of the cells. Thus the main energy-requiring process which occurs in the Malpighian tubule is the active transport of K^+ against a steep electrochemical gradient from the haemolymph to the lumen so that the insect is protected from excessive extracellular K^+. In some cases special modifications of the midgut (goblet cells) have been found to actively secrete K^+ from the haemolymph thus reducing haemolymph levels (Haskell *et al.*, 1965).

If the distribution of the mitochondria is indicative of the areas in which active transport occurs than both basal and apical surfaces are involved in this process. It has been demonstrated (Berridge, 1968;

Maddrell, 1969; Pilcher, 1970) that two separate components of K^+ transport exist. One requires the presence of Na^+ whilst the other functions in the absence of any other univalent cation (fig. 1.3.1.). These two components may well correspond to the basal and apical transporting systems. The presence of Na^+ ions allow K^+ ions to cross the cell at a high rate even when present in the bathing medium at a very low concentration. Sodium could be involved in a Na-K pump. Berridge (1968) proposes a hypothetical model. He suggests that K^+ secretion involves two pumps which are located on the basal and apical membranes. The former is a classical Na-K exchange pump which extrudes Na^+ from the cell into the blood in exchange for K^+. The latter is an electrogenic pump and transports K^+ from the cell into the lumen (fig. 1.3.1.). However, in this hypothetical model Na^+ ions have to cross the basal membrane, but there is some evidence that the basal membrane is impermeable to Na^+ ions (Maddrell, 1971).

Berridge (1968) showed that the rate of fluid secretion in Calliphora tubules depends on the concentration of K^+ ions in the bathing medium. In the absence of K^+ ions fluid secretion is slowed to less than 10% of its normal rate. Coast (1969) showed that the rate of secretion of tubular fluid was proportional to the osmotic pressure of the bathing fluid if a constant K^+

Fig. 1.3.1. Diagram to illustrate the model of ionic secretion by Malpighian tubules. a, coupled Na-K exchange pump on the basal membrane; b, 'electrogenic' potassium pump on the apical membrane. (After Berridge, 1968).


Lumen
Cell
Blood
Copper
Cl
Cl
K
b
K
Na
a
K
H2O
H2O
Pi
Y
Pi
YPi
Pi
X
Pi
XPi
Arsenate
Alkaline
phosphatase

concentration was used. If the osmotic pressure was kept constant the rate of secretion was related to the K^+ concentration. These findings suggest that water movements are secondary to the movements of K^+ and thus K^+ is actively transported by an electrogenic K^+ pump found on the apical cell membrane. Passive transport of K^+ is probably from the basal side due to the potential drop on this side of the cell.

If the Malpighian tubules merely removed substances from the haemolymph to the lumen the substances would accumulate and thus a mechanism must exist which would rapidly flush out the tubule contents and eliminate the excretory products. Potassium ions are thus actively secreted into the urine to make up the osmotic difference and thereby maintain a flow of urine.

1.3.2. Active sodium transport.

The Malpighian tubules of *Carausius*, *Rhodnius*, *Calliphora* and *Calpodes* also transport Na^+ into the lumen, in many cases actively. The absence of competition between Na^+ and K^+ transport suggests the presence of a specific Na^+ pump. It is possible that active transport of Na^+ is primarily a property of the apical microvilli. Maddrell (1969) showed that at the distal region of the tubule in *Rhodnius* where Na^+ concentrations are high the apical microvilli are up to 10 μm long and

are associated with many mitochondria whilst the basal infoldings are not emphasized. In Calliphora however the apical microvilli are only 3 μm long and associated with fewer mitochondria. Here Na^+ transport occurs slowly (Berridge, 1968).

Gee (1976a) has shown that tubules from Glossina transport Na^+ against a concentration gradient whereas K^+, although its presence is required, does not play an active role in fluid secretion. The rate of fluid secretion by Glossina tubules is therefore dependent on the Na^+ concentrations rather than the K^+ concentration. This Na^+ pump however is shown by Gee (1976a,b) to be insensitive to ouabain. Gee suggests that the presence of K^+ is essential in order to supply a pump on the basal cell membrane which maintains high intracellular K^+ concentrations (i.e. the Na-K-ATPase pump, fig. 1.3.1.). He also showed that the secretion of fluid can be inhibited by ethacrynic acid and amiloride (both compounds are Na^+ pump inhibitors in vertebrates) and these observations further confirm that fluid secretion by Glossina tubules is dependent on a Na^+ electrogenic pump.

1.3.3. Movement of other ions.

Chloride movements are thought to be passive but in Rhodnius a Cl^- pump has been shown to occur. A potential

gradient exists on the luminal side which would encourage movement into the lumen. The addition of copper ions which inhibit passive entry of anions (perhaps by clogging up the apertures or 'pores' of the membrane) prevents the tubules from secreting chloride ions (Maddrell, 1971).

Phosphate ions, however, appear to be transported either actively or by facilitated diffusion. Generally, smaller ions allow a faster secretion than do larger ones. Phosphate ions would be expected to cross slowly but this does not happen, phosphate ions allow secretions at a faster rate than any other anion. In addition, phosphate ion secretion in *Calliphora* is not prevented by the addition of copper ions. Fig. 1.3.1. shows the scheme proposed by Berridge (1968). It is postulated that the ions enter the cell by means of a carrier X situated on or in the basal membrane. This process may involve phophorylation on the outside of the basal membrane and dephosphorylation on the inside. This explains the fact that arsenate ions inhibit phosphate transport. Phosphate would thus leave the cell by combining with a carrier Y on the apical membrane and split off by a phosphatase. A non-specific alkaline phosphatase has been localized in the apical cell membrane (Berridge, 1967).

Transport of magnesium is thought to be passive however, Phillips & Maddrell (1974) demonstrated the active transport of Mg^{2+} by Malpighian tubules of the larva *Aedes campestris*. Unlike the tubules of other insects, e.g. *Carausius* where Mg^{2+} concentration in the secreted fluid is always lower ($<10\%$) than the bathing fluid, Mg^{2+} is transported at high rates against electro-chemical potential gradients. This has been calculated to be fast enough to meet the excretory requirements of the insect.

Berridge (1969) showed the inability of sulphate ions in supporting urine formation and it was suggested that the large sulphate anion could not pass rapidly enough through aqueous-filled channels or pores in the cell membrane. However, Knowles (1975) showed that the tubules of *Calliphora* secrete both phosphate and sulphate at comparable rates. He argues that although sulphate ions cannot support fluid secretion it is still possible that the transported sulphate ion cannot be utilized in the establishment of osmotic gradients and suggests a different site of transport for sulphate ions. The possibility exists that sulphate secretion by the tubules is linked to the removal of β-ecdysone conjugates (Price & Russel, 1975). Active sulphate transport has been shown to occur in *Aedes campestris* larvae (Maddrell & Phillips, 1975).

1.3.4. Movement of solutes.

Ramsay (1958) showed that various organic solutes of low molecular weight when added to the bathing medium of isolated Carausius tubules are concentrated in the secreted fluid. Ramsay found that DL-alanine, L-arginine, glycine, L-lysine, L-proline, DL-valine, D-glucose, D-fructose, sucrose and urea could easily cross the wall of the tubule. Maddrell (1972) pointed out that there could be frictional effects between the fluid moving into the lumen and these substances but nevertheless it is clear that the tubule wall must be permeable to these substances. Ramsay also provides evidence to support passive diffusion as the mechanism of transport of these substances. Thus metabolically useful substances are found in the secreted fluid of the tubules and would have to be actively reabsorbed. The major advantage of such a system, as explained by Ramsay, is that there is an automatic excretion of any unwanted substances simply by not providing a specific mechanism for its reabsorption.

Maddrell & Gardiner (1974) showed that tubules from a range of insects are permeable to a range of organic solutes even to molecules as large as inulin. The hindgut of insects is lined with cuticle and would have a much lower permeability to organic solutes than the tubules. Useful as well as toxic substances would

therefore be too large to cross the cuticular lining of the rectum and so could not be reabsorbed there. Absorption of water in the hindgut must lead to a greater concentration of substances dissolved in the rectal fluid and if the rectal wall was permeable to these substances there would be a strong tendency for them to diffuse back in to the haemolymph. In addition, the rectal cells would be bathed by highly toxic substances. Thus the rectal wall impermeability is necessary and useful substances such as disaccharides could be reabsorbed before they reach the rectum. The continuous flow of fluid into the lumen of the tubule ensures that a concentration gradient always exists between haemolymph and the primary excretory fluid and in turn ensures the passive transport in the fluid of substances that are able to diffuse across the wall.

Insect Malpighian tubules are also known to concentrate certain toxic or useless substances that are larger in size than the above considered molecules. Lison (1938) found that molecules with strongly acidic groups are actively transported by the tubules. Molecules with both acid and basic groups cannot cross the basal membrane and molecules weakly acidic can cross the basal membrane but are not concentrated in the lumen although they may stain the cytoplasm. Palm (1952) provides support to Lison's work. Lison and Palm provide data for an

active transport of a range of dyes by various insects but they do not provide the reason for a specific mechanism for their excretion. Ramsay (1958) provides a possible explanation: "It may be that the ability to concentrate dyes is an incidental property of some other feature of the excretory mechanism". In mammalian physiology it appears that dye molecules are concentrated by a mechanism normally used to excrete such non-metabolisable aromatic residues as hippuric acid (Smith, 1951). It is therefore feasible that dyes are excreted because they resemble other molecules normally excreted by the insect.

Pilcher (1970) showed that the rate of dye secretion is very little affected by changes in the rate of fluid secretion. At low rates of fluid secretion, the dye is concentrated by the tubules but higher rates of fluid secretion do not speed up dye secretion. Maddrell et al. (1974) showed two separate mechanisms for the active transport of two types of organic anions: acylamines and sulphonates. In the more permeable tubules of Calliphora dye transport depends on the rate of fluid secretion whereas in the less permeable tubules of Rhodnius and Carausius dye secretion is not affected by the rate of fluid secretion. Two separate mechanisms were deduced from the fact that competitive inhibition could not be demonstrated between members of the two groups whereas the presence of one compound

will interfere with the secretion of another of the same group. Maddrell & Gardiner (1975) showed that the ability to transport organic anions can be induced by feeding. They also showed (1976) the ability of tubules from Rhodnius, Pieris, and Manduca to excrete alkaloids. Many potentially toxic molecules are altered biochemically to form less toxic molecules (Maddrell, 1974) e.g. hippuric acid, ethereal sulphates, β-glucosides and β-glucuronides, acetamide derivatives and methylated compounds. It is for such compounds that the tubules possess an active transporting system. These compounds are too large and would diffuse at a relatively slower rate and thus a mechanism for their transport speeds up their excretion. Chapters 4 and 5 describe experiments showing transport of toxins such as ouabain and phlorizin which may be compared to the above mechanisms.

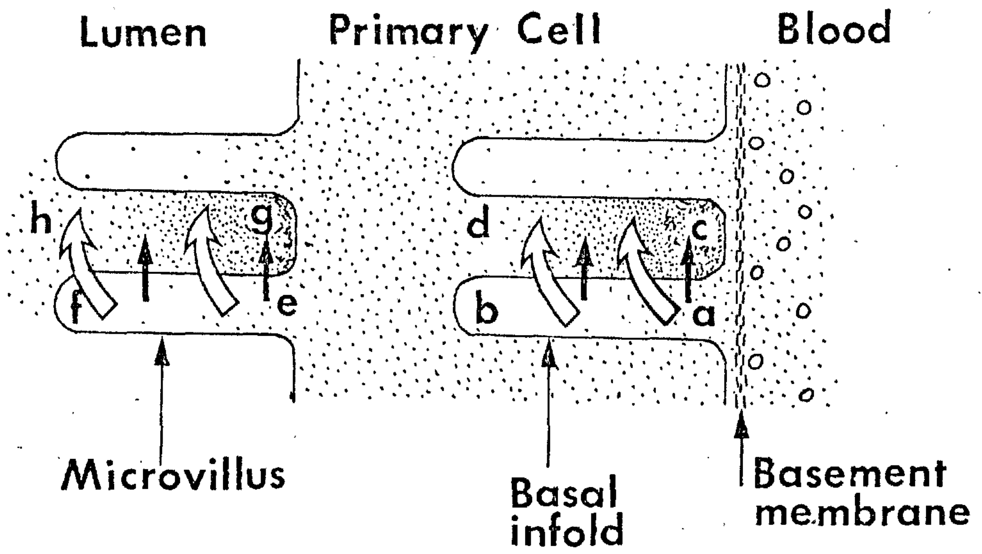
There is evidence that several of the useful metabolic substances occurring in the primary secretion of the tubules may be reabsorbed by the tubules themselves as well as the hindgut. This may be attributed to different regions or different cell types (see section 1.1.1.). Knowles (1975) provides evidence for glucose reabsorption in Calliphora tubules which, similar to the vertebrate system, is phlorizin sensitive. Further evidence for this reabsorption in Locusta tubules is presented and discussed in Chapter 6.

1.3.5. Movement of water.

The fluid secreted by the tubules initially is hyper-osmotic to the bathing medium as active secretion of various solutes have taken place. In addition the rate of fluid secreted by the tubules is inversely related to the osmotic concentration of the bathing medium (Maddrell, 1971). It thus seems possible that ion movements are the driving force for water movement which is consequently a passive mechanism.

The surface amplifications of the basal and apical membranes provide a large surface area to facilitate transport and to increase the overall water permeability of the tissue but Taylor (1971) suggests that these functions are incidental, and that the basal and apical membrane amplifications merely increase the effective passive permeability of the cells to solutes. As the tubules produce a fluid containing all substances of low molecular weight which are present in the haemolymph this explanation seems probable. The presence of the apical and basal infoldings provide blind ending channels in which standing concentration gradients could be formed if the compartments are unstirred (Berridge & Oschman, 1969; Fig. 1.3.5.1.). Local osmotic gradients are set up by the basal folds (a-b) and apical micro-villi (e-f). Isotonic fluid enters the open ends of the basal channels (a) and becomes hypotonic towards

Fig. 1.3.5.1. Application of the standing gradient hypothesis of solute-linked water transport (after Berridge & Oschman, 1969). For explanation see text.


Lumen
Primary Cell
Blood
h
g
f
e
d
c
b
a
Microvillus
Basal infold
Basement membrane

the closed ends (b) as solutes are pumped into the cell (closed arrows). As solutes enter the cytoplasmic compartments (c-d) a parallel osmotic gradient is set up. A combination of these two parallel standing gradients brings about a coupling across the surface and it is thought that both are important for inducing water transport. Fluid is hypertonic in the closed ends (c) but becomes isotonic at the open end (d) as water enters the cell (open arrows) from the hypotonic fluid in the basal folds. Similar osmotic gradients are set up in the microvilli. This results in an isotonic secretion in the absence of an osmotic gradient between the lumen and the blood.

Before accepting the standing gradient osmotic flow hypothesis several questions arise. One of the main objections raised in other tissues, the presence of leaky junctions rather than tight junctions (see Section 1.2.2.3.), does not apply to the Malpighian tubules as the channels are blind ending and cell junctions are not involved. The osmolarity of the secretions must be independent of the rate of fluid transport if standing osmotic gradient are predicted. Berridge (1968) found that isolated Dysdercus tubules produce urine with a constant osmotic pressure even when a diuretic hormone was added to increase urine production. Although these points support the standing

gradient hypothesis several features of the structure of the Malpighian tubules do not coincide directly with the hypothetical mathematical model of Diamond & Bossert (1967). The channels of the model systems were up to 100 µm long as compared with those of the Malpighian tubules which are 5-10 µm long (Maddrell, 1971). However Maddrell points out that in the Malpighian tubules both forward and backward channels occur together in series: one set is the basal infolds and the other is the cytoplasmic processes between the infolds. Together they may develop gradients large enough to offset the short channel lengths. Taking the mitochondria as indicative of active transport, the active transport occurs over a large proportion of the length of the channel whereas in the model osmotic equilibration is more complete if the transport is restricted to the blind end of the channel. No experimental evidence exists for the presence of active sites in the blind end only. The assumption of unstirred compartments is not valid as the tubules contract by muscular action causing the effective stirring of the contents (Taylor, 1971). Berridge & Oschman (1969) however, point out that as the infolds are close together the folds of the cytoplasm between them have dimensions which could effectively slow down the rapid mixing of transported ions with the main body of the cytoplasm. These points clearly indicate an inconclusive explanation of water and ion coupling, more experimental evidence

is required before the hypothesis can be accepted. In particular there is little evidence that the intercellular channels have the necessary high osmotic pressure, in fact, Gupta et al. (1976), using electron probe X-ray microanalysis, showed that gradients of Na^+, K^+, and Cl^- do not appear in the extracellular spaces between the microvilli as would be expected for the standing gradient hypothesis.

The standing gradient hypothesis could explain hypertonic fluid secretion by the tubules but some insects e.g. *Carausius* (Ramsay, 1954) secrete a hypotonic urine. It is possible that this is caused by solute reabsorption in the proximal region of the tubule provided the region is relatively impermeable to water. Proximal reabsorption of K^+ is thought to occur in tubules of *Calpodes* (Irvine, 1969) and *Rhodnius* (Ramsay, 1952). Berridge & Oschman (1969) attribute Na^+ reabsorption to the stellate cells having shorter and wider basal infoldings than the primary cells. This agrees with the Diamond & Bossert model and could explain the hypotonic urine of *Carausius*. These cells could return Na^+ to the blood without returning as much water as originally followed Na^+ in the primary cells. Thus if it can be confirmed that the tubules under certain conditions can produce a hypotonic urine and if this cannot be attributed to proximal reabsorption then the standing gradient hypothesis for water movement would have to be rejected.

1.3.6. Summary.

This section has dealt mainly with the 'typical' insect. The Malpighian tubules of blood-sucking insects differ in that they secrete at a high rate after a blood meal but much slower in the unfed insect where in vitro preparations require stimulation (diuretic hormones, 5-hydroxytryptamine or cyclic-AMP). On the other hand isolated tubules from other insects e.g. Locusta and Carausius maintain a constant basal rate of fluid secretion even in the absence of stimulatory chemicals or when starved (Mordue, 1969a; Pilcher, 1970a). Mordue (1969a, 1972) showed that Schistocerca tubules can be stimulated by c-AMP and adrenalin but not 5-HT. Maddrell & Klunsuwan (1973) report that Locusta tubules are unaffected by 5-HT but stimulated by c-AMP. This effect and the effect of various other stimulatory chemicals on Locusta tubules is discussed in Chapter 3 and 5. In Rhodnius and Glossina Na^+ transport plays an important role in secretion and secretion is not slowed down by reducing the K^+ concentration in the bathing medium (Maddrell, 1969; Gee, 1976a) whereas they are sensitive to changes of Na^+ concentrations. In other insects e.g. Calliphora and Locusta the tubules depend on higher K^+ concentrations in the bathing medium to maintain a maximum secretion rate (Berridge, 1967, 1968; Maddrell & Klunsuwan, 1973). Rhodnius tubules also do not seem to have the facilitated transport of phosphate. Their tubules are slowed down by a much larger decrease

in chloride concentrations than required to slow down other tubules. This means that either _Rhodnius_ tubules are more permeable to chloride or that chloride ions are actively transported. Maddrell suggests the possibility of an active transport mechanism for K^+, Na^+ and Cl^- in _Rhodnius_. It is thus well established that many insect Malpighian tubules secrete fluid in a similar fashion. The tubules of _Rhodnius_ are different in that their secretion requires stimulation, whilst those of _Aedes_ secrete magnesium and sulphate actively and _Glossina_ tubules depend on a Na^+ electrogenic pump. These species differ in accordance with their habitat.

Locusta tubules however, conform much more to the 'typical' generalized tubule where there is a Na^+ dependent K^+ entry on the basal side and an electrogenic K^+ pump on the apical side of the tubule cell. This active secretion is the basis for water transport which accompanies the ions in amounts which compensate the osmotic difference. Organic compounds in solution in the haemolymph enter the tubule lumen both passively and actively. The small molecules occur at high concentrations and this reflects the high permeability of the tubule to those substances. There is a possibility that the actively secreted organic compounds may act to further water movements and this requires

further validation (Chapters 4 and 5). The question of reabsoprtion by the tubules themselves is not well understood as it has been attributed to the stellate cells in some insects e.g. Calliphora whereas these stellate cells are not found in other insects e.g. Rhodnius. Is reabsorption confined to such cells, or does reabsorption still occur in the tubules without such cells? This question is expanded in Chapter 6 where evidence of reabsorption in Locusta tubules is given.

An attempt has been made to review the recent discoveries in this field and emphasize the areas in which more research is required as well as those areas discussed in the proceeding Chapters. Research on other insect Malpighian tubules would allow us to see whether the generalizations which apply to the tubules already examined are firmly based or not.

2. MATERIALS AND METHODS.

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2.1. MATERIALS

Locusta migratoria migratorioides (R.&F.) and *Zonocerus variegatus* (L.) were used as experimental animals. *Locusta* male adults (one week old) were obtained from a laboratory culture kept at 29-30°C and reared under a 16 h photo-regime on a diet of bran, fresh grass and lettuce. *Zonocerus* were obtained as first and second instars from the Centre for Overseas Pest Research, London and reared on a diet of bran, cabbage and the weed *Senecio jacobae*. This weed, which contains several poisonous alkaloids (Aplin & Rothschild, 1972), was collected from various sites in London and Oxfordshire. The adults were used in the experiments.

In some experiments an artificial diet containing glycosides was given to *Zonocerus* adults. The diet used was one devised by B.O.C. Gardiner (personal communication) to which ouabain (10^{-3}M) was added. It contained the following solid ingredients which were premixed thoroughly and stored in the refridgerator:

Casein	350 g
Bemax	750 g
Sugar	300 g
Dried yeast	150 g
Wessons salts	100 g
Sorbic acid	15 g
Cholesterol	10 g
Methyl-4-hydroxybenzoate	10 g
Choline chloride	15 g

The composition of Wessons salts was as follows:

$CaCO_3$	600.0 g
$CuSO_4$	0.6 g
$ZnCl_2$	0.5 g
$MnSO_4$	10.0 g
KI	1.6 g
Ferric citrate	55.0 g
$CaHPO_4$	150.0 g
NaCl	335.0 g
$MgSO_4$	240.0 g

The above salts were ground up in a pestle and mortar where necessary before thoroughly mixing in a 2 l beaker. This mixture was stored until required for use. When required for use a quantity of the complete salts was made up by adding 64.5 g K_2HPO_4 to 135.67 g of the above prepared mixture and thoroughly mixing by shaking in a bottle.

To prepare the diet 20 g of agar was added to 500 ml of water and allowed to dissolve over boiling water. 168 g of the solid premixed ingredients was added to 350 ml of a 10^{-3}M solution of ouabain. This was mixed thoroughly using a magnetic stirrer and the following ingredients were added:

Corn oil	2.0 ml
Formaldehyde	4.5 ml
Liquid vitamin mixture	2.0 ml

The dissolved agar was cooled down to 70°C and mixed in to the above mixture. When mixed, 4.0 g Vitamin C and 2.0 g Aureomycin were added to the mixture. This

was then poured out into plastic dishes and allowed to set. Each dish contained 0.0275 g of ouabain which was enough to feed a cage of about 30 individuals for two days.

2.2. TISSUE PREPARATIONS.

2.2.1. *In vitro* tubule preparation.

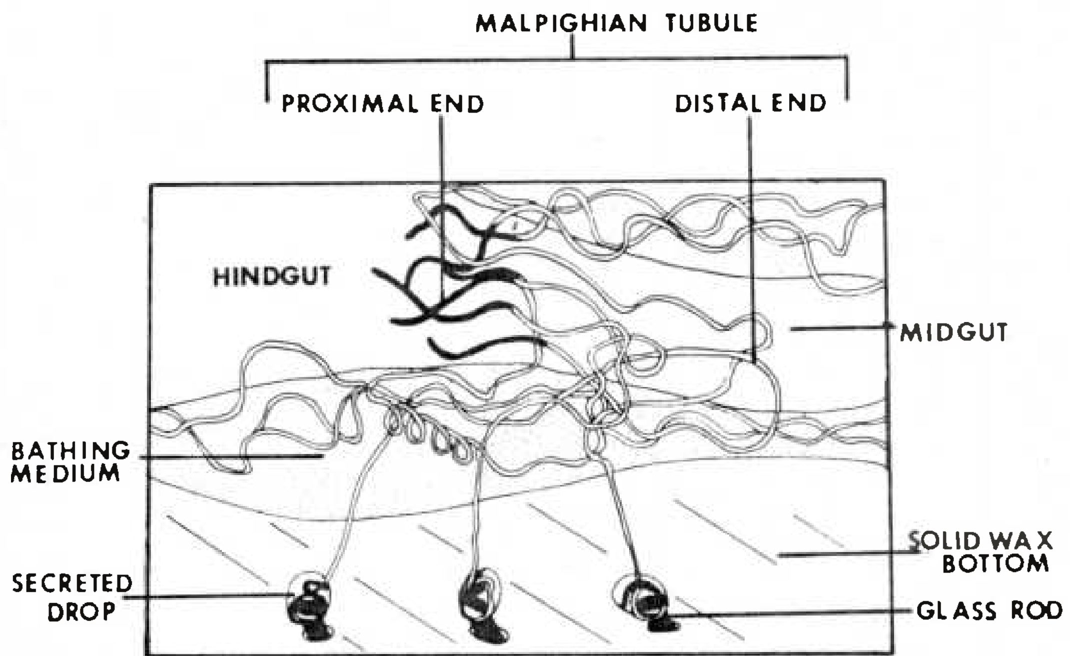
The *in vitro* technique for locust tubules devised by Maddrell & Klunsuwan (1973) was used to investigate Acridid tubular function. This method involves a transverse incision of the tip of the insect's abdomen followed by a gentle pull of the head away from the thorax. This tears the thin cuticle of the neck and the gut with the Malpighian tubules still attached to it is drawn out. The posterior cut end of the gut is ligatured and the whole length of the gut is immersed in 250-300 µl of bathing Ringer under liquid paraffin in a wax bottomed petri dish. The head is held down by a saddle-shaped pin to insure that no regurgitated fluid contaminates the bathing fluid. When this occurred the bathing fluid discoloured and the tubules immediately ceased to secrete. Tubules were cut at their point of entry into the midgut-hindgut junction and wound around fine coloured-glass rods

which were held by the wax in the petri dish (Plate 2.2.1.). This method was later modified to the use of Perspex dishes with drilled holes into which stainless steel pins were inserted to replace the coloured glass rods. Secreted drops accummulated around the rods and could be measured and collected for analysis.

There are many advantages to this method: it is easy to make any desired number of tubule preparations which run simultaneously. The tubules themselves are subjected to the minimum amount of handling and therefore they are less likely to have been ruptured along their lengths during the preparation as, in fact, the lengths of the tubules bathed in the Ringer solution are not touched at any stage during the setting up. In this way the distal ends of the tubules are not interfered with and their close association with the trachae is still maintained.

The standard Ringer solution used was that used by Maddrell & Klunsuwan (1973) which was found to maintain a constant rate for several hours. This contained: 5.73 g/l NaCl, 1.50 g/l KCl, 0.30 g/l $CaCl_2$, 0.41 g/l $MgCl_2$, 1.80 g/l glucose, 1.86 g/l $NaHCO_3$, 1.09 g/l NaH_2PO_4, 0.83 g/l sodium glutamate, 0.88 g/l sodium citrate, and 0.37 g/l malic acid. The concentrations of the following main ions were: Na^+ 142 mM, K^+ 20 mM, Ca^{2+} 2 mM, Mg^{2+} 2 mM, Cl^- 126 mM, HCO_3^- 22 mM, and $H_2PO_4^-$ 7 mM. The glucose concentration was 10 mM.

Plate 2.2.1. Photograph and explanatory diagram of the *in vitro* tubule preparation.


MALPIGHIAN TUBULE
PROXIMAL END
DISTAL END
HINDGUT
MIDGUT
BATHING MEDIUM
SOLID WAX BOTTOM
SECRETED DROP
GLASS ROD

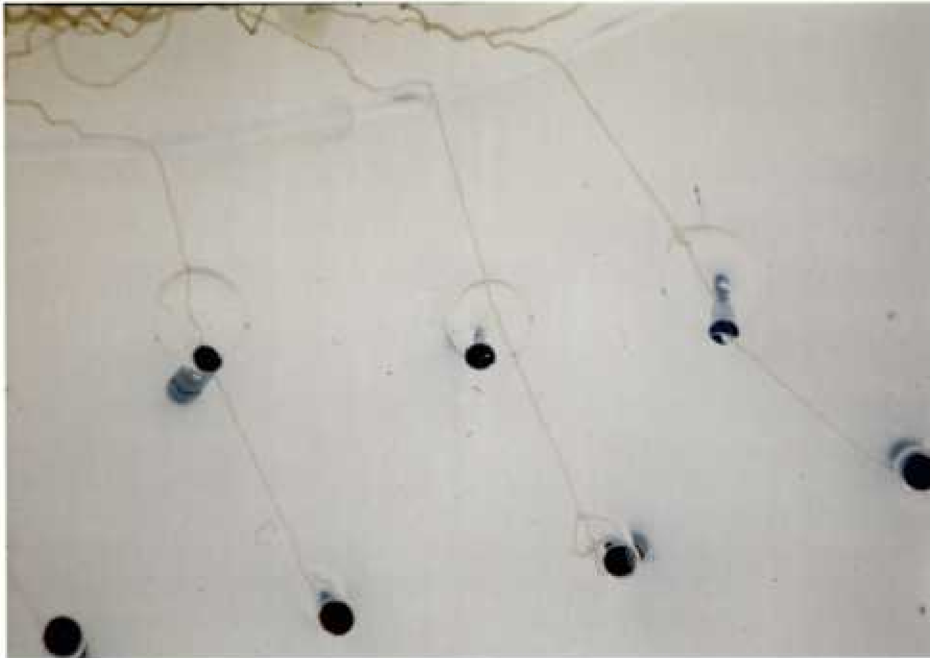
2.2.2. The double droplet preparation.

The *in vitro* technique was carried out as described above (2.2.1.). Tubules, when extended out to be wound around the glass rods, were passed through another bathing medium droplet (M_2) (Plate 2.2.2.). This method is essentially the one used by Ramsay (1958) adapted for the locust preparation.

2.2.3. The *in vitro* hindgut preparation.

Isolation of the rectal hindgut complex was performed by decapitating adults. The abdomen was then opened to expose the gut. The gut contents were then flushed out *via* a polythene tube ligatured at the top of the hindgut section. This was facilitated by using locusts which were starved overnight. The anal region was then ligatured and a 1% amaranth solution in Ringer was injected through the polythene tube into the hindgut sac to monitor any leakages. Fifty microlitres of the test solution was then injected into the sac and the sac was immersed in a 1 ml Ringer solution appropriate to the test substance within centrifuge tubes set up in a water bath at 29°C and saturated with bubbling O_2 (Fig. 2.2.3.). Several preparations could be set up in this way and the O_2 passed to each vial *via* T-tubes.

Plate 2.2.2. Photograph and explanatory diagram of the *in vitro* double droplet tubule preparation.



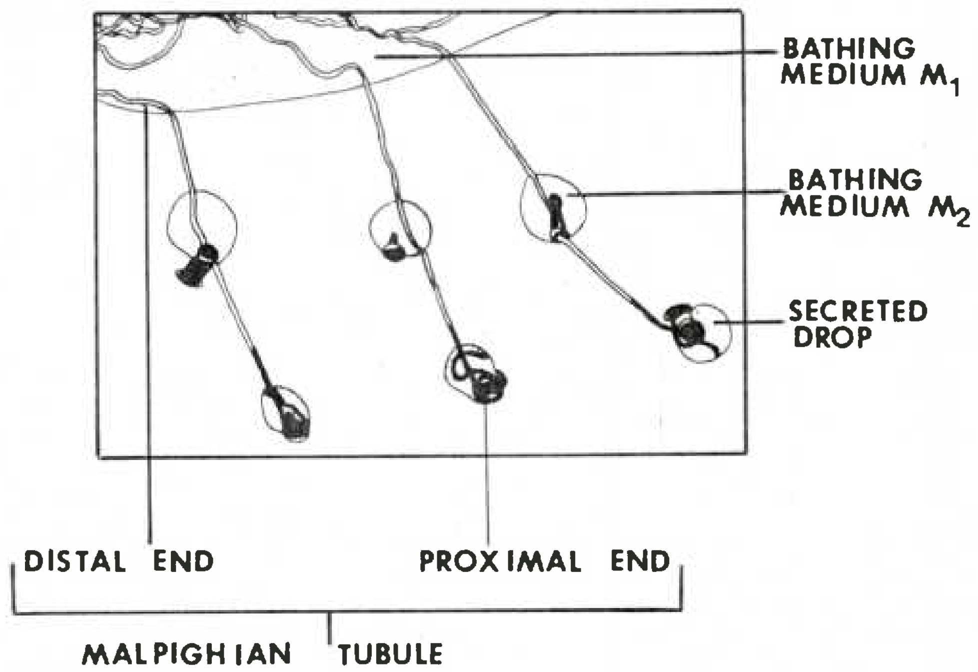
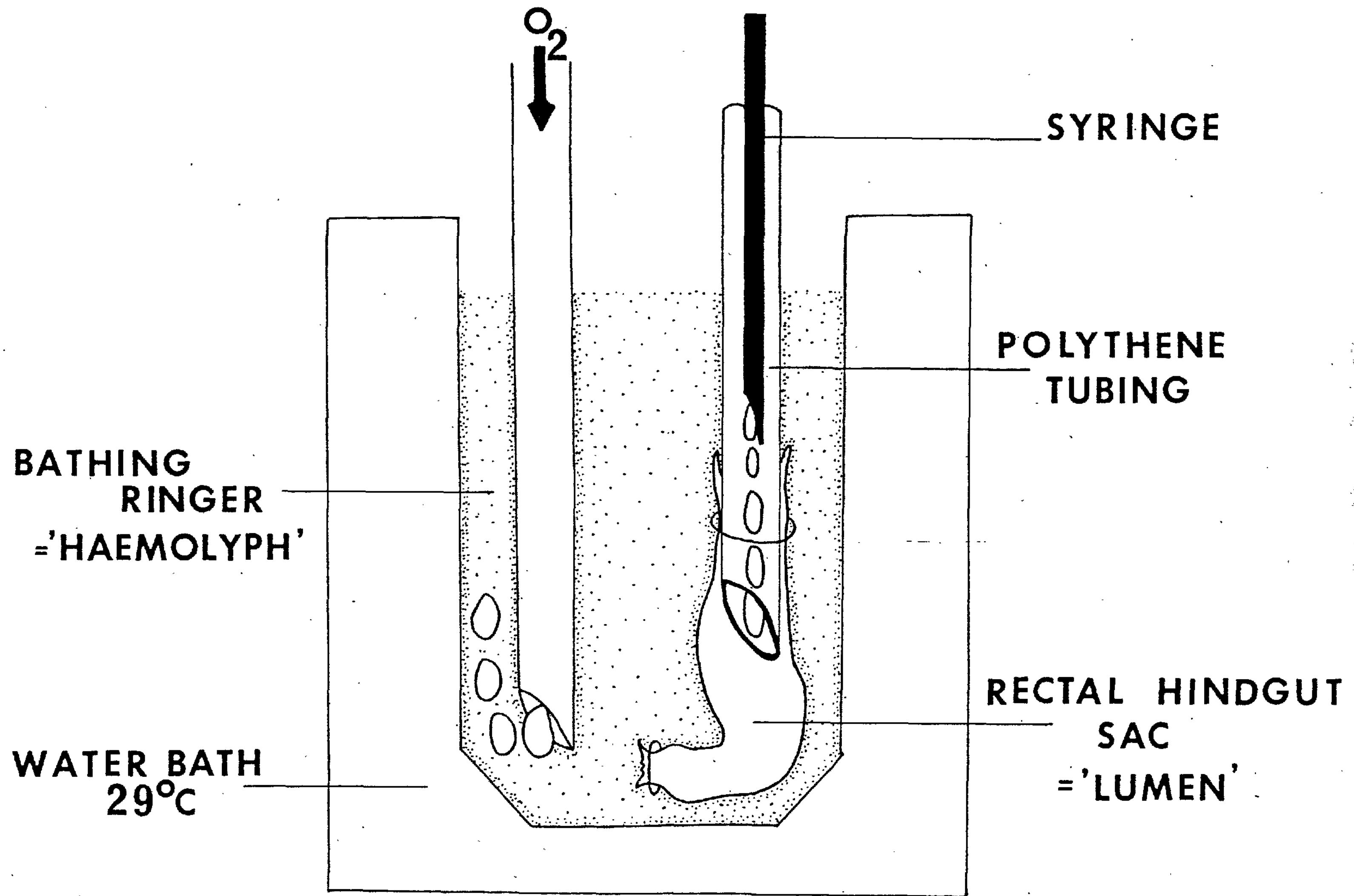

BATHING MEDIUM M_1
BATHING MEDIUM M_2
SECRETED DROP
DISTAL END
PROXIMAL END
MALPIGHIAN TUBULE

Fig. 2.2.3. Diagrammatic representation of the *in vitro* hindgut preparation.


O2
SYRINGE
POLYTHENE
TUBING
BATHING
RINGER
='HAEMOLYPH'
RECTAL HINDGUT
SAC
='LUMEN'
WATER BATH
29°C

2.2.4. Hormonal extract preparations.

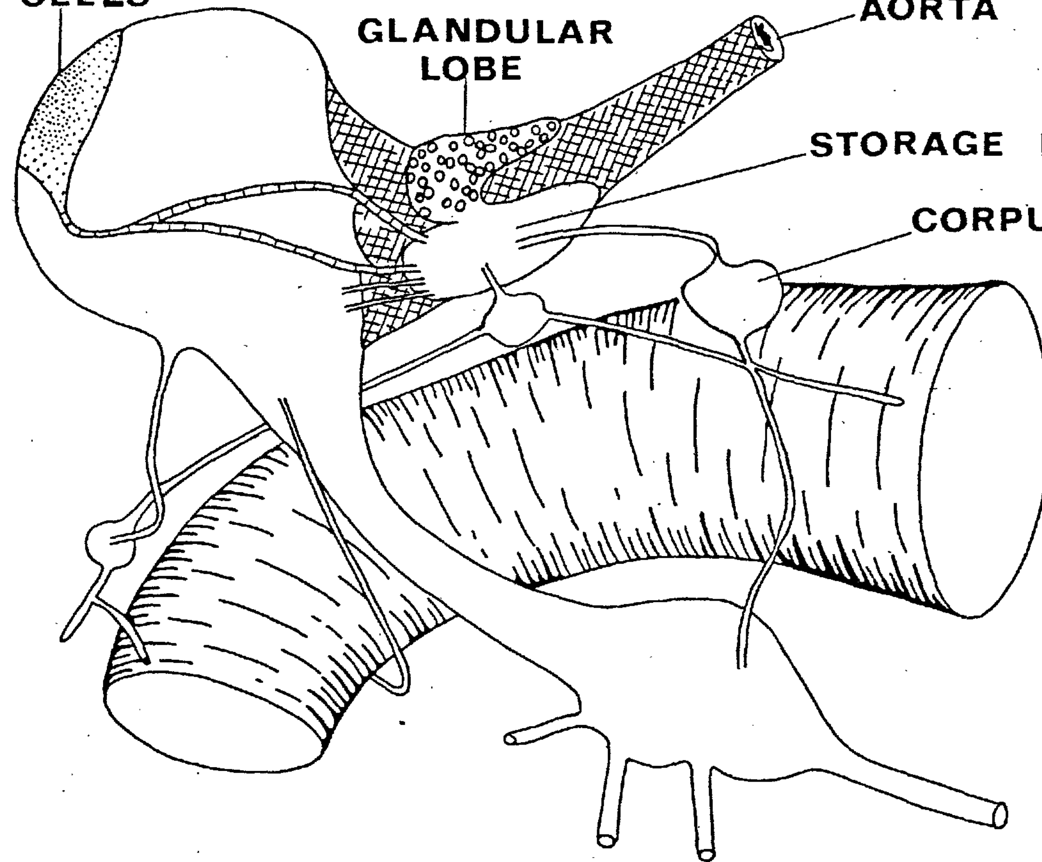
A diuretic factor was obtained from extracts of storage lobes of locust corpora cardiaca (Fig. 2.2.4.). Extracts were prepared by dissecting the storage lobes and sonicating them in either methanol or saline for 5 min. The resultant solution was centrifuged for 5 min and the supernatant removed and used as the assaying source in the case of saline extracts. The methanol extract was first evaporated under a stream of N_2 and then redissolved in saline for assaying.

2.3. ASSAYS.

2.3.1. Rate of fluid secreted.

The rate of fluid secreted was computed from measurements of the volume of the secreted drop and the time interval during which the drop was produced. The volume of the drop of secreted fluid formed at the cut end of a tubule was calculated from its diameter assuming it to be equivalent to a sphere ($V = 4/3 \pi r^3$). The diameter was measured by a micromètre eyepiece graticule. The assumption that the drops were spherical was tested by

Fig. 2.2.4. The neuroendocrine system in *Locusta*.
(After Cazal, 1971).


BRAIN NEUROSECRETORY
CELLS
GLANDULAR
LOBE
AORTA
STORAGE LOBE
CORPUS ALLATUM

comparing the size of drops of known volume delivered by a microlitre syringe to those expected from the above formula. No significant difference was found with drop sizes encountered in the experiments (Table 2.3.1.1.).

The rate of secretion by the Malpighian tubules was found to vary according to the time of the year, age of the insect, sex, type of diet and whether the insect was well fed or fasting. In order to provide a reasonably standard assay for diuretic activity only one week old, fed, male adults were used as experimental animals. Even then, tubules taken from single insects were found to vary in their secretion rates and a single mean value for the basal rate of tubules in general was unreliable. It was found, therefore, necessary to obtain a basal secretion rate for each tubule for the first 20 min of secretion before any tests were made on the tubules. Thereafter, each tubule was handled separately, each according to its own basal rate.

The experimental procedure of using 10 tubules from one individual and using these 10 individual values as a reflection of the behaviour of tubule populations was tested by performing a two way analysis of variance test. As this showed an insignificant difference between means (Table 2.3.1.2.) the

TABLE 2.3.1.1.

Chi-squared test of goodness of fit testing the assumption that the drops of secreted fluid are spherical.

VOLUME (µl)	EXPECTED DIAMETER ($V=4/3\pi r^3$)	OBSERVED DIAMETER	$\frac{(O-E)^2}{E}$	P
0.1	10.4	13	0.65	0.5
0.2	12.8	16	0.80	0.5
0.3	14.8	18	0.69	0.5
0.4	16.2	20	0.89	0.5
0.5	17.6	21	0.66	0.5
0.6	18.6	22	0.66	0.5
0.7	19.6	22	0.29	0.5
0.8	20.6	25	0.95	0.5
0.9	21.4	25	0.61	0.5
1.0	22.2	30	2.74	0.2

All values of P give no significant difference. Values of $\frac{(O-E)^2}{E}$ greater than 3.84 will give a significant difference. Drop sizes greater than 1.0 µl (value of 2.74) were rarely encountered in the experiments.

TABLE 2.3.1.2.

Two-way analysis of variance to test the assumption that one set of data from one insect is a reflection of the tubule behaviour from locust populations. Using data from three individuals on the increase in diuretic activity as a response to 2.5 gl concentration of storage lobes.

SOURCE OF VARIATION	SUM OF SQUARES	DEGREES OF FREEDOM (f)	MEAN SQUARE	VARIANCE RATIO (F)
Between rows	56.58	2	28.2917	0.802736
Between columns	577.83	7	82.5476	2.342170
Residual	493.42	14	35.2440	
Total	1127.83	23		

The variance ratio both between rows and between columns, i.e. between tubules and between individuals is not significant. (F = 4.74 for a 5% probability).

procedure of using 10 tubules of one insect as a reflection of tubule populations was subsequently used.

2.3.2. <u>Transport of radiochemicals by tubules</u>.

Table 2.3.2.1. lists the radioactive chemicals used. The measured secreted drops were taken up in glass capillaries (melting point tubes) and transferred into scintillation vials containing 5 ml of scintillation fluid containing 5.5 g/l PPO, and 1 g/l POPOP in a 2:1 Toluene:Triton-X 100 medium.

Radioactivity was measured, using a liquid scintillation counter (ICN Tracerlab) either by the pulse height shift method (Wang & Willis, 1965) which provided a value, corrected for quenching (Figs. 2.3.2.1., 2.3.2.2., and 2.3.2.3.), for the concentration of the secreted chemical, or by comparing the cpm of the secreted fluid with the cpm of the bathing medium. This provided a value for activity ratio (cpm/µl secreted fluid/cpm/µl bathing medium). Similarly, reabsorption ratios were determined by using M_2 bathing media droplets of the same specific activity and concentrations as M_1 and comparing these M_2 values with M_1 (cpm/µl in M_2/cpm/µl in M_1).

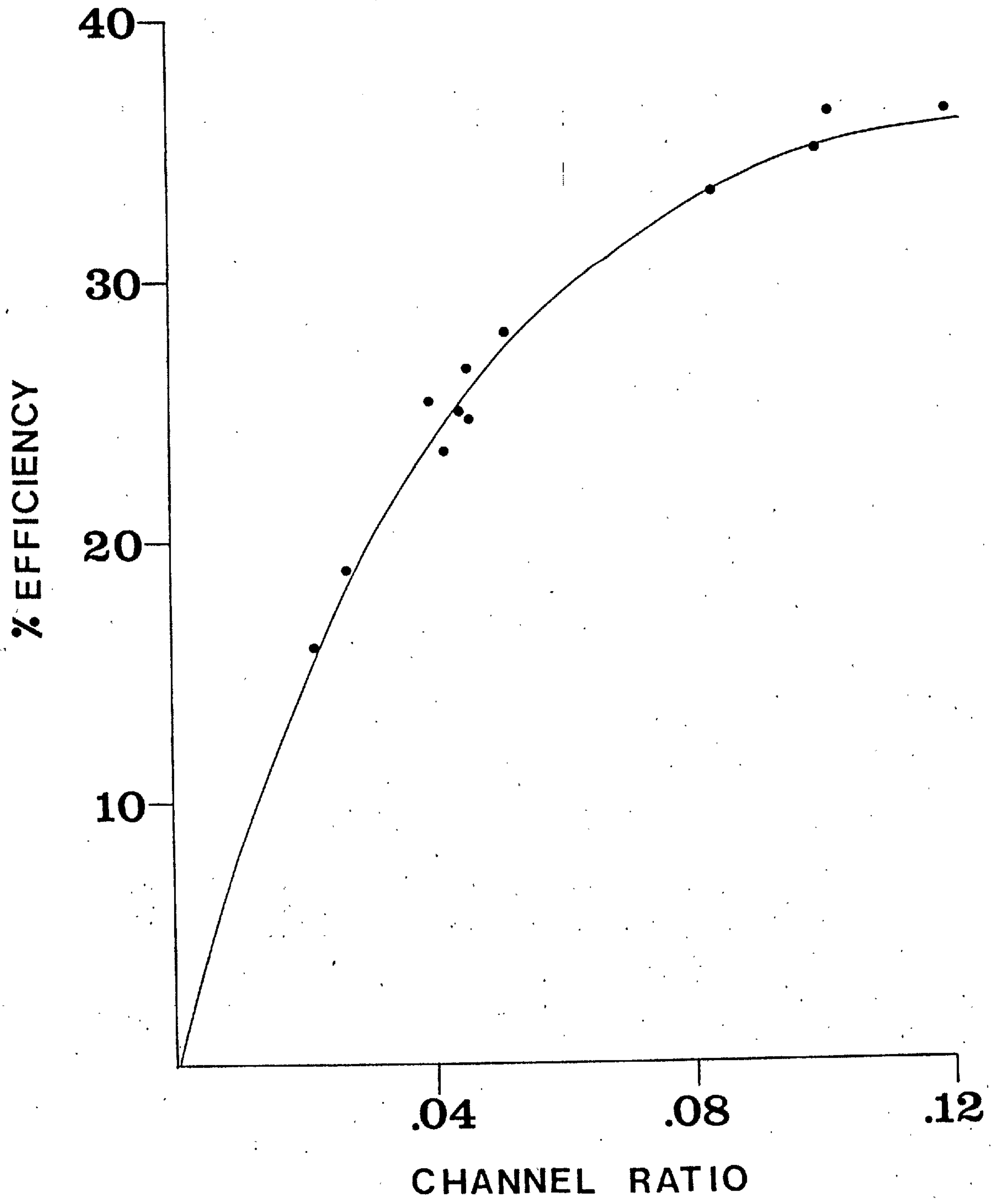
A two way analysis of variance was also performed as described above (2.3.1.) for transport of radiochemicals (Table 2.3.2.2.).

TABLE 2.3.2.1.

Radiochemicals used in the experiments.

ISOTOPE	CHEMICAL FORM	MANUFACTURERS	SPECIFIC ACTIVITY
^{22}Na	NaCl	Radiochemical Centre Ltd., Amersham	0.43 µCi/mM
^{3}H	Ouabain	"	6.70 Ci/mM
^{14}C	Trehalose	"	0.54 Ci/mM
^{14}C	Sorbose	"	0.07 Ci/mM
^{14}C	N-acetyl glucosamine	"	0.05 Ci/mM
^{14}C	Maltose	"	0.72 Ci/mM
^{14}C	Fructose	"	0.30 Ci/mM
^{14}C	Glucose	New England Nuclear, Mass., U.S.A.	0.33 Ci/mM
^{3}H	Phlorizin	"	6.57 Ci/mM

Fig. 2.3.2.1. Quench correction curve for ^3H.
Curve was fitted by eye.



40
30
20
10
% EFFICIENCY
.04
.08
.12
CHANNEL RATIO

Fig. 2.3.2.2. Quench correction curve for ^{14}C.

Curve fitted by eye.

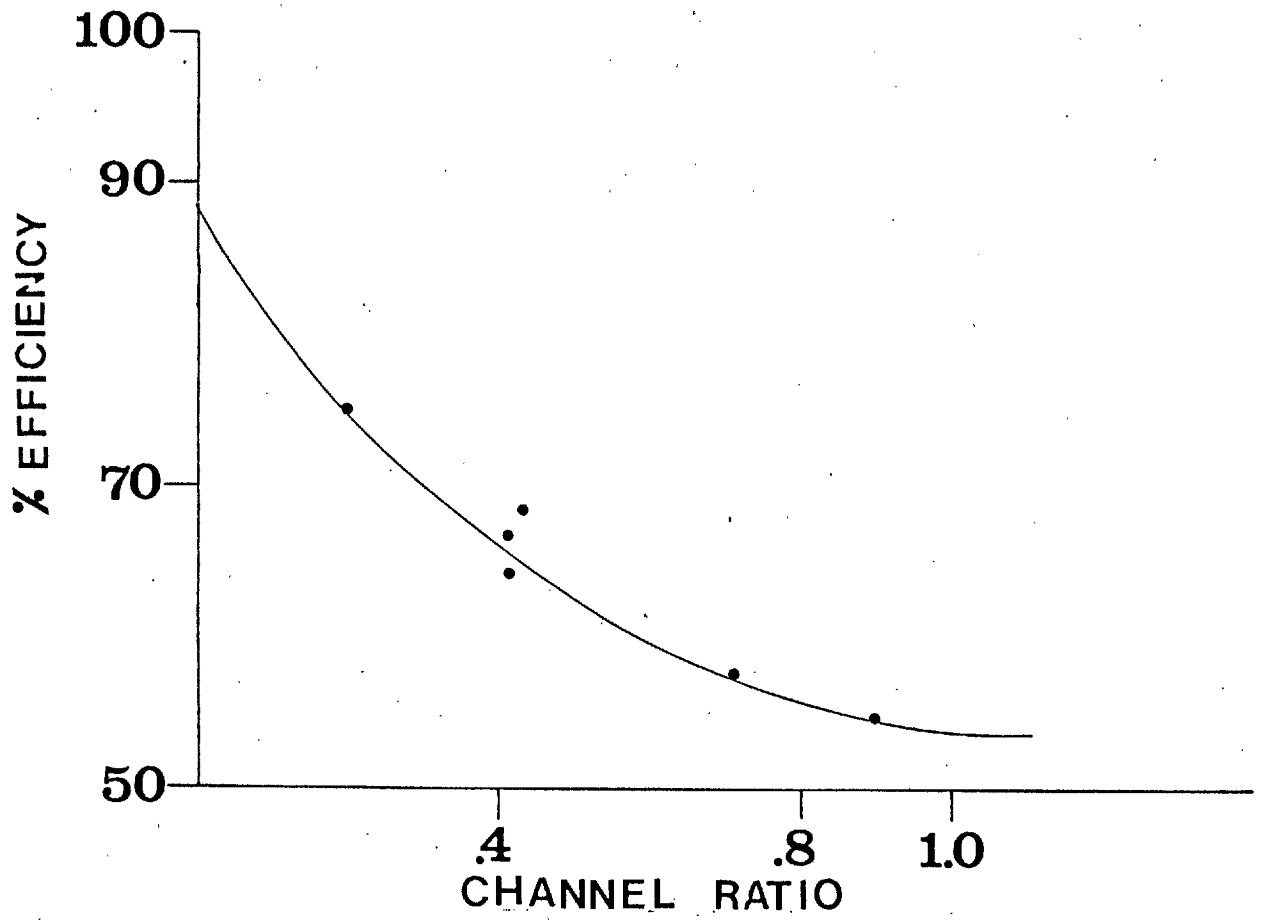

100
90
70
50
% EFFICIENCY
.4
.8
1.0
CHANNEL RATIO

Fig. 2.3.2.3. Quench correction curve for ^{22}Na.

Curve fitted by eye.

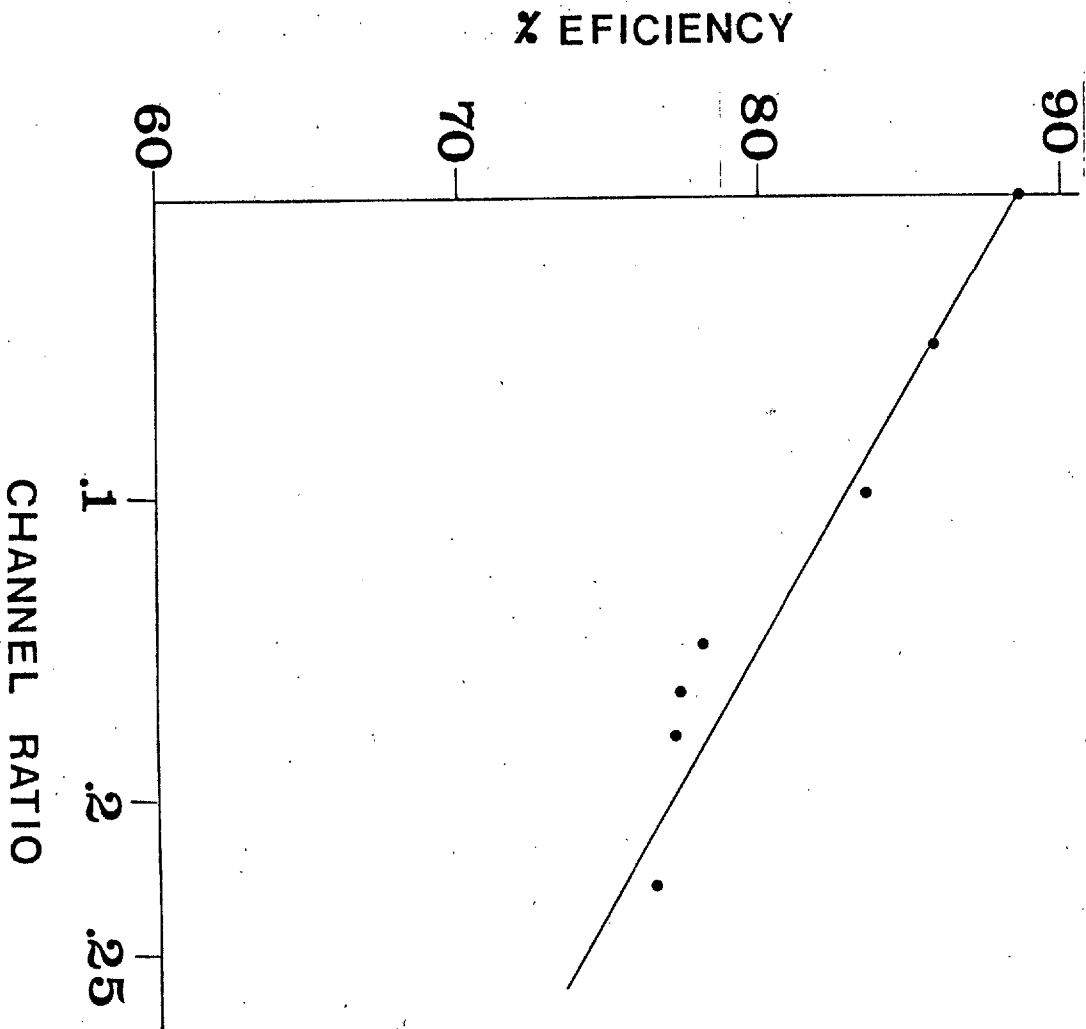

% EFICIENCY
60
70
80
90
.1
.2
.25
CHANNEL RATIO

TABLE 2.3.2.2.

Two-way analysis of variance to test the assumption that one set of data from one insect is a reflection of the tubule behaviour from locust populations. Using data from two individuals on the activity ratios of the secreted fluid containing ^{3}H-phlorizin.

SOURCE OF VARIATION	SUM OF SQUARES	DEGREES OF FREEDOM(f)	MEAN SQUARE	VARIANCE RATIO (F)
Between rows	0.00653	1	0.0065	0.0456898
Between columns	1.37080	5	0.2741	1.91729
Residual	0.71497	5	0.1430	
Total	2.09230	11		

The variance ratio both between rows and between columns, i.e. between tubules and between individuals is not significant. (F= 6.61 for a 5% probability).

2.3.3. Transport of radiochemicals by the hindgut.

The chemicals used were those listed in Table 2.3.2.1. Radioactivity was measured by monitoring the progress of radiolabelled material passing through the lumen into the 'haemolymph' contained in the centrifuge tubes. From the cpm obtained either the actual value for the concentration of the chemical was determined as described in 2.3.2. or the reabsorption ratio was calculated:

$$R.R. = 10/d.f.\ \frac{(cpm/\mu l_{\text{'haemolymph'}} - \text{zero } cpm/\mu l)}{(cpm/\mu l_{\text{lumen}})}$$

where R.R. = Reabsorption ratio

10 = 10 μl aliquot samples

d.f. = dilution correction factor which corrected for the amount of fluid removed when two samples were taken at time intervals.

zero cpm/μl = counts per minute at zero time in the 'haemolymph'.

2.3.4. *In vivo* tracer experiments.

Insects were injected with 50 μl of the radiochemical and at time intervals individuals were sacrificed. Five μl samples of haemolymph were taken up into glass micropipettes and transferred into scintillation vials. The faeces, hindgut and tubules were separately

dissected out and placed in centrifuge tubes containing 1 ml 80% methanol. These were then sonicated for 5 min, centrifuged for 5-6 min and 200 µl aliquots of the supernatant were transferred into the bottom of clean scintillation vials. The supernatant was allowed to air dry before scintillant was added and the vials were counted for radioactivity.

2.3.5. Thin layer chromatography.

Silica gel F254 (ICN Pharmaceuticals, GmbH & Co.) thin layer chromatography plates were used to investigate the composition of the secreted fluid. For phlorizin detection the plates were run in a chloroform: methanol:water (65:25:4) solvent system, dried at 100°C for 15-30 min and observed under ultra violet radiation. When using radioisotopes, centimetre squares were scraped and transferred into scintillation vials, scintillant was added and the vials were counted. Phenol groups were localized, when not using radioisotopes, by spraying with diluted Folin-Ciocalteu reagent (1 vol to 10 vols), drying, and spraying again with 20% aqueous sodium carbonate (Alvarado & Crane, 1964). Commercially obtained phlorizin was used as a standard. For a standard source of the aglycone, phloretin, phlorizin was hydrolysed in a 0.2N sulphuric acid solution

which was heated in a water bath for 90 min, filtered, and the resultant crystalline phloretin was dried and redissolved for spotting (Müller & Robertson, 1933).

3. DIURETIC ACTIVITY.

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3.1. INTRODUCTION

3.1.1. Evidence for a diuretic hormone

In many insects the functioning of the Malpighian tubules can be regulated by a neurosecretory diuretic hormone. Attempts have been made by several workers (Table 3.1.1.) to determine which part of the endocrine system is responsible for the control of tubule activity. In *Rhodnius* Maddrell (1963) found diuretic activity in breis of the pars intercerebralis and the large fused ganglionic mass situated in the mesothorax. The distribution of neurosecretory cells was examined in an attempt to locate the diuretic activity. It was found that 97% or more of the diuretic activity was contained within the posterior neurosecretory cells of the mesothoracic ganglion. In *Glossina* diuretic activity was found in extracts of thoracic ganglia (Gee, 1975a,b).

Extracts of the protocerebrum and corpora cardiaca have shown a diuretic effect on the Malpighian tubules in *Carausius* (Raabe, 1959; Pilcher, 1970a). Similar results were obtained for *Dysdercus* (Berridge, 1966), *Anisotarsus* (Nunez, 1956), *Pieris* (Nicolson, 1976) and *Schistocerca* (Highnam *et al.*, 1965; Mordue,

TABLE 3.1.

The activity of different parts of the nervous system in several insects.

MNSC = median neurosecretory cells, CC = corpora cardiaca, CA = corpora allata, MAG = mesothoracic abdominal ganglion, TG = thoracic ganglia, AG = abdominal ganglia, TAG = terminal abdominal ganglion, D = diuretic, AD = antidiuretic.

1. Altman, G. (1956)
2. Berridge, N.J. (1966)
3. De Besse, N. & Cazal, M. (1968)
4. Gee, J. (1975a,b)
5. Gersch, M. (1967)
6. Highnam, K.C. *et al*. (1965)
7. Jarial, M.S. & Scudder, G.G.E. (1971)
8. Maddrell, S.H.P. (1963)
9. Mills, R.R. (1967)
10. Mordue, W. (1969a,b,c)
11. Nicolson, S.W. (1976)
12. Nunez, J.A. (1956)
13. Pilcher, D.E.M. (1970 a,b)
14. Raabe, M. (1959)
15. Wall, B.J. & Ralph, C.L. (1964)

SPECIES	MNSC	CC	CA	TG	MAG	AG	TAG
Rhodnius[8]	D	D			D		
Dysdercus[2]	D	D					
Schistocerca[6,9]	D	D					
Locusta[3,10]	D	D & AD					
Anisotarsus[12]	D	D					
Gryllus[3]	D	AD					
Periplaneta[3,9,15]	AD	AD	D & AD				D&AD
Corethra[5]				AD	D		
Carausius[13,14]	D	D					
Apis[1]		AD	D				
Clitumnus[3]		AD					
Glossina[4]				D			
Pieris[11]			D				
Cenocorixa[7]	AD						

1969a,b,c; 1971). Highnam *et al.*, (1965) showed that weight loss in hydrated animals was inhibited when the neurosecretory cells in the pars intercerebralis were removed causing distention of the abdomen. In *Locusta* and *Schistocerca* selective cautery of the cerebral neurosecretory cells resulted in water retention and a dramatic increase in blood volume. This was shown to be a result of the removal of a diuretic hormone (Cazal & Girardie, 1968; Mordue, 1971). Mordue (1971; 1972) has shown that this hormone is concentrated in the storage lobes of the corpora cardiaca where a potent effect was observed, whereas only a slight increase in the rate of dye excretion was observed from brain extracts. This may indicate that the hormone is complexed with a carrier protein in the brain. On the other hand glandular lobes of corpora cardiaca only produced a very weak increase in the rate of dye excretion by the tubules, but were shown to contain an antidiuretic factor which increased the rate of reabsorption by the rectum (Mordue, 1972). Cazal & Girardie (1968) showed the presence of an antidiuretic factor in corpora cardiaca which inhibited the fluid secretion by *in vitro* tubules. The diuretic hormone was also shown to have water retention properties on *in vitro* rectal preparations (Goldsworthy & Mordue, 1972). The *in vitro* semi-isolated preparation of the Malpighian

tubules described in Chapter 2 provides a direct method of assay and has been used in this study to examine in more detail these observations on <u>Locusta</u>.

The corpora allata, supraoesophageal ganglion and terminal ganglion in <u>Periplaneta</u> contain both an antidiuretic and diuretic factor (Wall & Ralph, 1964; Mills, 1967). This antidiuretic 'principle' may be produced in the supraoesophageal ganglion, transported to the corpora allata where it is stored until it is required to be released when a need to conserve water arises. This antidiuretic effect is not apparent in dehydrated or salt-loaded animals which may indicate that the hormone is used as quickly as it is produced under such conditions.

3.1.2. <u>Release of the diuretic hormone</u>.

In <u>Rhodnius</u> Maddrell (1963) found that diuretic hormone is released not from the mesothoracic ganglion but from the large number of swellings spread out over the surface of the abdominal nerves each acting as a neurohaemal area. This would provide an increase of the rate at which an effective concentration of the hormone can occur in the haemolymph. A large number of endings both reduce the speed at which replenishment of the hormone is necessary and increase, by summation of a

large number of axons acting at a relatively slow rate, the rate of the system as a whole. These neurohaemal areas have been shown to occur in several insects (Raabe *et al.*, 1971). Maddrell & Gee (1974) found that maximal *in vitro* release of the diuretic hormone in *Rhodnius* occurred when the neurohaemal areas were exposed to high K^+ concentrations (40 mM). They showed further that the release is dependent on Ca^{2+}. From these findings it is presumed that the release of neuro-hormones at these axon endings is due to potassium depolarization associated with the arrival of an action potential, the entry of Ca^{2+} controlling the release.

In both *Rhodnius* and *Glossina* release of diuretic hormone occurs after ingestion of a liquid (Maddrell, 1964; Gee, 1975a). *Periplaneta* (Mills, 1967) and *Carausius* (Ramsay, 1955; Pilcher, 1970a) tubules secrete much faster in fed than in fasting insects. Similarly, starved locusts show a slower rate of fluid secretion than well fed ones. The faeces produced after a short time of feeding were shown to have a much higher water content and amounts of water in faeces is highest in locusts with the more active neurosecretory systems e.g. in crowded conditions (Mordue, 1972). In *Dysdercus*, tubules taken on the

third day of the fifth instar, when the blood contains diuretic hormone, secrete whilst those taken from the sixth day are inactive (Berridge, 1966). Pieris adult tubules are responsive directly after pupal-adult ecdysis (Nicolson, 1976). It is possible that as long as the insects have access to food some hormone would be released into the circulation. The tubules could thus be activated to secrete and will inactivate the hormone at a rate equal to its release into the haemolymph. When the insects no longer feed, the release of hormone ceases or is reduced and the tubules inactivate any remaining hormone and thus cease to secrete.

The extent and duration of diuretic activity is not very marked indicating that the hormone is inactivated in some way or removed from the haemolymph. It has been shown that the Malpighian tubules are able to inactivate the diuretic hormone e.g. Rhodnius (Maddrell, 1964), Dysdercus (Berridge, 1966) and Carausius (Pilcher, 1970a). The Rhodnius hormone is stable at -20°C but loses most of its activity when boiled (Aston & White, 1974). Carausius hormone, on the other hand, is stable at room temperatures for several days (Pilcher, 1970a). When Carausius hormone is used to induce active secretion in Rhodnius

tubules, secretions of much lower concentrations that would stimulate *Carausius* tubules stimulate *Rhodnius* tubules. As the hormone differs in *Rhodnius* and *Carausius* (Maddrell *et al*., 1969) it is thought that the higher response of *Rhodnius* tubules may be due to their inability to inactivate the *Carausius* hormone.

Aston & White (1974) found that chromatographic analysis showed this diuretic hormone as a low molecular weight which closely chromatographed with cyclic-AMP. They suggest that hormone that is stored could be an aggregation of 5-HT with a small molecule such as ATP, of a high molecular weight form, whilst the low molecular weight form is that which is released into the haemolymph to bring about diuresis. Hughes (1977) working on the high molecular weight form showed that much of the activity is associated with aggregates of high molecular weight, the low molecular weight being more unstable. In *Glossina* Gee (1975b) found that methanol extracts of thoracic ganglia were stable at room temperatures after boiling. In *Locusta* extracts were found to be stable in methanol (Mordue & Goldsworthy, 1969). The stability of locust diuretic hormone and the ability of other insect diuretic hormones to stimulate locust Malpighian tubules is further investigated in this study.

3.1.3. The diuretic response

Following the addition of diuretic hormone from extracts of the appropriate centres, no change in osmotic pressure occurs. Both an increase in rates of Na^+ and K^+ secretion occurs in Carausius (Pilcher, 1970b) and in most other species. There is no significant change in the concentration of these ions only an increase in the rate of secretion occurs with the increase in urine flow. Secretion of Na^+ remains constant in the absence of the diuretic hormone but that of K^+ decreases in the absence of the hormone. The rate of urine secretion is influenced by the concentration of Na^+. Sodium in some way allows more K^+ to be pumped. Pilcher (1970b) measured the potential difference across the tubule membrane on adding diuretic hormone and found that if the external K^+ concentration is decreased while internal K^+ concentration remains constant the potential change can be recorded. On addition of diuretic hormone there is an increase in the lumen positive potential. A change in potential thus occurs without a change in the concentration of the ions. The increase, on addition of the diuretic hormone, in the membrane potential without the subsequent change in ionic concentrations of either side of the membrane tends to indicate that it might

be due to an electrogenic ion pump. It is known that the trans-wall potential is dependent for its maintenance on metabolic energy. It thus seems that the diuretic hormone affects the Malpighian tubules by stimulating active K^+ transport. In *Rhodnius* the trans-wall potential first becomes more negative on addition of the diuretic hormone and then climbs (as Na^+ and K^+ pumps are stimulated) until the lumen is positive with respect to the haemolymph as the hormone is used up. It then reverses to reach a steady negative level (Maddrell, 1971). The lumen negativity was shown to correspond to a Cl^- pump. Flow of fluid begins as negativity develops as a result of these anionic and cationic pumps.

Anstee & Bell (1975) found evidence that Na^+-K^+-ATPase may play a role in fluid secretion by Malpighian tubules of locusts whereas in *Glossina* (Gee, 1976b), *Rhodnius* (Maddrell, 1971) and *Oncopeltus fasciatus* (Scudder & Rafaeli-Bernstein, in prep.) ouabain has no effect on the rate of fluid secreted and a Na^+-K^+-ATPase does not seem to be essential for fluid secretion (see Chapter 1). These apparently contradicting results are investigated in this Chapter.

Many hormones have been shown to act by way of a two-messenger system (see reviews by Robison et al., 1968; Nathanson, 1971). The hormones themselves act as first messengers and travel from their cells of origin to the target tissue where they stimulate the formation of a second messenger such as cyclic-AMP. Aston (1975) showed an increase in c-AMP levels corresponding to the increase in trans-wall potential in Rhodnius tubules. If the diuretic hormone acts as the first messenger and causes an increase in intracellular c-AMP then one would expect that an increase in c-AMP would result in an increase in secretion. This was found to be the case in several insects (Maddrell et al., 1969; Mordue, 1972). Serotonin (5-HT) has been found to simulate the diuretic response in Rhodnius and Carausius (Maddrell et al., 1969). In addition, 5-HT causes pronounced writhing movements of Carausius tubules (Pilcher, 1971) and Periplaneta americana tubules (Flattum et al., 1973). Maddrell et al. (1969) found that inhibitors of 5-HT also inhibit the diuretic hormone response in Rhodnius and Carausius. On the other hand the effect of corpora cardiaca extracts on Apis tubules is mimicked by adrenalin (Altman, 1956). 5-Hydroxytryptamine has been shown to have no effect on Glossina (Gee, personal communication)and Locusta (Mordue, 1972; Maddrell & Klunsuwan, 1973). However, dye excretion is increased

in *Locusta* in the presence of adrenalin but inhibited in the presence of acetylcholine (Mordue, 1972). *Glossina* tubules on the other hand can be stimulated by ecdysterone (Gee *et al.*, 1977). In contrast, ecdysterone switches off fluid secretion in the tubules of *Calpodes* at pupation which is in some way mediated by juvenile hormone (Ryerse, 1978).

In an attempt to enlarge upon the nature and action of the locust diuretic hormone an investigation is made here on the role c-AMP plays in stimulating fluid secretion and the effects of various chemicals on fluid secretion. A knowledge of mimics and inhibitors of the diuretic response could disclose some information about the receptor sites or active parts responsible for inducing tubule secretion and may throw further light on the possible structure of the diuretic hormone.

To summarize, this chapter reports on the investigation made on locust tubules by using an *in vitro* rather than an *in vivo* method to (a) substantiate the existance of the diuretic hormone, (b) investigate the stability of this hormone and compare its response to the responses obtained from other insect diuretic hormones, and (c) investigate the role of c-AMP and other pharmacologically active substances on diuresis.

3.2. RESULTS

3.2.1. Effect of diuretic hormones:

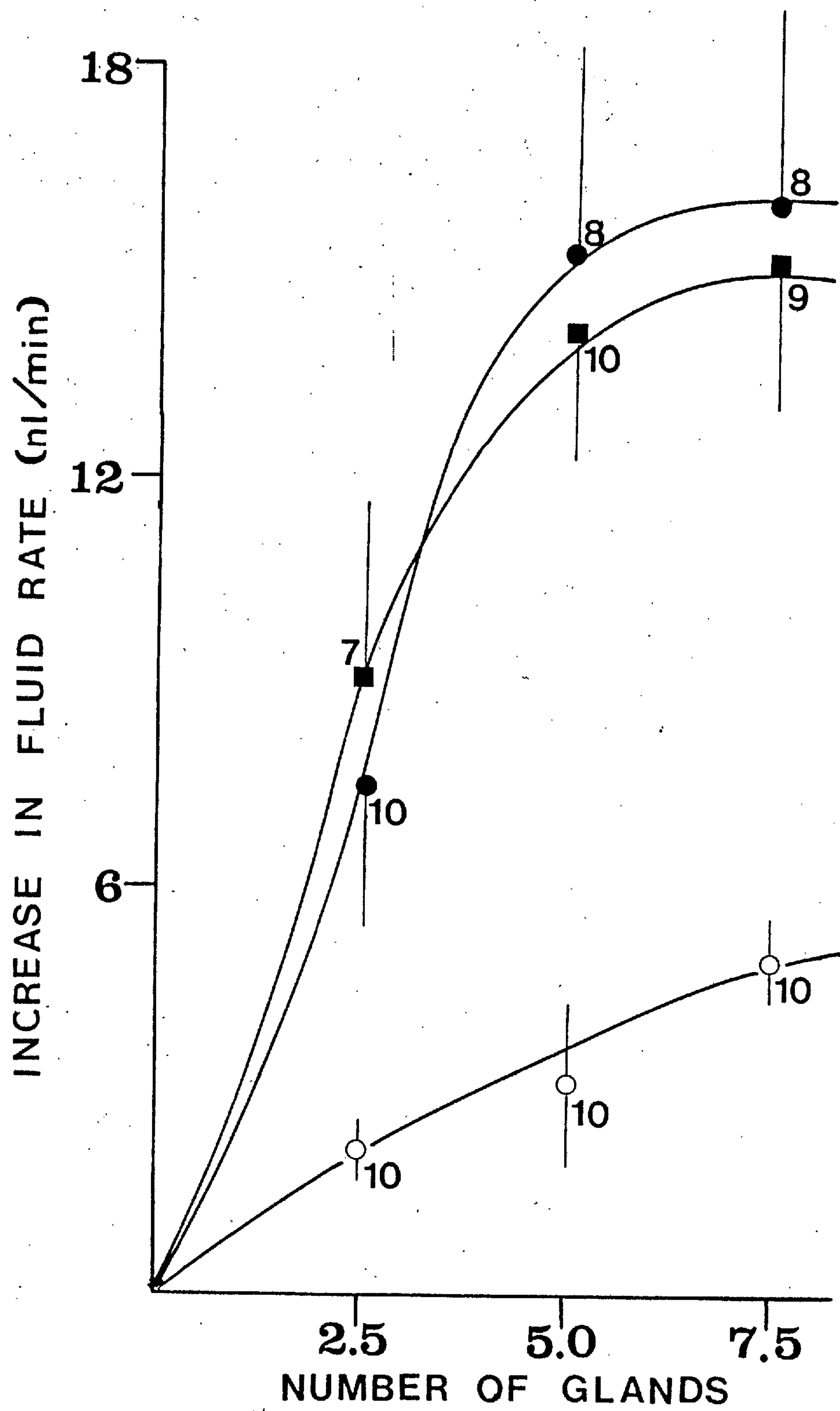
3.2.1.1. Dose-response of hormone extracts.

The response to different doses of both methanol and saline extracts was investigated. The increase in the rate of fluid secreted was measured as an indication of response by the tubules. Fig. 3.2.1.1. shows the results obtained. When using methanol and fresh saline extracts (kept frozen throughout the extraction procedure until ready to be assayed) no significant difference occurs in the response ($P>0.1$). However, the dose response curve of the saline extract which was not kept 'fresh' during the extraction procedure and was allowed to remain at room temperature for 24 hours was found to differ significantly from the above two curves and produced much lower responses to the same doses ($P<0.05$). The response to fractions eluted from a chromatographic column, equivalent to 4-5 glands, produced a mean increase in fluid rate of 10.82 $\pm$ 2.12 nl/min. This value can be seen to fall well within the response to the crude extracts.

3.2.1.2. Stability of the diuretic hormone extracts.

As a significant difference was found between the

Fig. 3.2.1.1. Dose-response curves for methanol ■ ,'fresh'saline ● , and saline ○ extracts of locust storage lobes. Vertical lines show extent of standard error and subscript figures indicate the number of observations.



18
12
6
INCREASE IN FLUID RATE (nl/min)
2.5
5.0
7.5
NUMBER OF GLANDS
8
8
9
10
7
10
10
10
10

response of methanol or fresh saline extracts and saline extracts kept at room temperatures a saline extract was tested at various time intervals after being kept at room temperature to determine its stability. A methanol extract was used as a control. Fig. 3.2.1.2. shows the decay curve of the saline extract. After 3 hours it had lost most of its activity. The methanol extract on the other hand shows no significant decay.

3.2.1.3. Response to other insect diuretic hormones.

Extracts from *Periplaneta* (provided by J. Jones, Imperial College, London), *Rhodnius* (provided by L. Hughes, ARC Unit, Sussex), and *Glossina* (provided by J. Gee, Bristol University) were assayed on locust tubules at different concentrations and compared to the locust diuretic hormone response. Fig. 3.2.1.3.1. shows the dose-response curve obtained for extracts from *Periplaneta* corpora cardiaca. Fig. 3.2.1.3.2. shows the dose-response curves obtained from extracts of *Rhodnius* mesothoracic ganglia and *Glossina* thoracic ganglia. More than 2.5 ganglia/300 µl of bathing fluid (=0.8 ganglia/ 100 µl) is required for a maximum response from the *Rhodnius* hormone. This figure also corresponds to the threshold concentration required for the

<u>Fig. 3.2.1.2</u>. Decay curve of a saline extract kept at room temperature. The dashed line represents the methanol control. Vertical lines indicate extent of standard error and subscript figures indicate the number of observations.

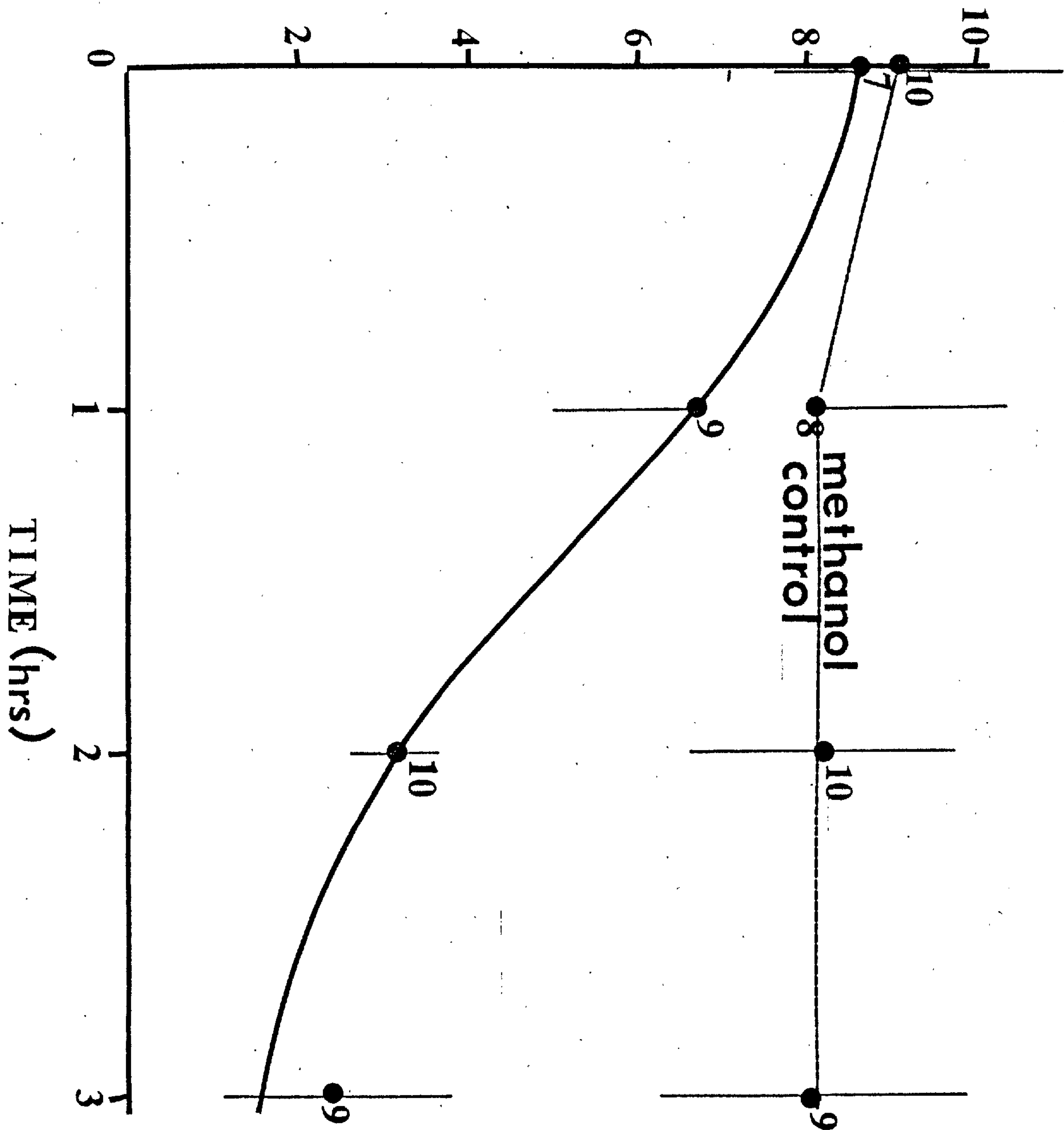
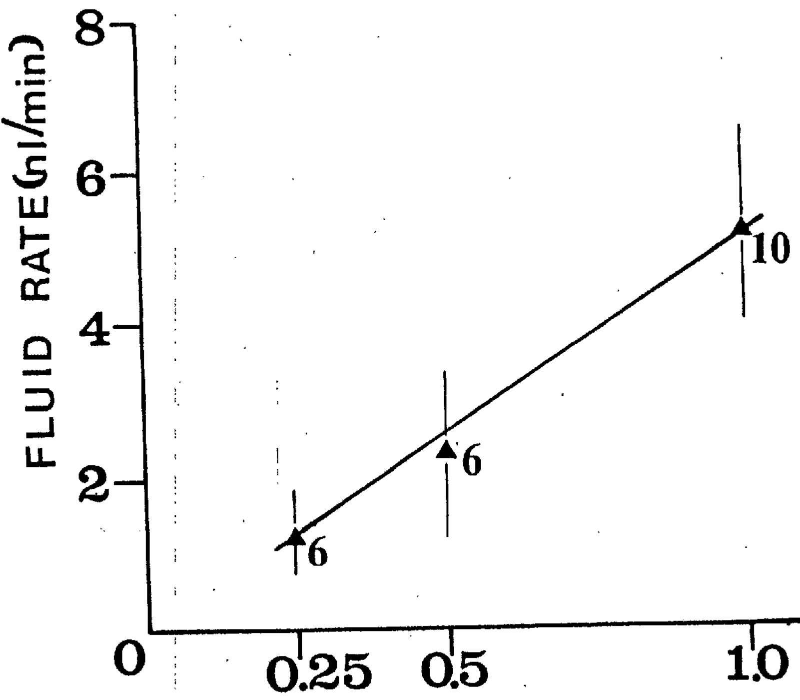
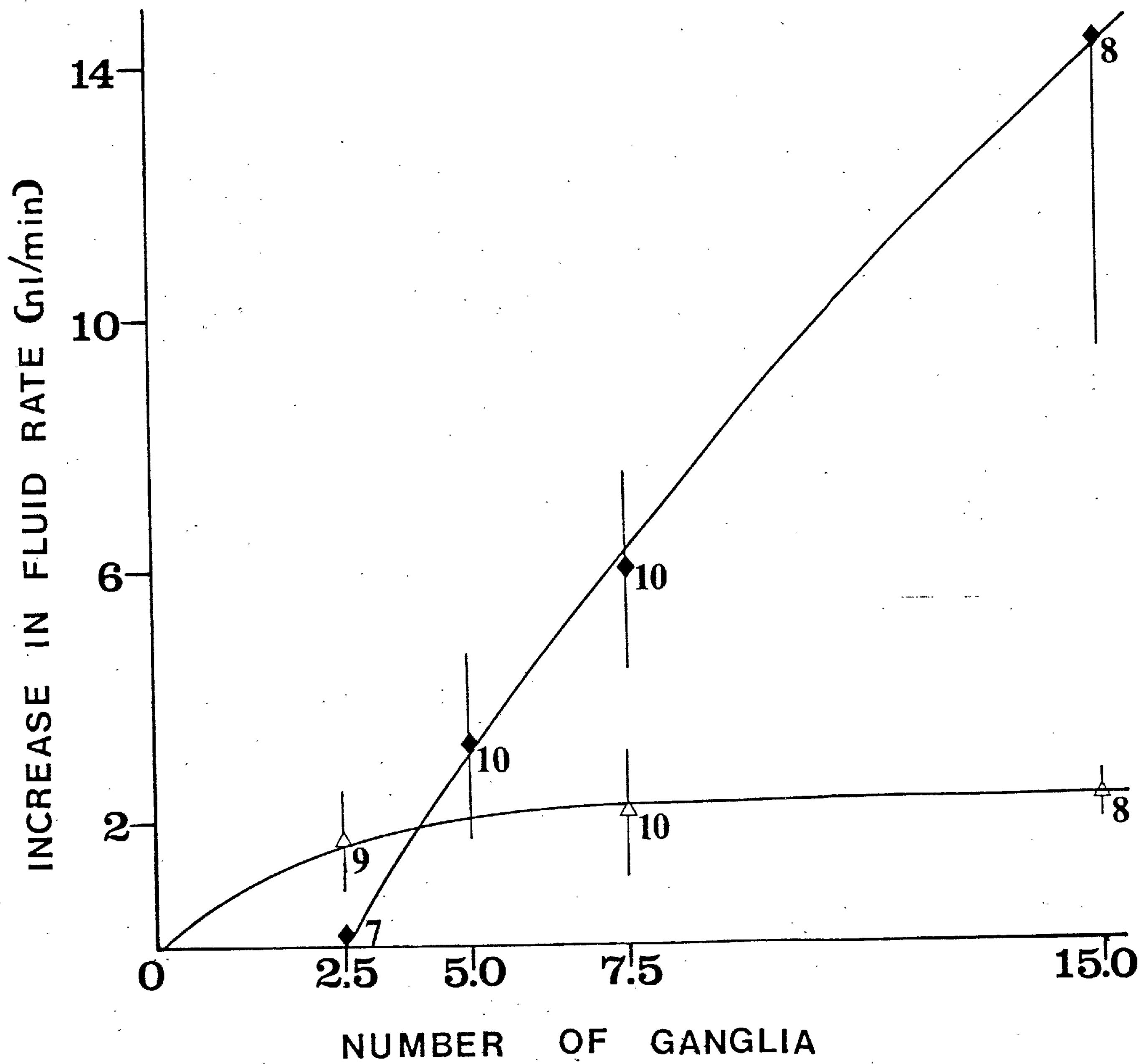

INCREASE IN NORMAL FLUID SECRETION RATE (nl/min)
TIME (hrs)
methanol control
0
1
2
3
2
4
6
8
10

Fig. 3.2.1.3.1. Dose-response curve for extracts of *Periplaneta* corpora cardiaca. Vertical lines show extent of standard error and subscript figures indicate the number of observations. Insert shows results using concentrations of glands lower than 2.5.



FLUID RATE (nl/min)
0
2
4
6
8
0.25
0.5
1.0
6
6
10
INCREASE IN FLUID RATE (nl/min)
0
4
8
12
20
2.5
5.0
7.5
7
4
7
NUMBER OF GLANDS

Fig. 3.2.1.3.2. Dose-response curves for extracts of *Rhodnius* *mesothoracic* ganglia △ and *Glossina* thoracic ganglia ◆ . Vertical lines show extent of standard error and subscript figures indicate the number of observations.


INCREASE IN FLUID RATE (nl/min)
14
10
6
2
0
2.5
5.0
7.5
15.0
NUMBER OF GANGLIA
7
10
10
8
9
10
10
8

Glossina hormone. Fig. 3.2.1.3.3. compares these responses with the response to locust diuretic hormone. Clearly, the locust tubules respond maximally to their own hormone when fresh.

3.2.2. Effect of pharmacologically active substances:

3.2.2.1. Effect of cyclic nucleotides.

Both cyclic-AMP and dibutyryl cyclic-AMP were assayed on locust tubules. After the tubules were allowed to secrete at their normal basal rate for 20 min the bathing medium was discarded and a fresh aliquot injected which contained 10^{-3}M cyclic-AMP or dibutyryl cyclic-AMP. In a control set of tubules the bathing medium was also changed but contained normal bathing Ringer. The results of these experiments is shown in Fig. 3.2.2.1.1. The rate of fluid secretion increases significantly in the presence of both compounds. Fig. 3.2.2.1.2. shows this increase expressed as the rate of change of fluid secretion ($\triangle$ RATE) provided by the slopes of the above graphs. It can be seen that these increases are significant when compared with the control.

3.2.2.2. Effect of biogenic amines and inhibitors.

The biogenic amines adrenalin (10^{-3}M), histamine (10^{-3}M), and 5-hydroxytryptamine (10^{-3}M) were assayed on

Fig. 3.2.1.3.3. The response of locust tubules to locust diuretic hormone (methanol extract ■, fresh saline extract ●, and saline extract ○) and to hormone extracts from *Periplaneta* ▲, *Rhodnius* △, and *Glossina* ◆.

Curves fitted by eye.

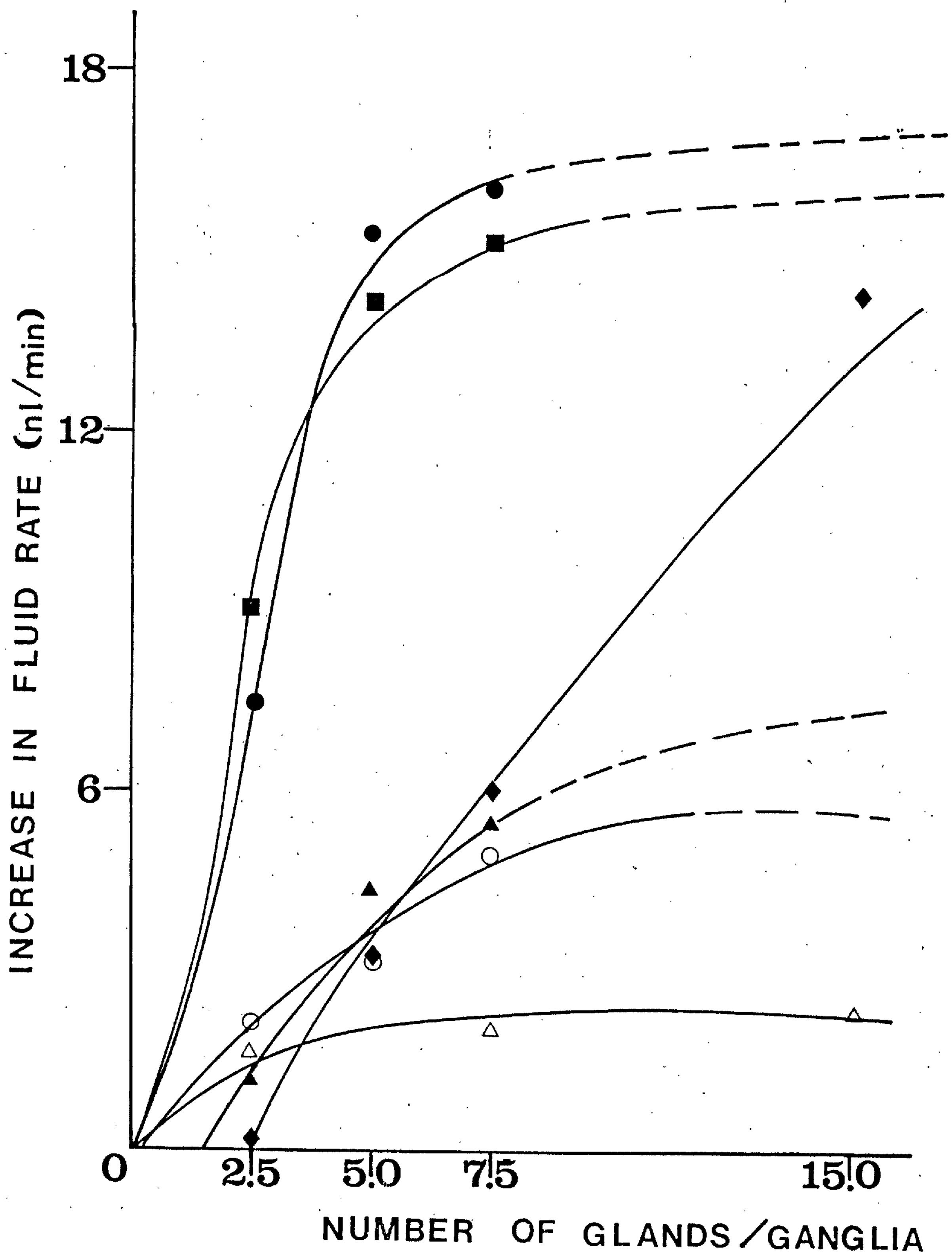
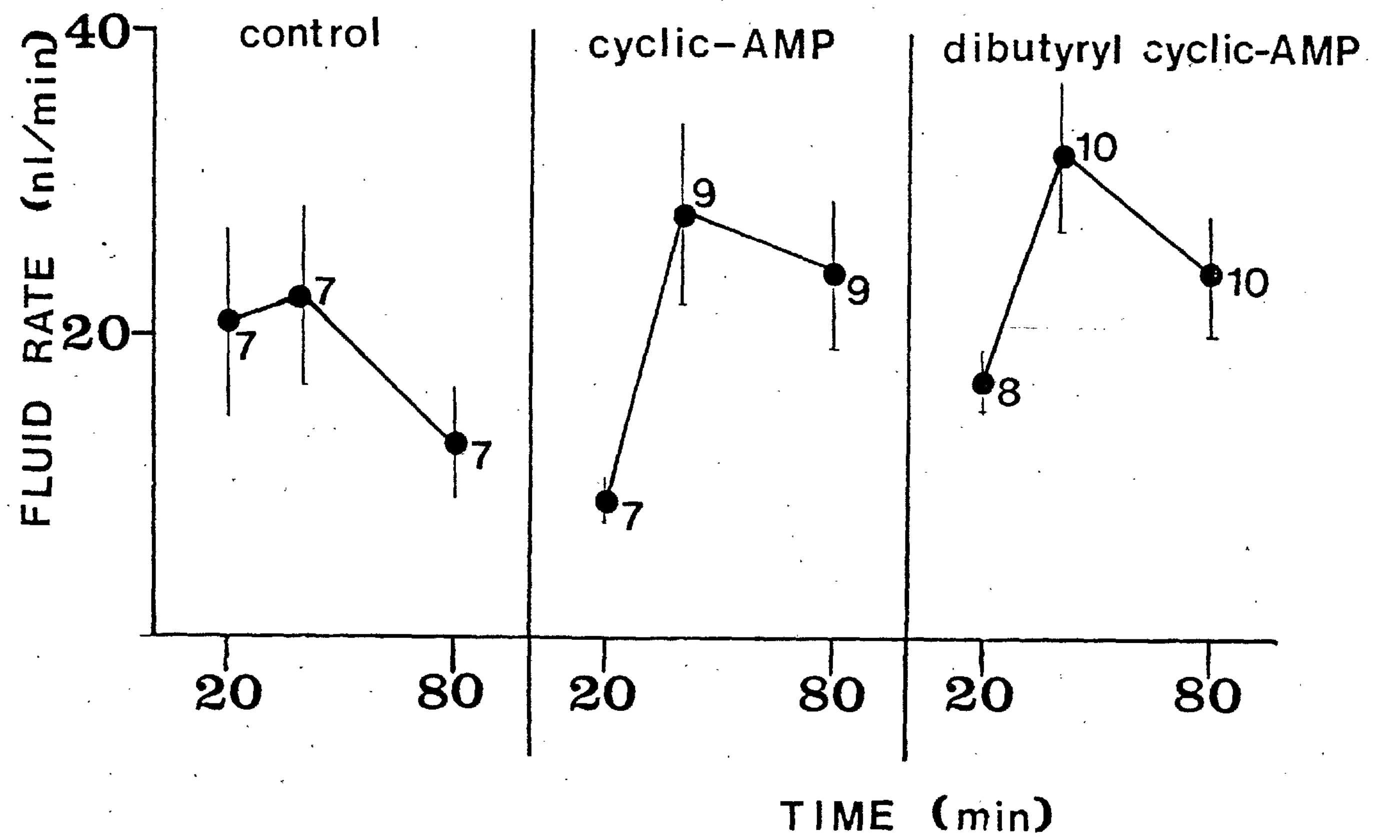
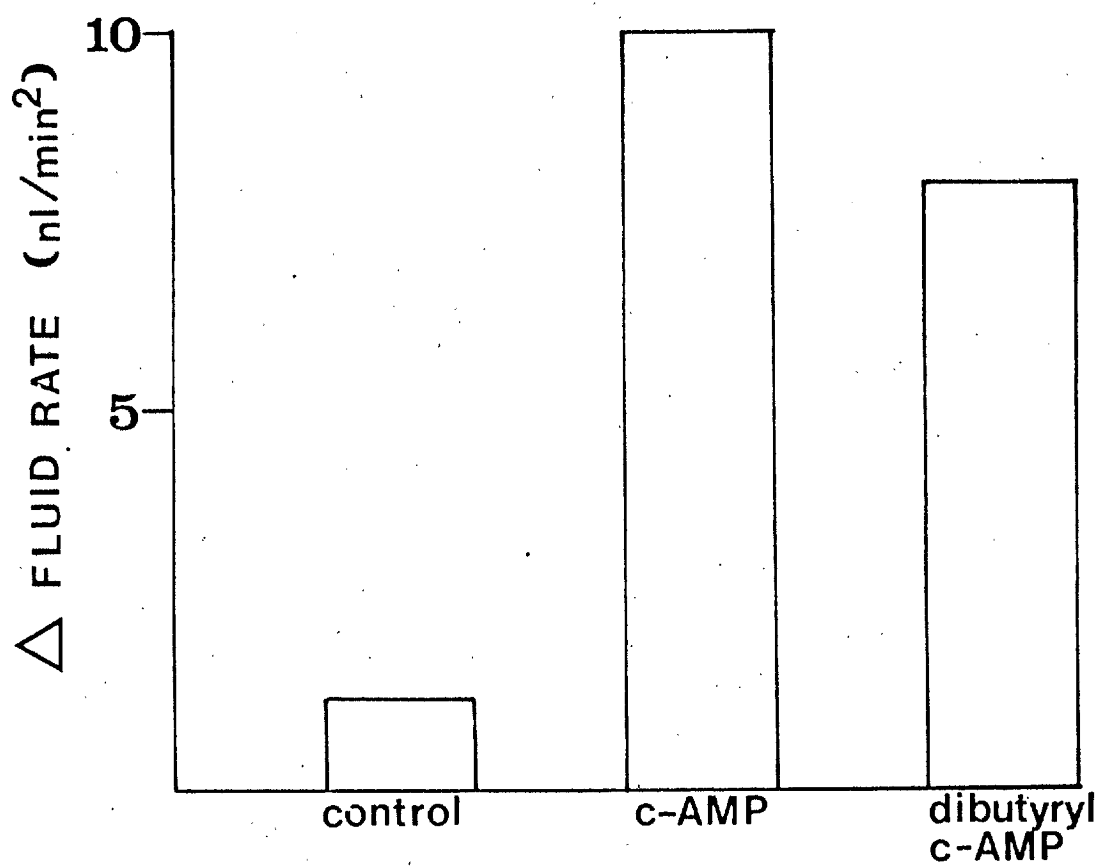

18
12
6
0
2.5
5.0
7.5
15.0
INCREASE IN FLUID RATE (nl/min)
NUMBER OF GLANDS/GANGLIA

Fig. 3.2.2.1.1. The effect of c-AMP and dibutyryl c-AMP on fluid secretion rate. Vertical lines show extent of standard error and subscript figures indicate the number of observations.



FLUID RATE (nl/min)
40
20
control
cyclic-AMP
dibutyryl cyclic-AMP
7
7
7
7
9
9
8
10
10
20
80
20
80
20
80
TIME (min)

Fig. 3.2.2.1.2. Changes in the rate of fluid secretion in the presence of c-AMP and dibutyryl c-AMP.

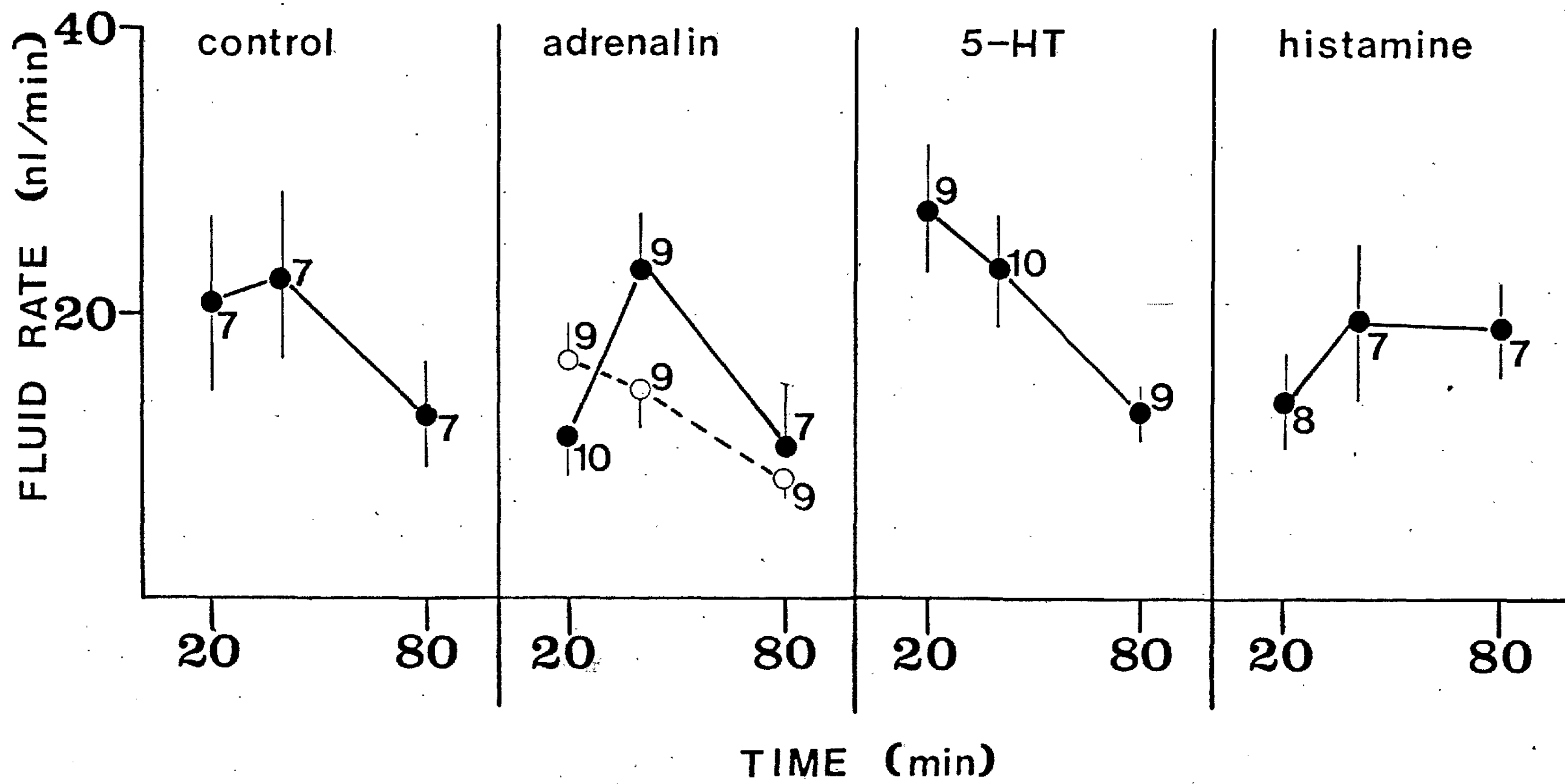


Δ FLUID RATE (nl/min²)
10
5
control
c-AMP
dibutyryl c-AMP

locust tubules, treated as the above experiments. The results are shown in fig. 3.2.2.2.1. Only adrenalin and histamine produce significant increases in the rate of fluid secreted when compared with the control (fig. 3.2.2.2.2.). The addition of 5-HT on the other hand produces a gradual decline in activity.

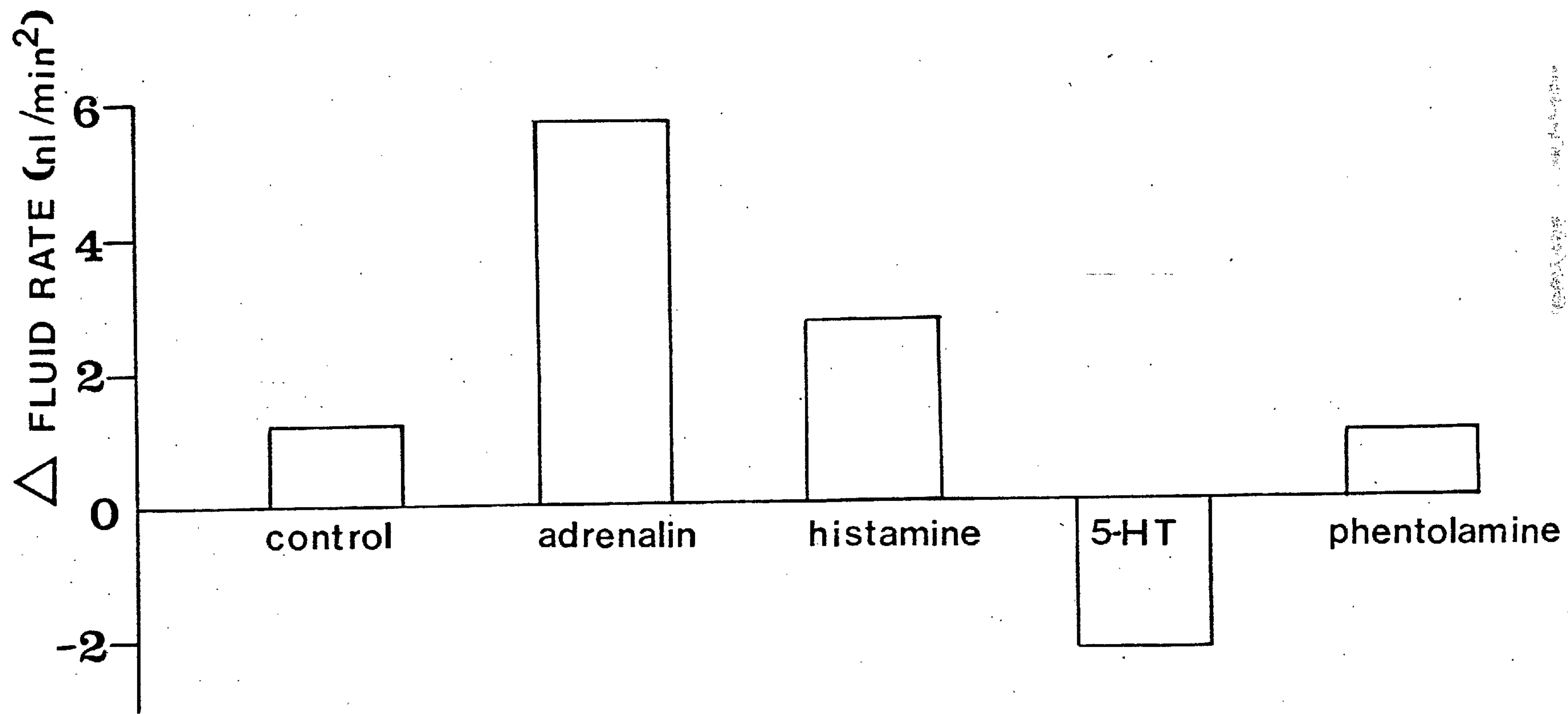
In an attempt to find an inhibitor of fluid secretion which might have an effect on these chemicals the adrenergic blocker, phentolamine, was used. On its own, phentolamine does not have a significant effect on diuretic activity (fig.3.2.2.2.2. and 3.2.2.2.3.). However, when added to the bathing medium in the presence of adrenalin, the expected increase in the rate by adrenalin is abolished. On the other hand phentolamine had no effect on the action of either c-AMP or the locust diuretic hormone (fig. 3.2.2.2.3.). When adrenalin is added to the bathing medium, in addition to causing an increase in fluid secretion, a pronounced writhing movement of the tubules was observed. This is not pronounced when either c-AMP or locust diuretic hormone is present. Phentolamine, in addition to abolishing any expected increases by adrenalin also abolishes these writhing movements. These writhing movements were also seen to be very pronounced when *Glossina* hormone was added to the

Fig. 3.2.2.2.1. The effect of adrenalin, 5-HT, and histamine on fluid secretion rate. Vertical lines show extent of standard error and subscript figures indicate the number of observations. Dashed curve indicates the presence of phentolamine.



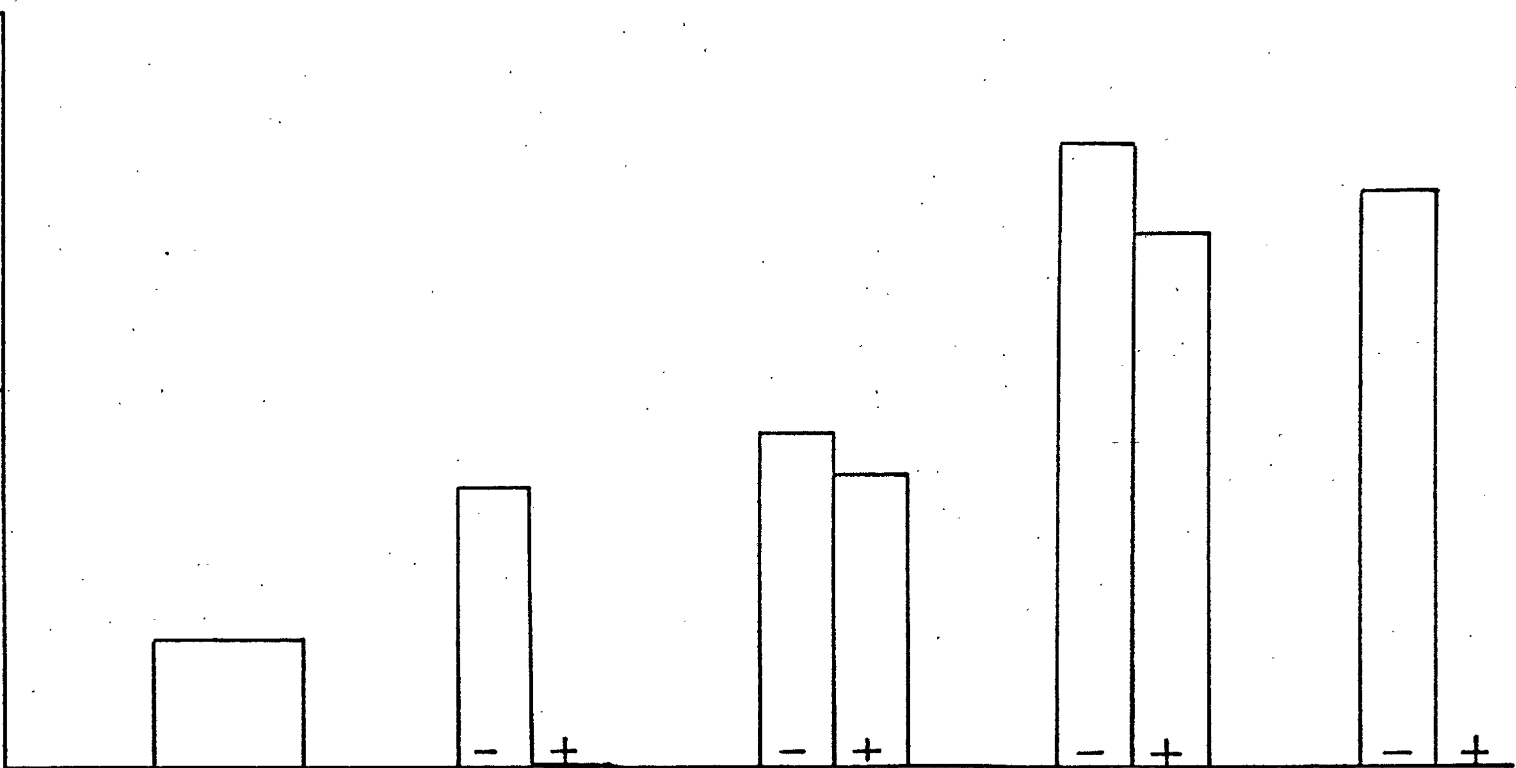
FLUID RATE (nl/min)
40
20
control
adrenalin
5-HT
histamine
20
80
20
80
20
80
20
80
TIME (min)

Fig. 3.2.2.2.2. Changes in the rate of fluid secretion in the presence of adrenalin, histamine, 5-HT, and phentolamine.



△ FLUID RATE (nl /min²)
6
4
2
0
-2
control
adrenalin
histamine
5-HT
phentolamine

Fig. 3.2.2.2.3. The effect of phentolamine on the increases in fluid secretion rate produced by adrenalin, c-AMP and diuretic hormones from *Locusta* and *Glossina*.


INCREASE IN FLUID RATE (nl/min)
10
5
−
+
phentolamine
control
adrenalin
c AMP
D.H.
Locusta
D.H.
Glossina

bathing medium. Phentolamine was therefore tested on the Glossina hormone effect and the expected increase of fluid rate plus the writhing movements were abolished (fig. 3.2.2.2.3.).

3.2.2.3. Effect of steroids and inhibitors.

The effect of the steroids cholesterol (10^{-6}M), ecdysterone (10^{-6}M) and aldosterone (10^{-3}M) was tested on locust tubules. Fig. 3.2.2.3.1. shows that after 20 min had elapsed, on addition of the test substance, both cholesterol and aldosterone caused an increase in the rate of fluid secreted, whilst ecdysterone was inactive producing a slight decline in the rate. However, after 40 min the tubules began reacting to the presence of ecdysterone. The extent of stimulation expressed as Δ rate is shown in fig. 3.2.2.3.2.

The inhibitor of Na^{+} electrogenic pumps, ethacrynic acid, was tested on the effect of aldosterone and was found to reduce any significant effect on diuretic activity (fig. 3.2.2.3.2. and 3.2.2.3.3.). When ethacrynic acid was added to the bathing medium containing c-AMP the increase expected by c-AMP was maintained and ethacrynic acid failed to affect the action of c-AMP. The slight reduction seen in fig. 3.2.2.3.3. on adding ethacrynic acid to a c-AMP preparation (=0.7 nl/min^{2}) can be explained by the slight change in the osmotic pressure of the bathing

Fig. 3.2.2.3.1. The effect of cholesterol, ecdysterone, and aldosterone on fluid secretion rate. The dashed curve represents the effect of ethacrynic acid on aldosterone's effect. Vertical lines show extent of standard error and subscript figures indicate the number of observations.

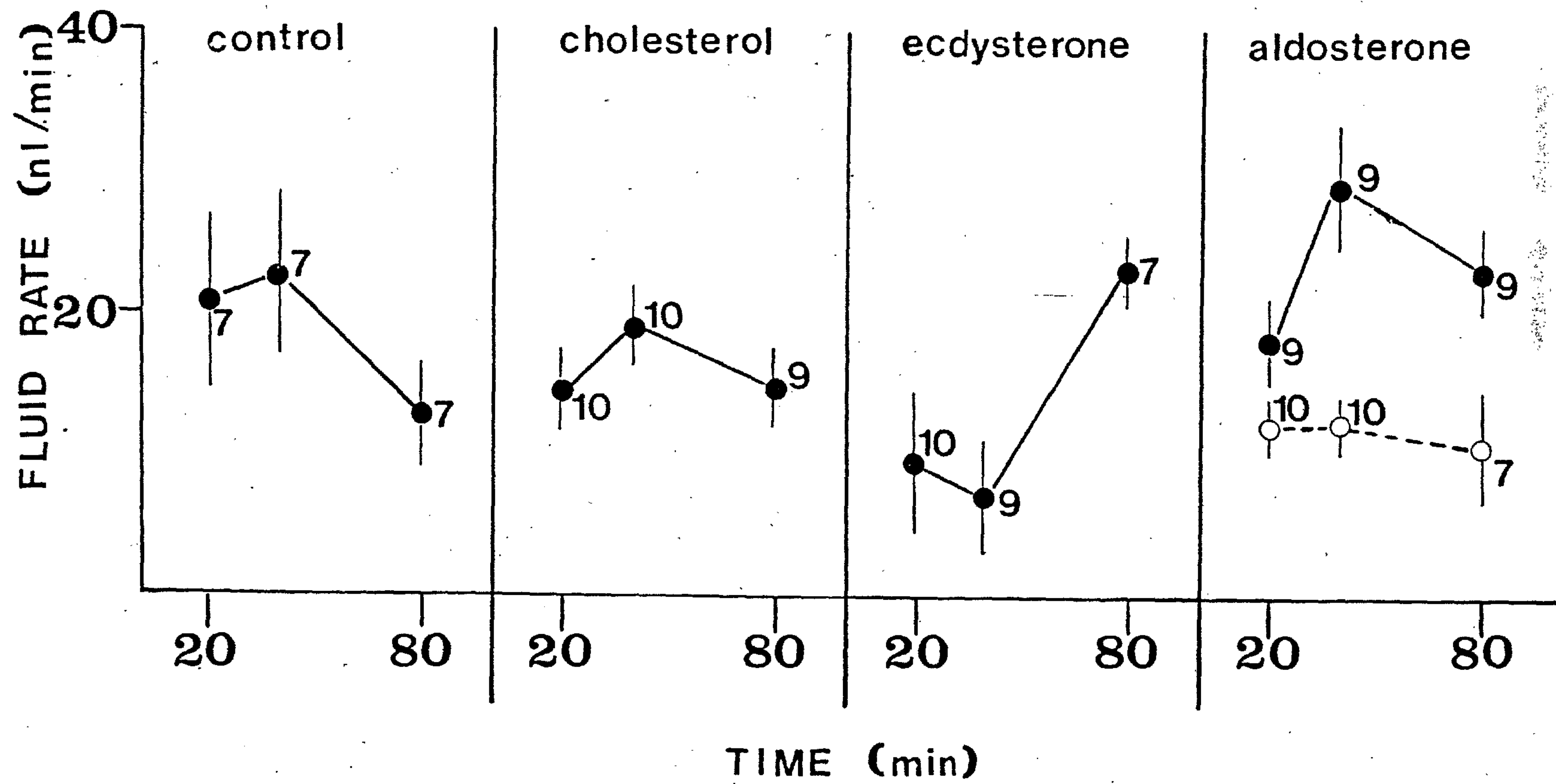

control
cholesterol
ecdysterone
aldosterone
FLUID RATE (nl/min)
40
20
7
7
7
10
10
9
10
9
7
9
9
9
10
10
7
20
80
20
80
20
80
20
80
TIME (min)

Fig. 3.2.2.3.2. Changes in the rate of fluid secretion in the presence of cholesterol, ecdysterone, aldosterone and ethacrynic acid.

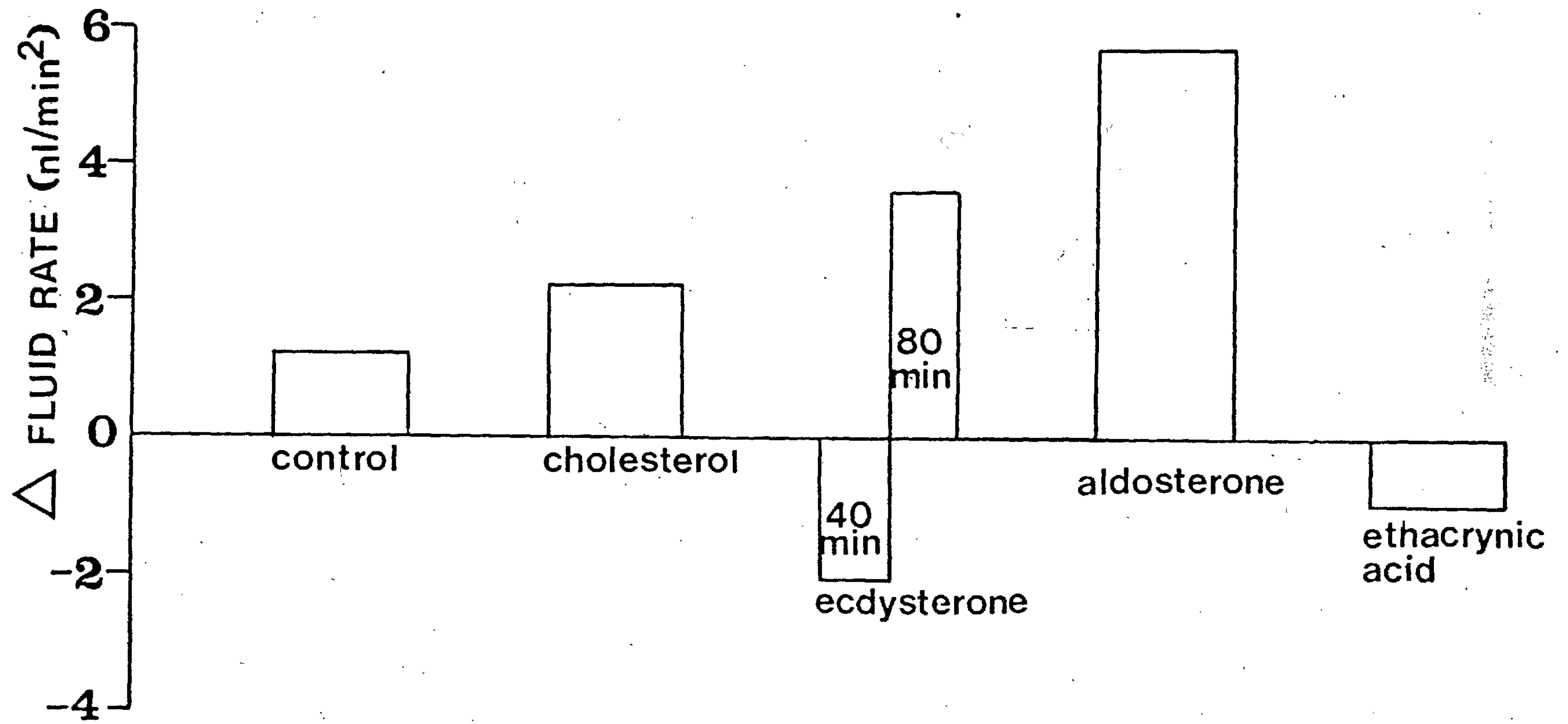
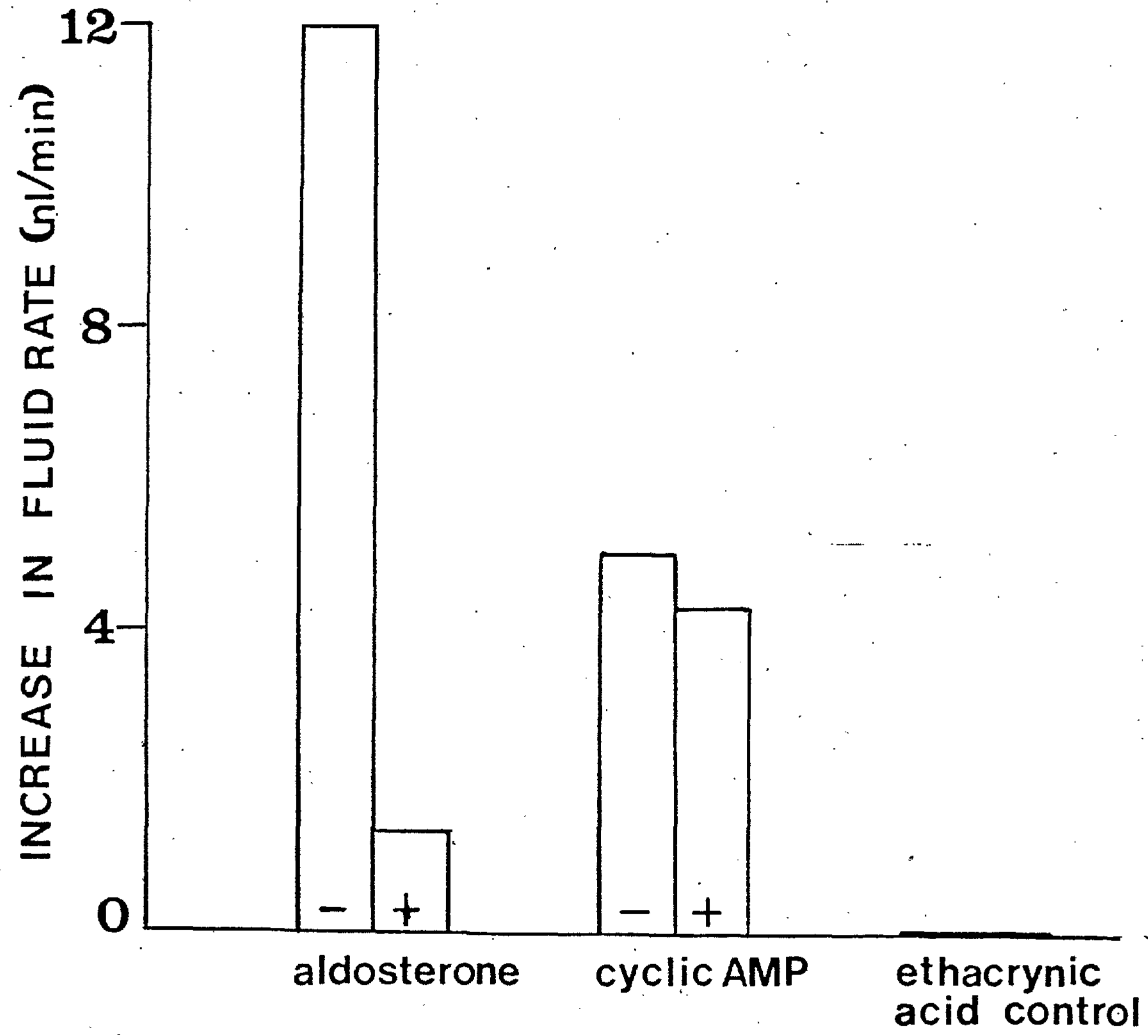

△ FLUID RATE (nl/min²)
6
4
2
0
-2
-4
control
cholesterol
80 min
40 min
ecdysterone
aldosterone
ethacrynic acid

Fig. 3.2.2.3.3. The effect of ethacrynic acid on the increases in fluid secretion rate produced by aldosterone and c-AMP.



12
8
4
0
INCREASE IN FLUID RATE (nl/min)
−
+
−
+
aldosterone
cyclic AMP
ethacrynic acid control

medium. The change in osmotic pressure however, cannot account for the large drop in activity caused in the presence of aldosterone.

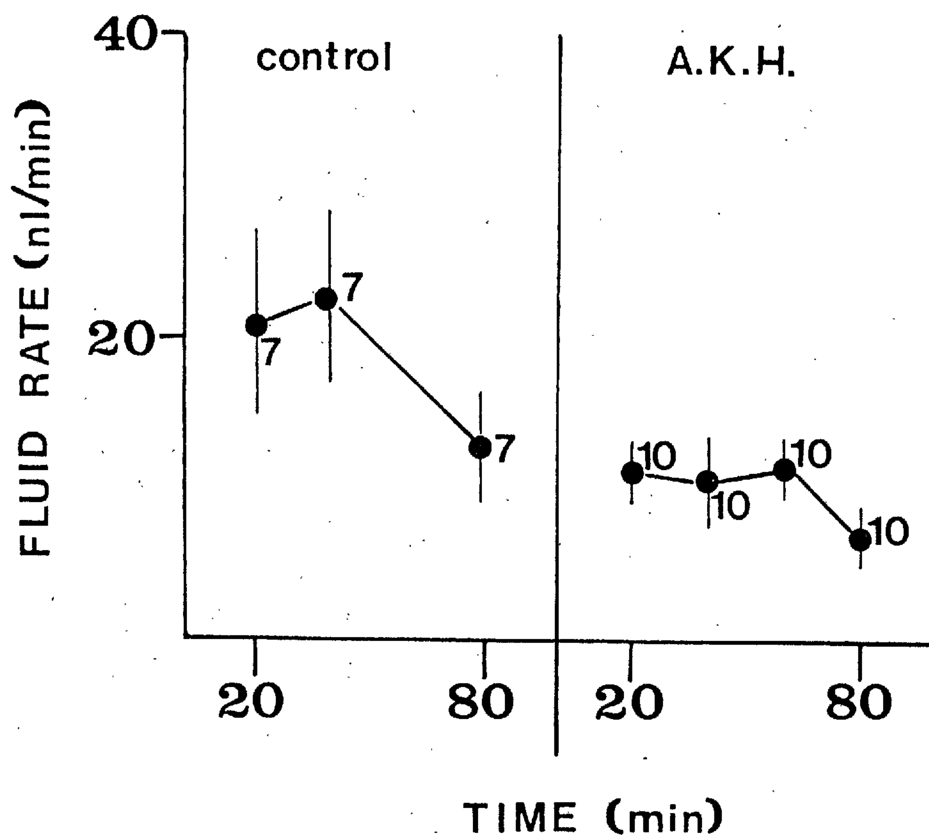
3.2.2.4. Effect of peptide hormones.

The effect of adipokinetic hormone, adrenocortico-trophic hormone, vasopressin and oxytocin was tested on locust tubules. These results are shown in figs. 3.2.2.4.1. and 3.2.2.4.2. Only ACTH produces an increase in diuretic activity. AKH has no effect whereas both vasopressin and oxytocin show a decrease in the rate of fluid secreted.

3.2.2.5. Effect of glycosides.

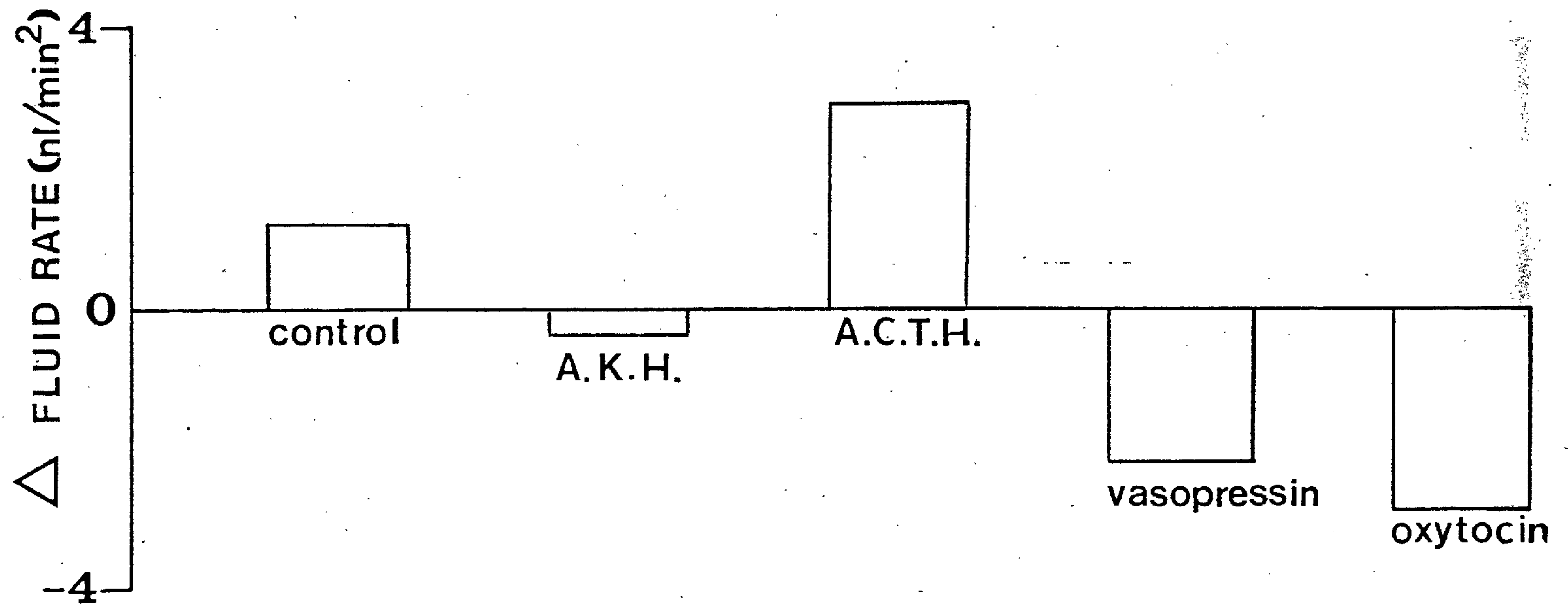
The effects of the cardiac glycoside, ouabain (10^{-3}M), as a possible inhibitor of fluid secretion and the plant glycoside phlorizin (10^{-3}M) as well as its aglycone phloretin (10^{-4}M) were investigated on locust tubules. These results are shown in fig. 3.2.2.5.1. No significant decrease in fluid rate occurs when ouabain is added to the bathing medium. Phlorizin and phloretin, on the other hand, caused marked increases in the rate (fig. 3.2.2.5.1. and 3.2.2.5.2). In addition, the increase caused by phlorizin can be repeated after allowing the tubules to return to their basal rate, although the second

Fig. 3.2.2.4.1. The effect of AKH on fluid secretion rate. Vertical lines show extent of standard error and subscript figures indicate the number of observations.



40
20
FLUID RATE (nl/min)
control
A.K.H.
7
7
7
10
10
10
10
20
80
20
80
TIME (min)

Fig. 3.2.2.4.2. Changes in the rate of fluid secretion in the presence of AKH, ACTH, vasopressin and oxytocin.



△ FLUID RATE (nl/min^2)
4
0
-4
control
A.K.H.
A.C.T.H.
vasopressin
oxytocin

<u>Fig. 3.2.2.5.1</u>. The effect of ouabain, phlorizin ● and phloretin ■ on fluid secretion rate. Vertical lines show extent of standard error and subscript figures indicate the number of observations.

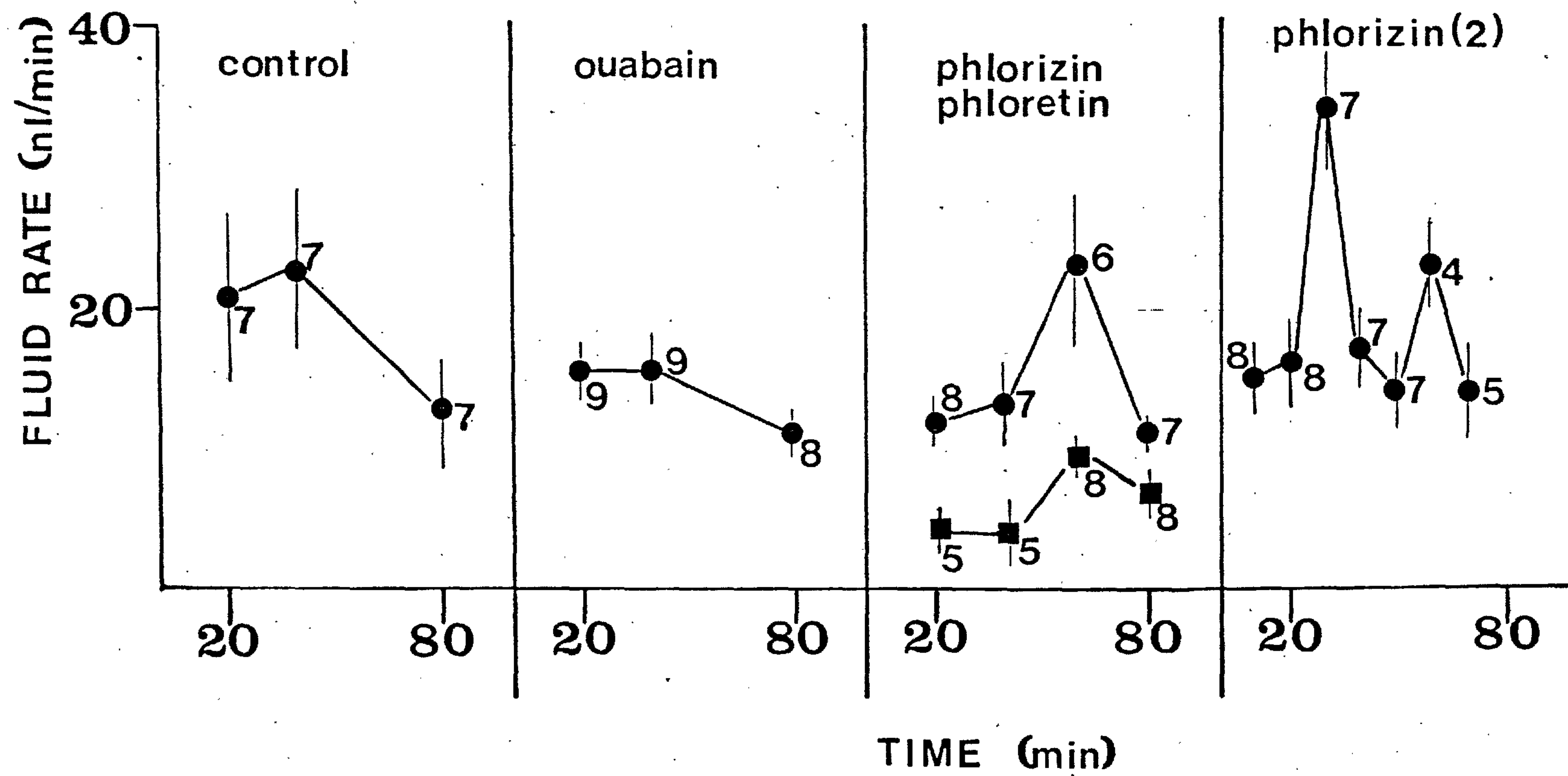
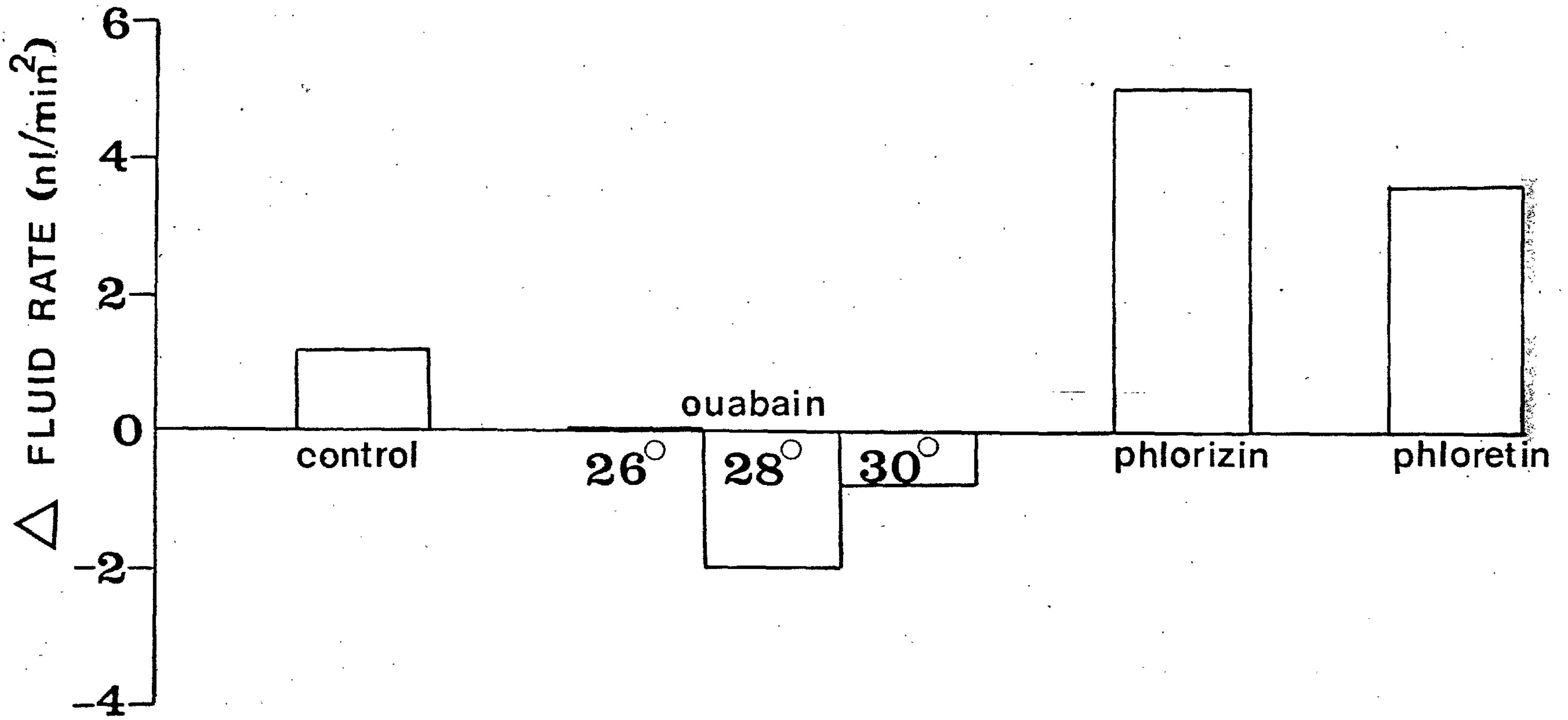

FLUID RATE (nl/min)
40
20
control
ouabain
phlorizin
phloretin
phlorizin(2)
20
80
20
80
20
80
20
80
TIME (min)

Fig. 3.2.2.5.2. Changes in the rate of fluid secretion in the presence of ouabain (at different temps.), phlorizin and phloretin.

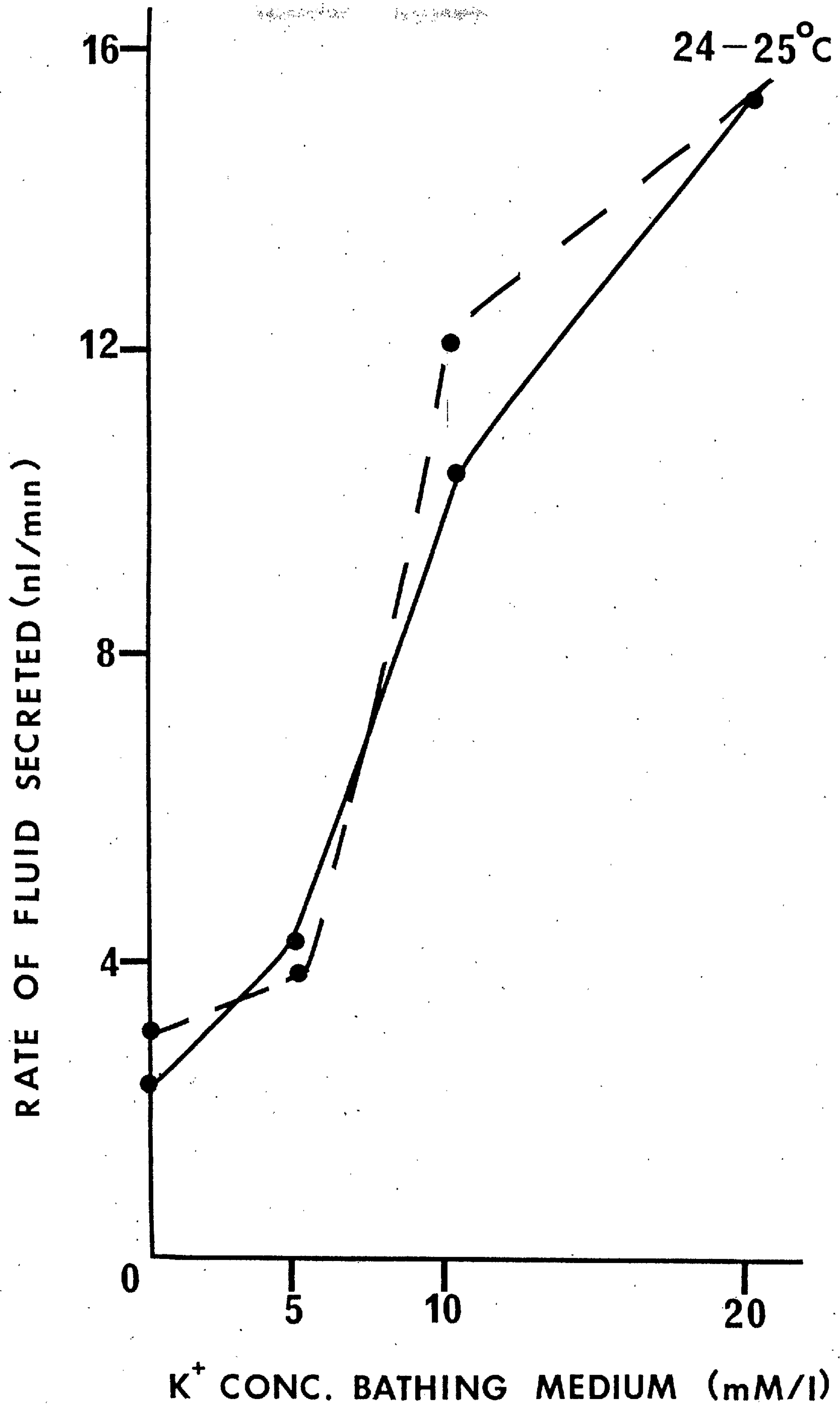


Δ FLUID RATE (nl/min^2)
6
4
2
0
-2
-4
control
ouabain
26°
28°
30°
phlorizin
phloretin

increase is smaller than the first (fig. 3.2.2.5.1.). Clearly phlorizin increases the rate of tubular secretion and this increase can be repeated after recovery of the tubules.

The possibility that the concentration of K^+ in the bathing medium may influence the extent of inhibition by ouabain, when the presence of a ouabain sensitive exchange pump will be most readily demonstrated if the bathing medium contained low K^+ concentrations, was tested. Ouabain was added to bathing media containing different K^+ concentrations. The results of these experiments are shown in fig. 3.2.2.5.3. From the curves it can be seen that the possibility was eliminated as even in a K^+-free bathing medium, although a very low rate of fluid secretion occurs, the addition of ouabain does not significantly lower this rate. The effect of temperature was also tested (fig. 3.2.2.5.2.), at room temperature in which the above experiments were performed no inhibition occurs, however, a slight inhibition was observed at 28°C and a smaller inhibition was observed at 30°C. Ouabain however, even at higher temperatures failed to abolish fluid secretion.

Fig. 3.2.2.5.3. The effect of ouabain over a range of K^+ concentrations on the rate of fluid secreted by Malpighian tubules of *Locusta*. Broken lines, tubules with ouabain (1 mM/l) present in the bathing medium; solid lines, control tubules in untreated Ringer. Cationic balance was made up with choline chloride.


24 – 25°C
16
12
8
4
0
5
10
20
RATE OF FLUID SECRETED (nl/min)
K+ CONC. BATHING MEDIUM (mM/l)

3.3. DISCUSSION

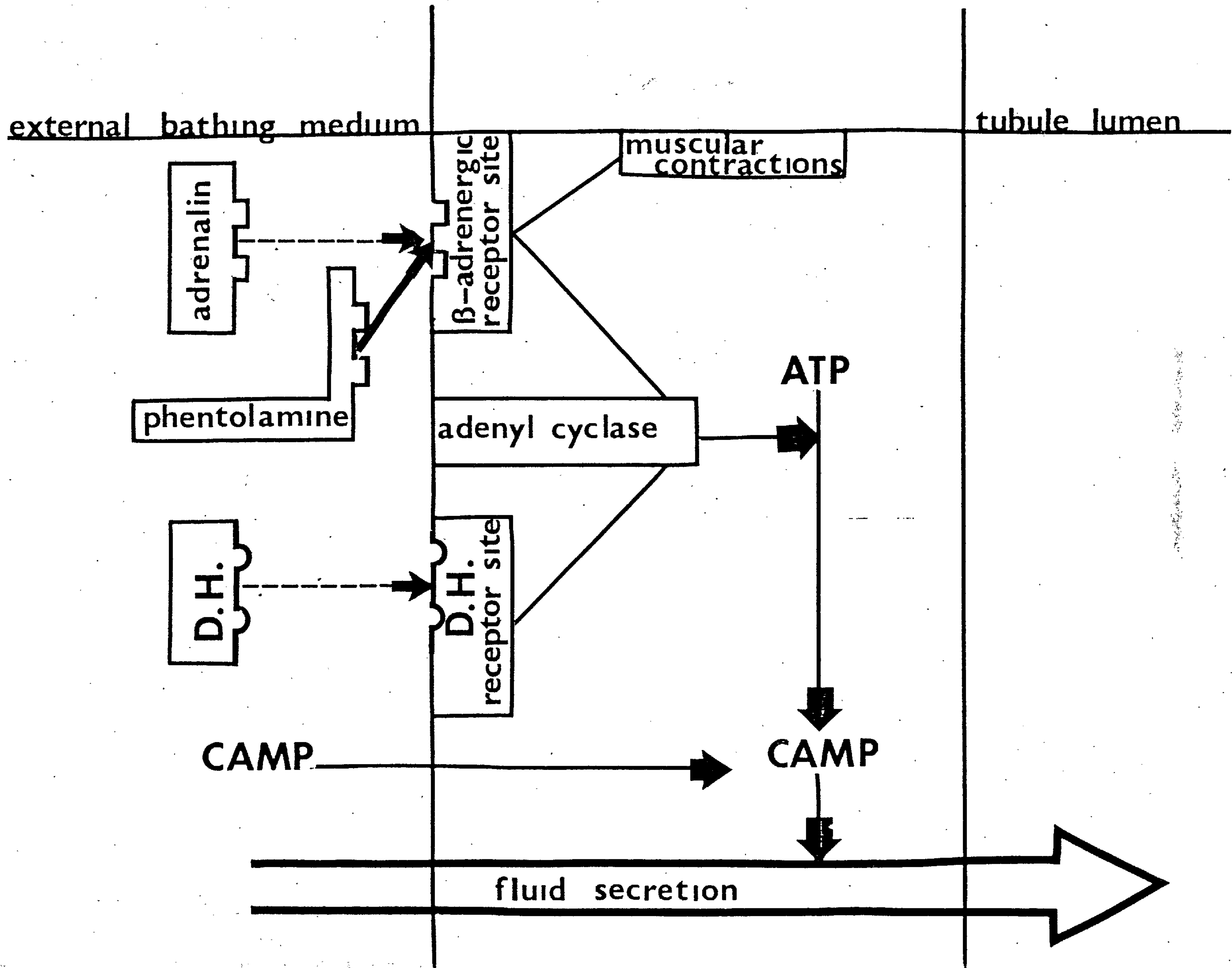
The results clearly show that *Locusta* tubules respond to fresh saline and methanol extracts from storage lobes of corpora cardiaca. These extracts were found to be stable only in methanol or if kept frozen. A saline extract kept at room temperature would loose more than 75% of its activity after as short a time as 3 hours. The explanation to the loss in activity can only be speculative: it is possible that the loss in activity is due to the presence of degradative enzymes which may be released with the diuretic hormone in some way. This may explain why no loss in activity occurs in methanol extracts.

Several chemicals were found to mimic the diuretic hormone action. Cyclic-AMP, dibutyryl cyclic-AMP, adrenalin, histamine, ecdysterone, cholesterol, aldosterone, phlorizin and phloretin produced significant increases in fluid secretion. It proved impossible to inhibit urine formation by isolated Malpighian tubules of *Locusta* with ouabain. The effect of ouabain was tested at the same molarity (10^{-3}M) as that used by Anstee & Bell (1975), using low K^{+} concentrations in the bathing medium. It was shown here that no inhibition occurs even with

varying K^+ concentrations in the bathing medium whilst maintaining the cationic concentrations constant with choline ions. The conclusions from this study are that ouabain does not reduce urine formation by _Locusta_ tubules which agrees with results obtained for _Rhodnius_ (Maddrell, 1971) and _Glossina_ (Gee, 1976a,b).

Known inhibitors of some of the stimulatory chemicals such as phentolamine (ß-adrenergic blocker) and ethacrynic acid (Na^+ electrogenic pump inhibitor) successfully inhibited the actions of adrenalin and aldosterone respectively, but failed to inhibit the action of either diuretic hormone or cyclic-AMP. Similarly, 5-HT and c-AMP were reported by Berridge & Prince (1972) to have different effects on the trans-wall potential difference in _Calliphora_ salivary glands. This suggests that c-AMP is involved as a second messenger. One can envisage a specific hormone receptor which exists in association with these chemical receptors which when stimulated could stimulate the second messenger. Another possibility is that a stimulation of these chemical receptors might produce not only the expected physiological response (muscular writhing in the case of adrenalin) but also a general increase in intra-cellular c-AMP levels which affects fluid secretion. Such a model (fig. 3.3.) can only be

Fig. 3.3. Model proposed to explain the action of stimulatory chemicals on fluid secretion by *Locusta* tubules.


external bathing medium
tubule lumen
muscular contractions
adrenalin
β-adrenergic receptor site
phentolamine
adenyl cyclase
ATP
D.H.
D.H. receptor site
CAMP
CAMP
fluid secretion

speculative, further investigation is required to substantiate it. However, the model serves as a basis for the design of such experiments.

The response of locust tubules to diuretic hormones derived from other insects is always lower than the response to its own hormone. Rhodnius tubules have been reported by Aston & White (1974) to require a threshold concentration of 0.07 ganglia/100 µl of bathing fluid. Locust tubules react only to a larger dose of 0.8 ganglia/100 µl. The same dose of 0.8 ganglia/100 µl is required from Glossina hormone to stimulate Locusta tubules, however, in this case the dose compares well with that required by Glossina tubules themselves, reported to be 0.5 ganglia/100 µl by Gee (1975 b).These comparisons however must be considered with caution as the concentration of the ganglia or glands may not be a true reflection of the actual hormonal concentration. Whilst there is an underlying basic similarity amongst species there are differences in detail. These differences reflect the adaptability of the insect to its water-balance problems encountered in different habitats. However, it still remains impossible to ascertain whether the same active factor is to be found in different species until the diuretic hormone is isolated and chemically analyzed even though the similarity of their effects on Malpighian tubules might tempt us to regard their secretion identical.

4. TRANSPORT OF THE CARDIAC GLYCOSIDE, OUABAIN.

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4.1. INTRODUCTION

In Chapter 3 the effect of ouabain on the rate of fluid secretion was discussed. It has been seen that ouabain does not affect the *in vitro* Malpighian tubules. In Chapter 6 it will be shown that it inhibits glucose reabsorption. Clearly, there is evidence that ouabain affects the functioning of the Malpighian tubules but it is yet not clear what mechanism is involved when Na^+-K^+-ATPase is inhibited in the tubules. In many insects the presence of ouabain or other cardenolide in the food plants does not have any deleterious effects but can affect insects which do not normally eat such plants.

The origin of resistance to such toxins is still imperfectly understood. Many insects such as *Poekilocerus bufonis* (von Euw *et al*., 1967), *Manduca saxta*, *Hyalophora cercopia* and *Danaus plexipus* (Rothschild, 1972; Vaughan & Jungreis, 1977) possess glands which are able to sequester glycosides present in their diets. The function of this accumulation has been disputed: it may be an active function, perhaps as a defence mechanism against predation, or it may be merely the storage of excess which cannot be excreted. The toxicity of these chemicals is renowned and the problem arises as to

how the insect handles such a high concentration of toxic substances in its haemolymph. Scudder & Rafaeli-Bernstein (in prep.) found that cardiac glycoside accumulation in _Oncopeltus fasciatus_ does have an active function and storage is not due to an inability in excreting these substances, in fact, there exists a competition between sequestration of dietary toxins in the dorso-lateral glands and their excretion by the Malpighian tubules.

The polyphagous feeding habits of the grasshopper, _Zonocerus variegatus_ (L.) (Plate 4.1.) have been described in some detail (Toye, 1971, 1974; Bernays _et al._, 1974; Youdeowei, 1974; Terry _et al._, 1976). Bernays _et al._ (1974) have shown that _Zonocerus_ are predisposed to feeding on toxic plants such as _Manihot_ and _Pergularia_ both of which contain glycosides toxic to most animals. _Zonocerus_ fed on _Datura_ accumulate glycosides in the repugnatorial gland (Rothschild _et al._, 1977). The possibility still exists that toxins can also be removed from the haemolymph by the Malpighian tubules.

This Chapter describes the investigation made on the ability of the tubules of _Zonocerus_ to secrete ouabain present in their diet. Comparisons are made with

Locusta (another acridid) which does not feed on toxic plants and therefore might not be expected to possess Malpighian tubules capable of excreting toxins.

PLATE 4.1. *Zonocerus variegatus* (L.).

4.2. RESULTS

4.2.1. The effect of ouabain on the rate of fluid secretion by tubules from *Locusta* & *Zonocerus*.

No effect of ouabain on the rate of fluid secretion was seen on tubules of *Locusta* (Chapter 3) when using normal bathing Ringer as well as in bathing Ringers containing differing concentrations of KCl. The same experiments were performed on the tubules of *Zonocerus*. Fig. 4.2.1. and Table 4.2.1. show that there is also no effect of ouabain on the rate of fluid secreted by the tubules of *Zonocerus*. No significant difference occurs on adding ouabain to the bathing medium, $P > 0.1$ in all cases.

4.2.2. The effect of injected ouabain on the survival of insects.

The survival rate of *Locusta* and *Zonocerus* adults (10 animals/group) following injection of ouabain into the haemolymph is shown in fig. 4.2.2. With daily injections of 10 μl of 10^{-8}M (5.84×10^{-9} μg, = 5.84 ng) ouabain the rate of survival of locusts was low and all locusts were dead within 4 days. When 10 μl of 10^{-12}M (0.0584×10^{-9} μg, = 0.0584 ng) ouabain was injected there was a higher rate of survival. The mortality following both treatments

Fig. 4.2.1. The effect of ouabain over a range of K^+ concentrations on the rate of fluid secreted by Malpighian tubules of *Locusta* ● and *Zonocerus* ▲ . Broken lines, tubules with ouabain (1 mM/l) present in the bathing medium; solid lines, control tubules in untreated Ringer. Cationic balance was made up with choline chloride.

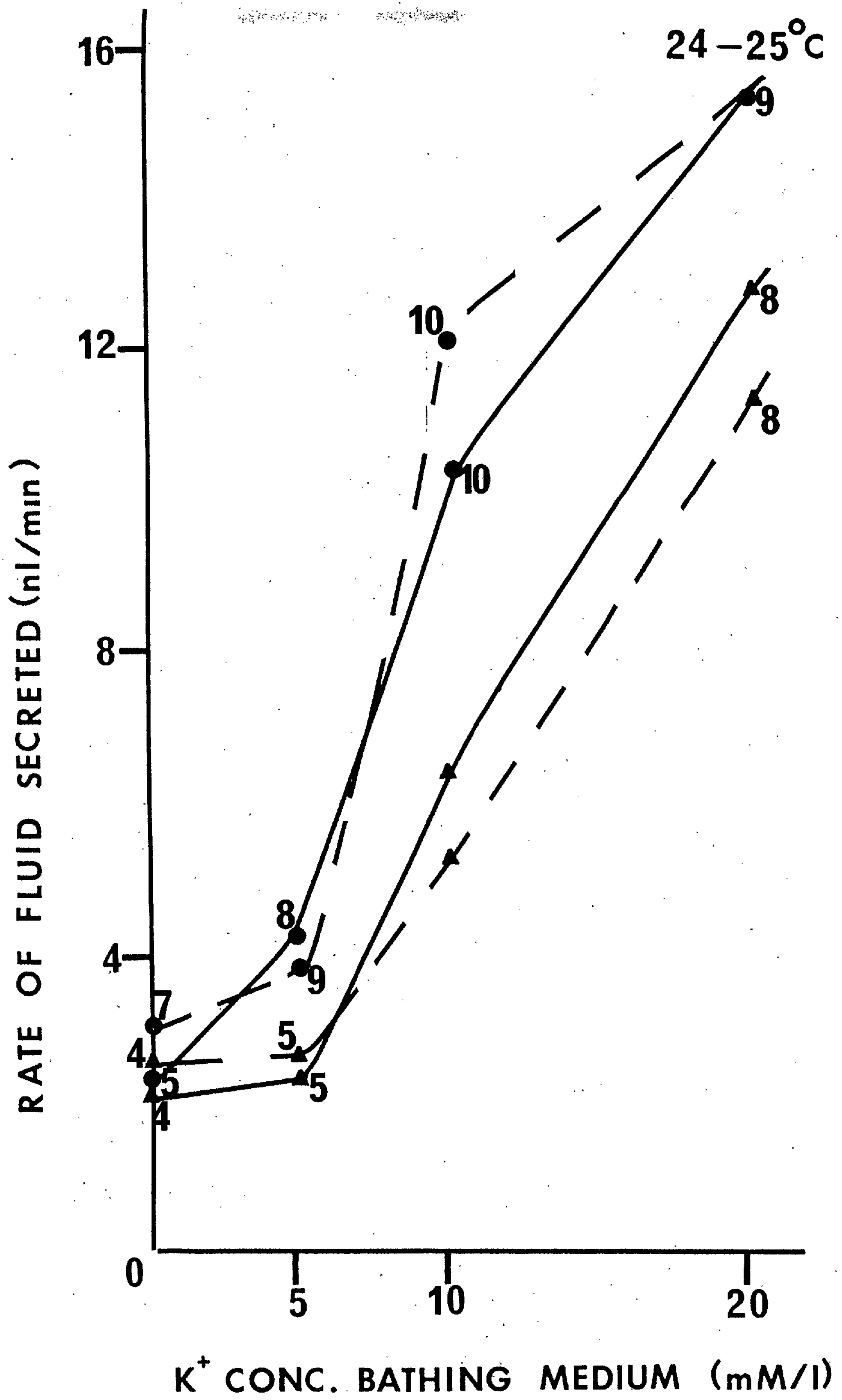

24 – 25°C
RATE OF FLUID SECRETED (nl/min)
16
12
8
4
0
5
10
20
K+ CONC. BATHING MEDIUM (mM/l)
9
10
10
8
8
8
9
7
4
5
5
5
4

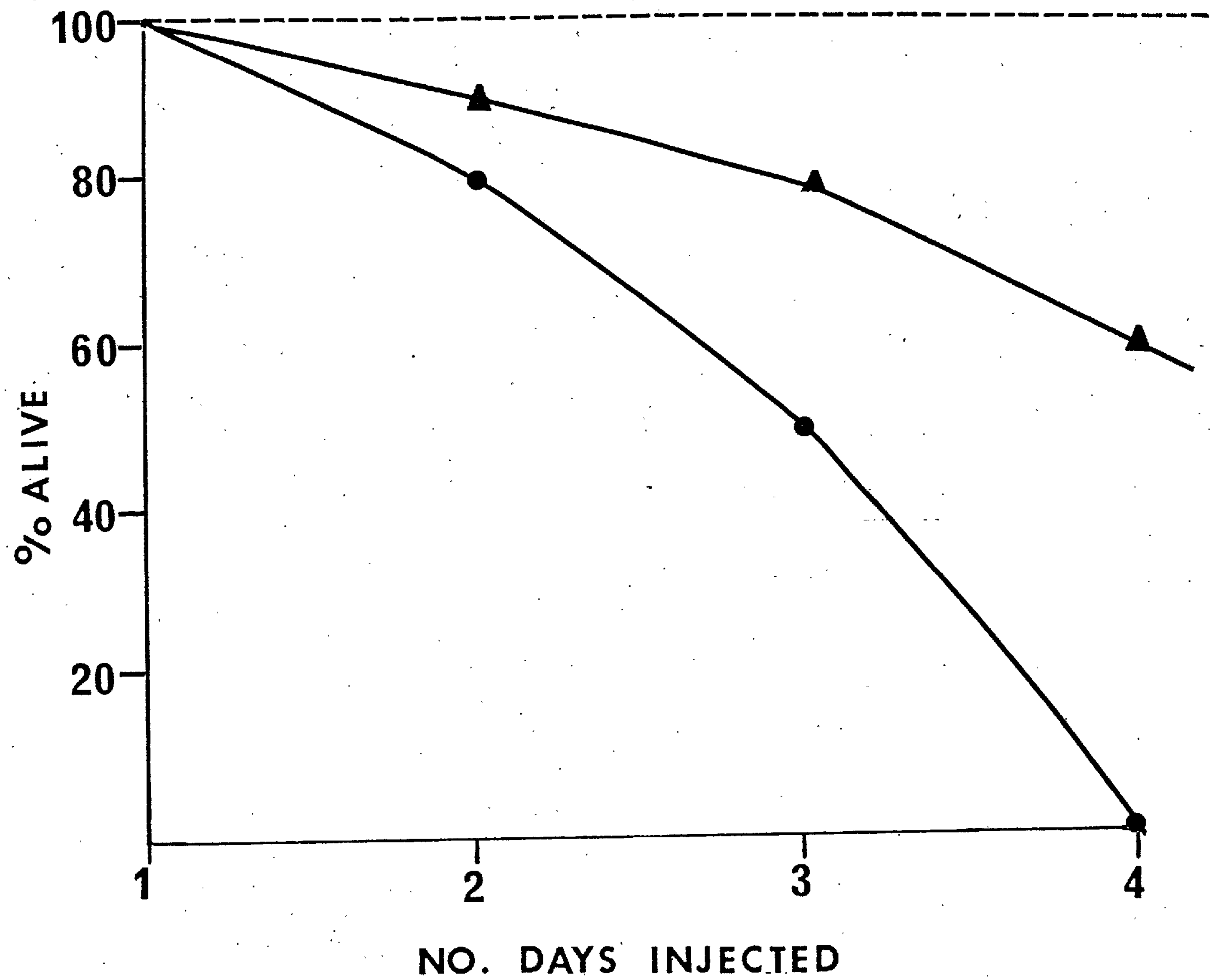
TABLE 4.2.1.

The effect of ouabain over a range of K^+ concentrations on the rate of fluid secreted by Malpighian tubules of *Locusta* and *Zonocerus*.

K^+ conc. bathing	RATE OF FLUID SECRETED means $\pm$ S.E.			
	LOCUSTA		ZONOCERUS	
	control	+ouabain	control	+ouabain
0 mM/l	2.2±2.2	2.4±1.2	2.2±0.4	2.6±0.3
5 mM/l	3.4±2.3	4.2±2.7	2.6±0.6	2.1±0.4
10 mM/l	12.1±6.2	10.5±9.9	5.3±1.8	6.3±1.9
20 mM/l	15.4±1.7	15.4±2.7	11.4±1.4	12.9±1.3

All P values >0.1 therefore no significant differences.

Fig. 4.2.2. Survival of insects injected with ouabain (10 µl/day; 10 animals/group).
● *Locusta* injected with 10^{-8}M ouabain;
▲ *Locusta* injected with 10^{-12}M ouabain;
dashed line, *Locusta* control, *Zonocerus* control, and *Zonocerus* injected with 10^{-8}M and 10^{-12}M ouabain.


100
80
60
40
20
% ALIVE
1
2
3
4
NO. DAYS INJECTED

was much higher than in control insects injected with 10 μl of saline and in Zonocerus individuals following the same ouabain treatment. None of the control Locusta nor the Zonocerus adults died within the experimental period.

4.2.3. Permeability of the tubules to ouabain:

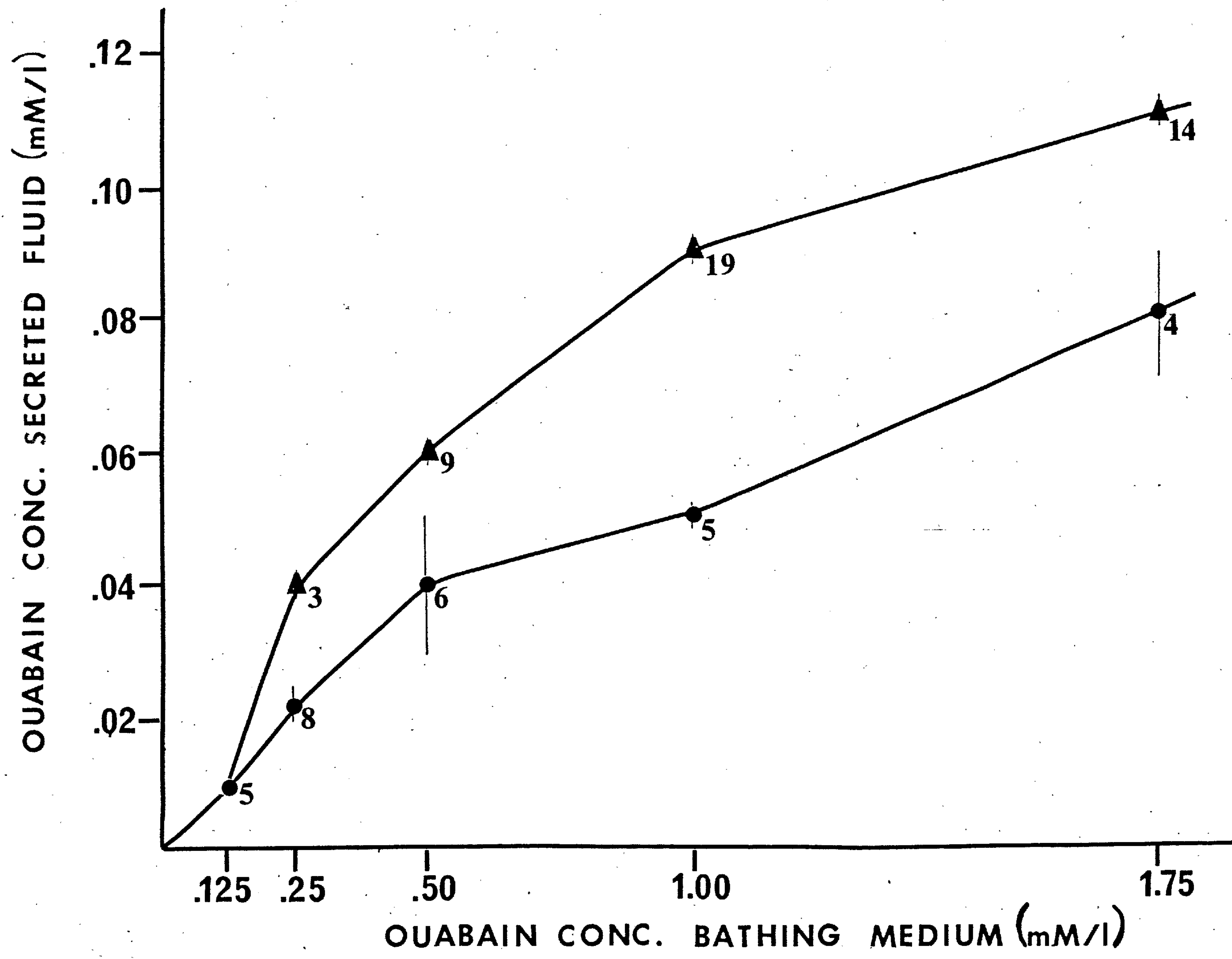
4.2.3.1. Tubules from untreated insects.

The permeability of the tubules to ouabain was tested by using ^{3}H ouabain in the bathing medium and monitoring the progress of radiolabel into the secreted drops. As it was not possible to identify the source of the radiolabel, the possibility that the radiolabel originates from a metabolite of ouabain still exists. The results of these experiments are shown in fig. 4.2.3.1. It can be seen that tubules from Zonocerus excrete higher concentrations of ouabain than those from Locusta at the same bathing concentration of ouabain. For example, at a ouabain concentration of 1 mM/l in the bathing medium the ouabain concentration in the urine secreted by the tubules of Locusta was 0.05 $\pm$ 0.001 mM whilst in Zonocerus the concentration in the secreted fluid was 0.09 $\pm$ 0.001 mM.

4.2.3.2. Tubules from injected Zonocerus.

Malpighian tubules from Zonocerus injected with 292 μg (10 μl of 10^{-3}M) ouabain per day show an

Fig. 4.2.3.1. Passive permeability to ouabain by tubules from untreated *Locusta* ● and *Zonocerus* ▲. Vertical lines show extent of standard error and subscript figures indicate the number of observations.


OUABAIN CONC. SECRETED FLUID (mM/l)
.12
.10
.08
.06
.04
.02
.125
.25
.50
1.00
1.75
OUABAIN CONC. BATHING MEDIUM (mM/l)
14
19
9
3
5
4
5
6
8

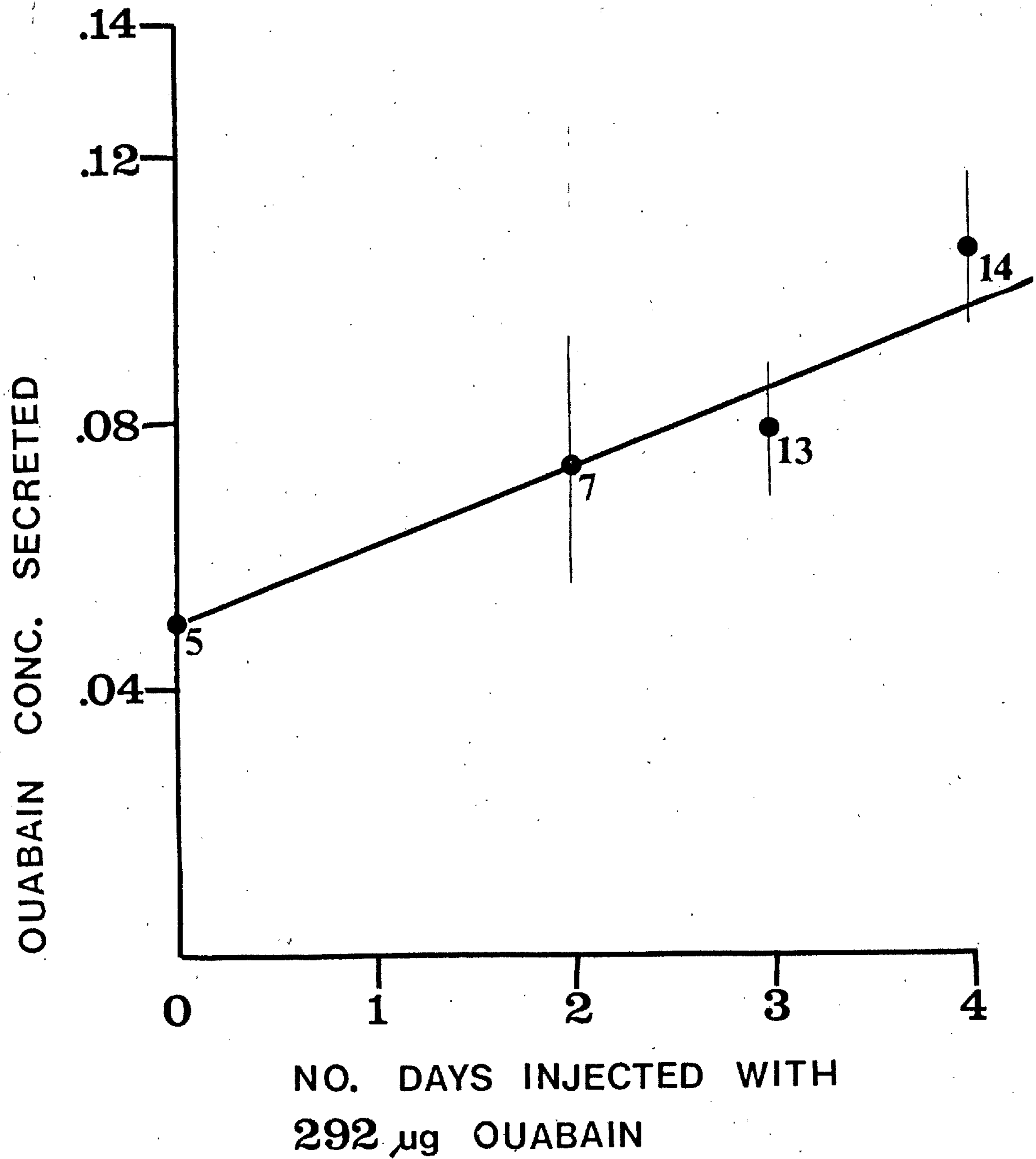
increase in the concentration of ouabain found in the secreted fluid (fig. 4.2.3.2.). Thus not only do they show 100% survival when injected with ouabain (4.2.2.) but the concentrations of ouabain in the urine increases when ouabain is present in the haemolymph. Clearly, Zonocerus is capable of handling the ouabain present in the haemolymph by excreting it faster than Locusta. The identical experiments could not be performed on Locusta as the injected ouabain killed them.

4.2.3.3. Tubules from Zonocerus fed on an artificial diet containing ouabain.

In the previous experiments, although an increase in the passive transport of ouabain is seen when Zonocerus adults are confronted with ouabain in the haemolymph, in all cases the concentration of ouabain in the secreted fluid never exceeded that in the bathing medium (figs. 4.2.3.2. & 4.2.3.1.). Although Zonocerus individuals were able to survive daily injections, after 4 days they only achieved an increase of 0.106 mM from the original level. To achieve a 10 fold increase so that activity ratios greater than 1 would occur in the secreted fluid would thus require a considerable amount of injections which invariably must affect the normal performance of individuals.

An artificial diet which contained ouabain was thus fed to adult Zonocerus. Fig. 4.2.3.3.1. shows that

Fig. 4.2.3.2. The effect of injecting ouabain into the haemolymph on the permeability of *Zonocerus* tubules to ouabain. Vertical lines indicate extent of standard error and subscript figures indicate the number of observations.


.14
.12
.08
.04
OUABAIN CONC. SECRETED
5
7
13
14
0
1
2
3
4
NO. DAYS INJECTED WITH
292 µg OUABAIN

Malpighian tubules from Zonocerus that were fed on this artificial diet were able to excrete ouabain at a concentration higher than that present in the bathing medium. This effect was detectable after 12 days (fig. 4.2.3.3.2.) and the proportion of insects able to excrete ouabain in this manner increases to some 90% after 24 days when activity ratios as high as 2.7 are achieved.

Fig. 4.2.3.3.1. The effect of feeding ouabain on the activity ratios of ouabain. Dashed line indicates the level of ouabain found in the bathing medium. Vertical lines show extent of standard error and subscript figures indicate the number of observations. Regression line fitted by the least square method.

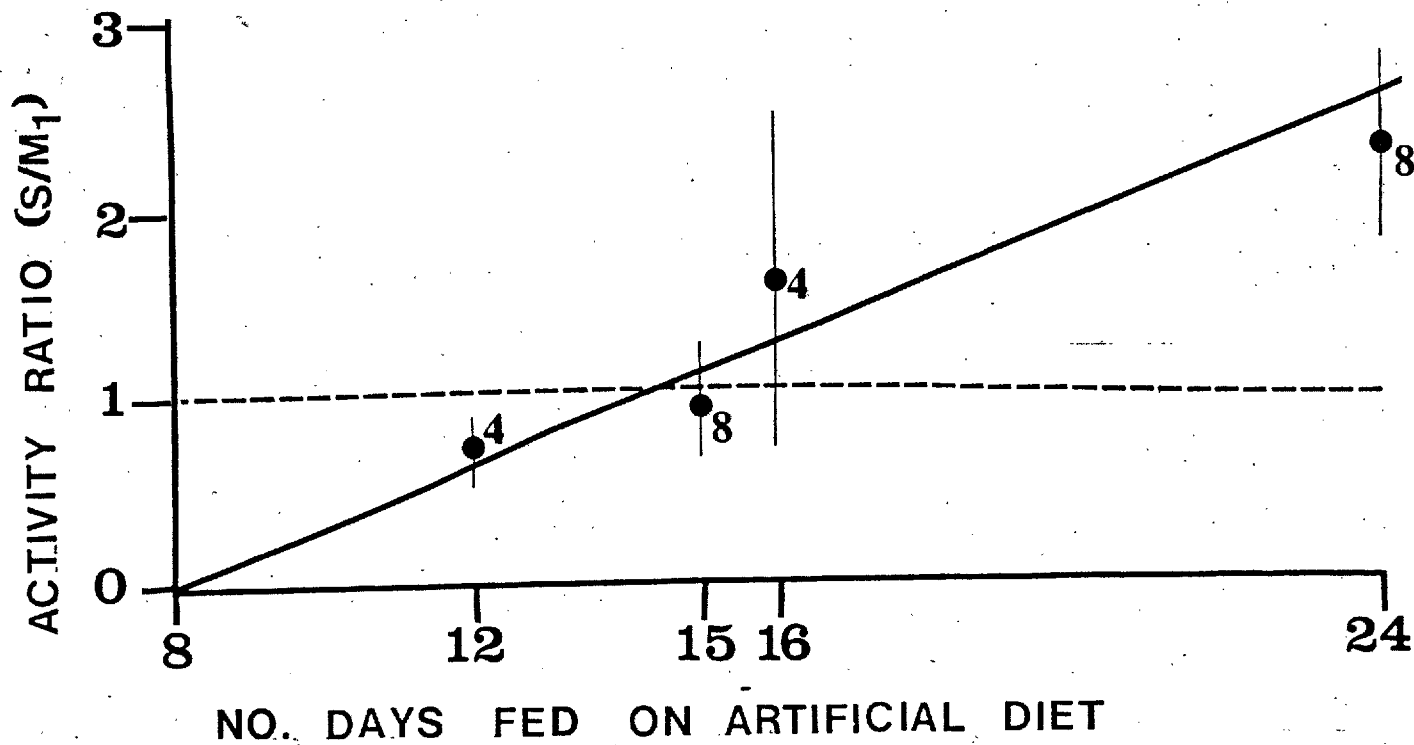
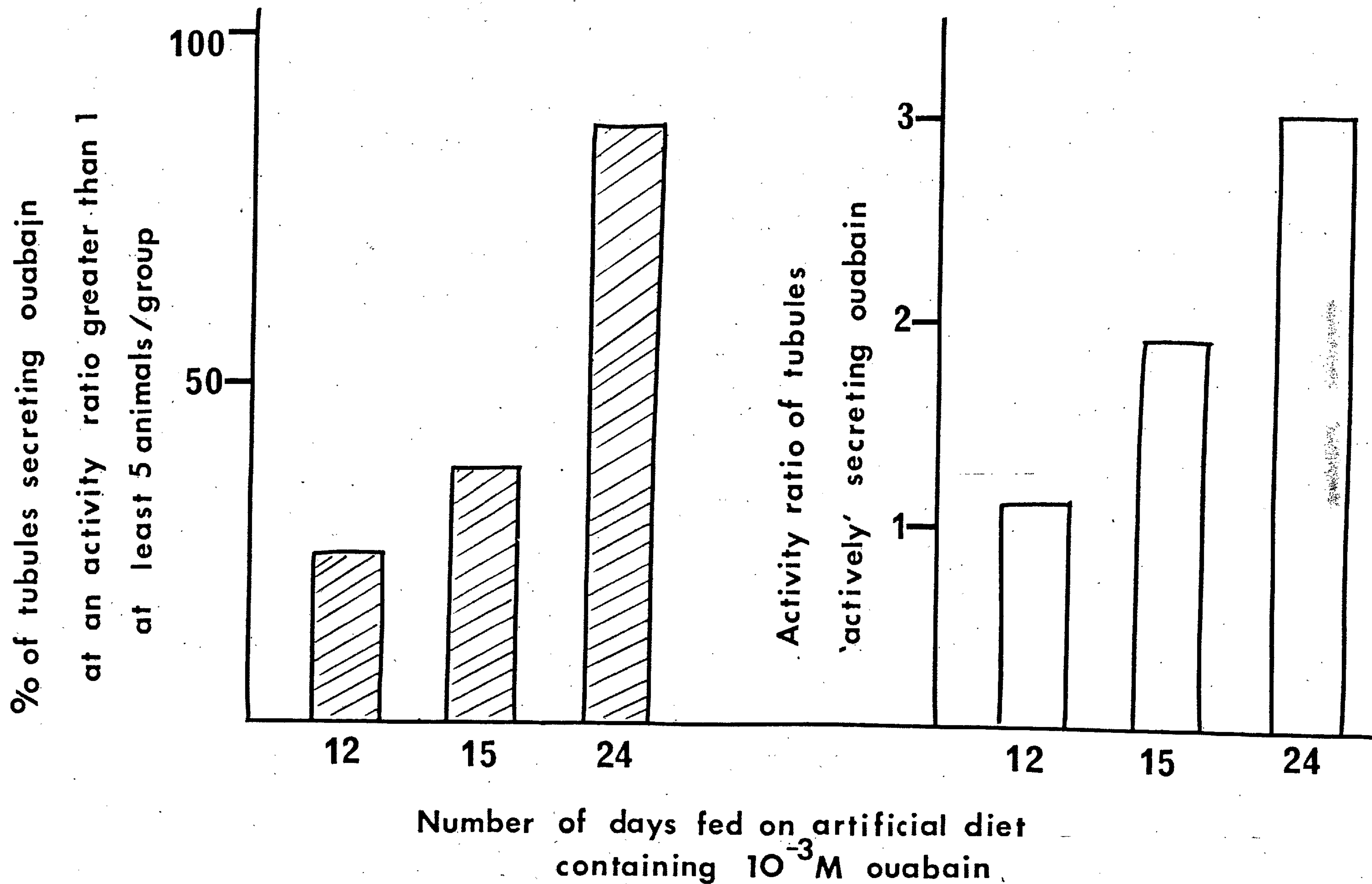

ACTIVITY RATIO (S/M$_1$)
3
2
1
0
4
8
4
8
8
12
15
16
24
NO. DAYS FED ON ARTIFICIAL DIET

Fig. 4.2.3.3.2. The effect of feeding ouabain upon the secretory activity of *Zonocerus* tubules. Cross-hatched columns, % of tubules secreting ouabain at a concentration greater than that in the bathing medium. Open columns, mean activity ratios of these tubules.



% of tubules secreting ouabain
at an activity ratio greater than 1
at least 5 animals/group
100
50
12
15
24
Activity ratio of tubules
'actively' secreting ouabain
3
2
1
12
15
24
Number of days fed on artificial diet
containing 10^{-3}M ouabain

4.3. DISCUSSION

Cardiac glycosides have been shown to be specific inhibitors of cation transport (Skou, 1965). Ouabain acts directly on the Na^+-K^+-ATPase by binding to the enzyme in competition to K^+. This Na^+-K^+-ATPase enzyme system plays a major role in both insect and vertebrate tissues (Treherne, 1966; Bergmann *et al.*, 1970; Gulati & Jones, 1971). O'Riordan (1969) found indications of ouabain effect on the midgut of *Periplaneta*, whereas Treherne (1966) and Haskell *et al.* (1965) found no effect on *Cercopia* midgut. It has been shown that it also plays a major role in renal Na^+ reabsorption but is not involved in the active transport of K^+ in insect Malpighian tubules (Maddrell, 1969; Keynes, 1972; Gee, 1976b). Grotthuss *et al.* (1974) showed a change in the structural characteristics of the Malpighian tubules of *Drosophila* which they attribute to ouabain treatment. Na^+-K^+-ATPases have been localized histochemically by Schulte (1972) working on *Drosophila* Malpighian tubules and by Anstee and Bell (1975) in microsomal preparations of Malpighian tubules of *Locusta*. They also found an inhibition of fluid secretion by ouabain. In this Chapter in both *Locusta* and *Zonocerus* ouabain

has been shown not to have an apparent effect on the normal functioning of the tubules, certainly fluid secretion is unaffected by the presence of ouabain in the bathing medium. Vaughan & Jungreis (1977) have suggested that a high K^+ concentration blockades the ouabain binding to Na^+-K^+-ATPase. However, even at low K^+ concentrations it proved impossible to inhibit fluid secretion in both Locusta and Zonocerus. The possibility, however, that an ATPase is involved in maintaining the ionic battery of the tubule cells, as it does in other cells, still exists.

The results in this Chapter clearly show that Zonocerus is capable of handling ouabain more efficiently than Locusta. The response to ouabain by the tubules themselves differs from the response obtained when ouabain is injected into the live insect in Locusta. Locusta are incapable of handling low amounts of ouabain (0.0584×10^{-9} μg) injected into the haemolymph and die whereas Zonocerus are capable of handling amounts as high as 292 μg. This survival rate is reflected in the passive permeability of the tubules to ouabain, Locusta tubules secrete lower concentrations than Zonocerus tubules.

Not only are _Zonocerus_ tubules capable of secreting ouabain passively at a higher level than _Locusta_ but the presence of ouabain in the haemolymph, whether injected or originating in the diet of the insect, induced the tubules to secrete even higher levels eventually reaching levels in the secretions which are greater than those found in the bathing medium. This indicates that exposure to ouabain over periods of 12 days induces a pump in the Malpighian tubules which is capable of secreting ouabain against a concentration gradient. It would be of considerable interest to know the effects of prolonged feeding on a diet containing glycosides, during the whole of the postembryonic stage, upon the levels of glycoside secreted by the tubules. This induction may well be an explanation to the origin of the development of insect resistance to many insecticides but whether or not this tubular mechanism is _the_ or _a_ fundamental reason for the tolerance of _Zonocerus_ to cardiac glycosides and other toxins awaits further investigation.. Nevertheless as a result of this investigation it is clear that the physiology of the Malpighian tubules of _Zonocerus_ is well adapted to its diet. Toxins ingested in the diet can be removed from the insect via the urine without any harmful effects upon the Malpighian tubules.

5. TRANSPORT OF THE PLANT GLYCOSIDE, PHLORIZIN.

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5.3. DISCUSSION 148

5.1. INTRODUCTION

In Chapter 6 the mode of action of phlorizin on glucose transport will be discussed. It will be shown to inhibit reabsorption of glucose by the tubules. In Chapter 3 it has been shown that phlorizin as well as its aglycone, phloretin, increase the rate of fluid secretion by the tubules. Moreover, a second dose of phlorizin produced an additional, though smaller, increase which may indicate that the tubules somehow either destroy or remove the phlorizin to make available sites for a second response. The possibilities exist that the increase in rate of fluid secretion, maybe due to interference with the mechanism of fluid secretion, or be a consequence of increased osmotic flow associated with the transport of phlorizin against a concentration gradient. An investigation of the permeability of the tubules to phlorizin itself would thus throw further light onto the mechanism of phlorizin in inhibiting glucose transport and stimulating fluid secretion.

To date, active phlorizin transfer in tissues sensitive to it has not been firmly established. The two alternative interpretations offered to explain glucose inhibition in the vertebrates are still unresolved:
(a) the glucose carrier may be immobilised by the action of phlorizin binding to some immobile component of the

membrane or (b) phlorizin enters the cells in competition with glucose. Alvarado & Crane (1964) were unable to detect phlorizin in intestinal epithelial cells following incubation. Binding of the aglycone portion of phlorizin when observed has been strong and irreversible by glucose. On the other hand, Wilbrandt & Rosenberg (1961) pointed out that with compounds of low saturation constants a maximal outward flow will occur at low concentrations in the cell and thus one would not expect to find measurable concentrations of phlorizin within the cell.

Landau *et al*. showed that several aliphatic and aromatic glycosides are actively transported by hamster small intestine *in vitro*; and Alvarado & Crane (1964) have shown that some phenylglycosides are actively transported in the small intestine of hamster and rat along pathways common with those used for sugars.

When considering the Malpighian tubules the possibility of active transport of phlorizin is more tenable than in vertebrate tissues. The tubules have been shown to be freely permeable to many organic solutes (Ramsay, 1958; Maddrell & Gardiner, 1974; Knowles, 1975a), even to inulin with a molecular weight of over 5,000. Ramsay (1958) pointed out the great advantage in this apparent excretion of useful substances which are later reabsorbed. The system provides an automatic

excretion of toxic chemicals simply by the fact that no reabsorption of these chemicals occurs. Not only are the tubules freely permeable to organic solutes but they have been shown (Maddrell et al., 1974) to actively secrete certain organic anions such as acylamides and acidic dyes. This ability was later shown (Maddrell & Gardiner, 1975) to be inducable by feeding insects. Maddrell & Gardiner suggest that the active transport depends on the continued presence in the haemolymph of some product of digestion. Similarly it has been shown in Chapter 4 that tubules of Zonocerus can be induced to excrete ouabain when it is present in the insect's diet. Maddrell & Gardiner (1976) have also shown active transport of several alkaloids which are potential plant toxins available in an herbivorous diet. The possession of systems which can actively secrete such compounds are of obvious advantage in the rapid clearance of potentially harmful substances which may be products of metabolism or taken up in the diet. Phenols and other reactive and harmful molecules have been reported to be metabolised to less harmful compounds, such as β-glucosides, (Gilmour, 1965) but these in turn must be removed.

This Chapter reports the investigation made on the permeability of the tubules to phlorizin and shows that mainly the aglycone, phloretin, is removed by the Malpighian tubules from the bathing medium against concentration gradients.

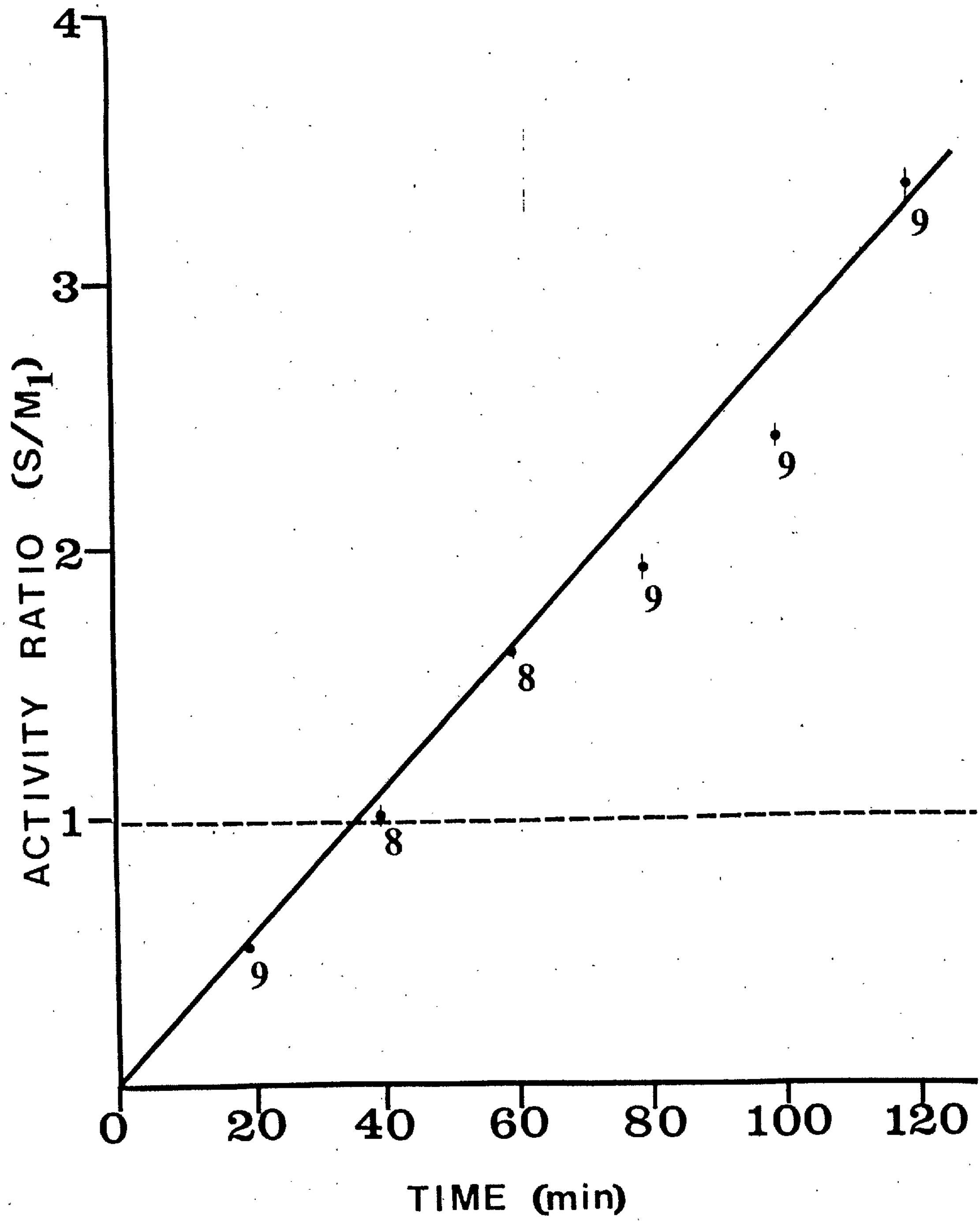
5.2. RESULTS

5.2.1. Permeability of the tubules to phlorizin.

Phlorizin ($-^{3}H$) (specific activity, 6.57 Ci/mM) at a concentration of 1 mM/l (10^{-3}M) was added to the bathing medium after normal secretion was obtained. Samples of secreted fluid were taken at 20 min intervals and were measured for radioactivity. The results are shown in Fig.5.2.1.1. The dashed line represents the level of concentration of phlorizin found in the bathing medium. After 40 min of secretion the tubules reach levels of activity ratios which exceed 1, that is, they exceed the levels found in the bathing medium.

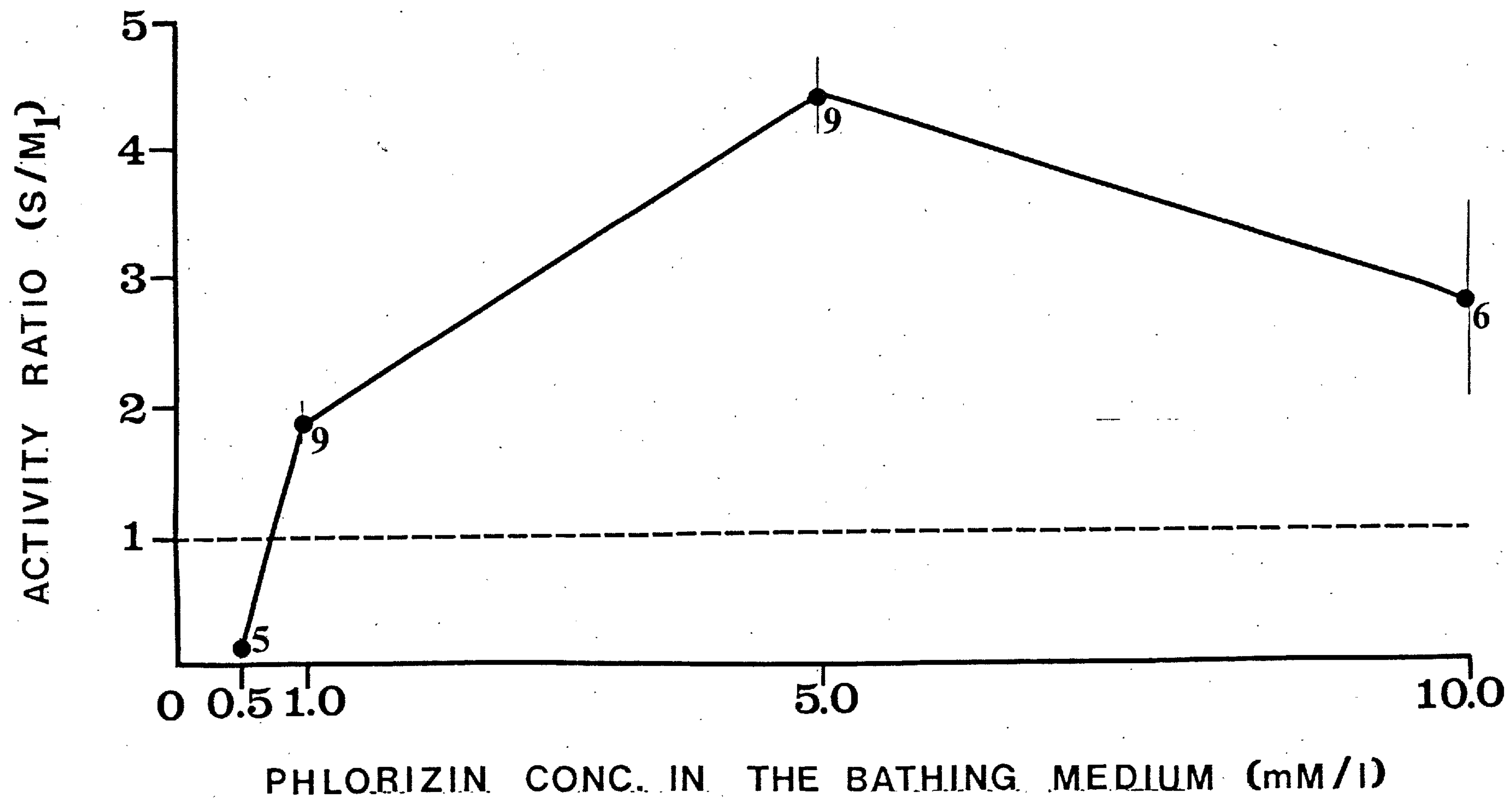
Similar experiments were performed to obtain results for varying concentrations of phlorizin in the bathing medium. Bathing media containing 0.5, 5.0, and 10.0 mM/l phlorizin ($-^{3}H$) were added to tubule preparations after normal secretion was established. Samples of secreted fluid were collected after 80 min of secretion for each of the bathing media concentrations. These results are represented in Fig.5.2.1.2. At concentrations in the bathing medium of greater than 0.75 mM/l activity ratios rise well above 1 and therefore show a high accumulation of phlorizin in the secreted fluid.

Fig. 5.2.1.1. The permeability of the tubules to 1 mM/l phlorizin. The dashed line represents the levels of phlorizin in the bathing medium. Vertical lines indicate extent of standard error and subscript figures indicate the number of observations.



4
3
2
1
ACTIVITY RATIO (S/M$_1$)
9
8
8
9
9
9
0
20
40
60
80
100
120
TIME (min)

Fig. 5.2.1.2. The permeability of the tubules to different concentrations of phlorizin. The dashed line represents levels of phlorizin in the bathing medium. Vertical lines indicate extent of standard error and subscript figures indicate the number of observations.


5
4
3
2
1
ACTIVITY RATIO (S/M$_I$)
0 0.5 1.0
5.0
10.0
PHLORIZIN CONC. IN THE BATHING MEDIUM (mM/l)
5
9
9
6

5.2.2. Kinetics of phlorizin transport.

The results obtained when testing the permeability of the tubules to phlorizin (5.2.1.) suggested an apparent active transporting system for phlorizin. To test this the data was converted from cpm/µl, which yielded the relative amount of radioactivity in the secreted fluid when compared with the bathing medium, to dpm/µl and thus to actual molar concentrations (2.3.2.). These results are represented in the saturation curve of Fig.5.2.2.1. The curve shows that the phlorizin concentration in the secreted fluid is dependent on the phlorizin concentration of the bathing medium. The dashed line represents the passive permeability of phlorizin which would be expected for simple passive leakage.

Initial velocities (v_o) were calculated according to Kotyk & Janacek (1975) where $v_o = \Delta^1 - \Delta^2/2 + \Delta^3/3 - \Delta^4/4$ (Table 5.2.2.1.). Taking v_o as the calculated value of 0.2087, values for $v_1 ....v_n$ were calculated. The rate of secretion of phlorizin as a function of the concentration of phlorizin in the bathing medium is shown in Fig.5.2.2.2.

Values for K_m and V_{max} were calculated using the double reciprocal plot of Lineweaver-Burk. These are shown in Fig.5.2.2.3. The value for K_m can be

Fig. 5.2.2.1. The effect of the phlorizin concentration in the bathing medium on its concentration in the secreted fluid. The dashed line is the relationship where both the bathing medium and the secreted fluid contain equal concentrations of phlorizin. Vertical lines show the extent of standard error and subscript figures indicate the number of observations.

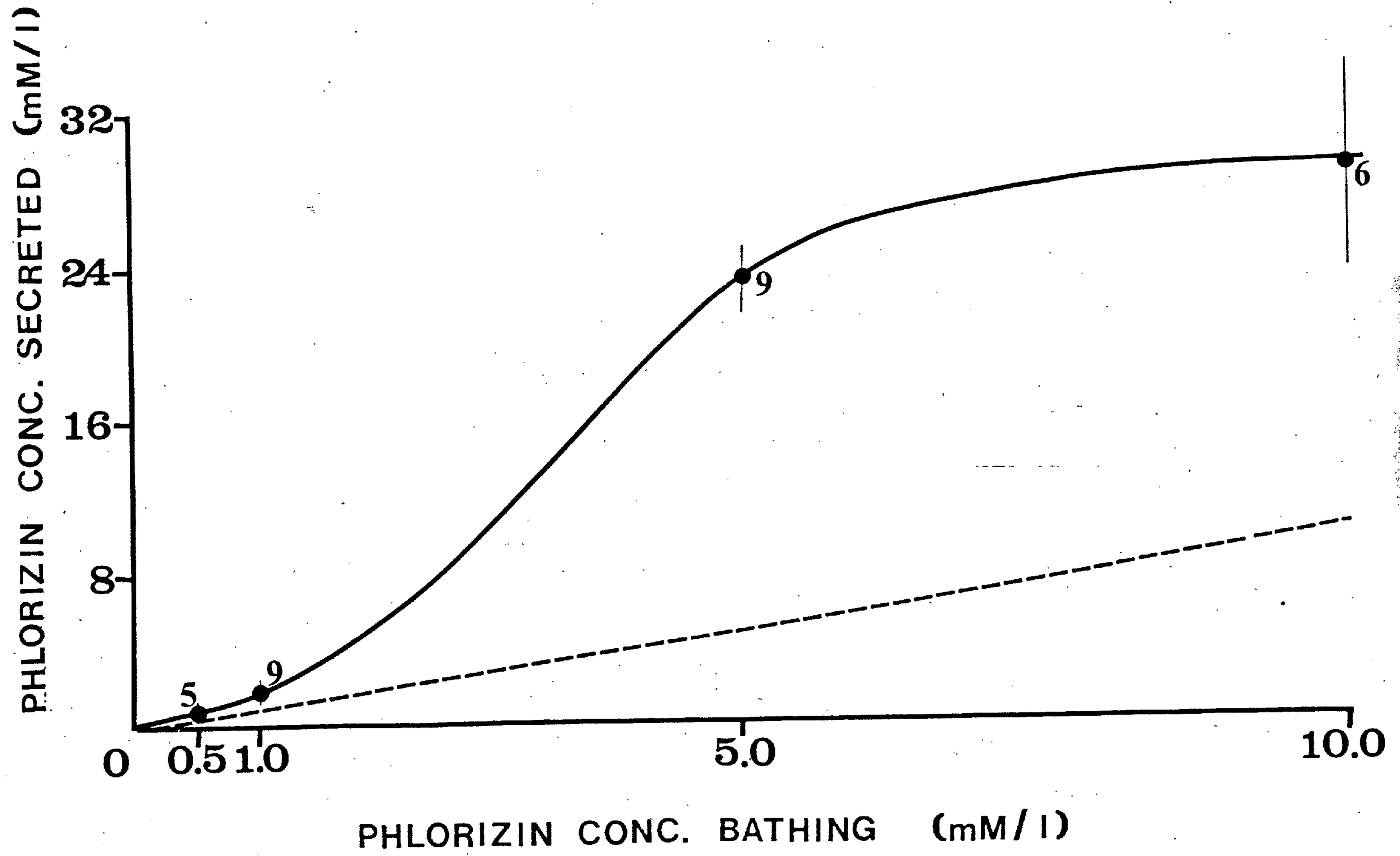

PHLORIZIN CONC. SECRETED (mM/l)
32
24
16
8
0
0.5
1.0
5.0
10.0
5
9
9
6
PHLORIZIN CONC. BATHING (mM/l)

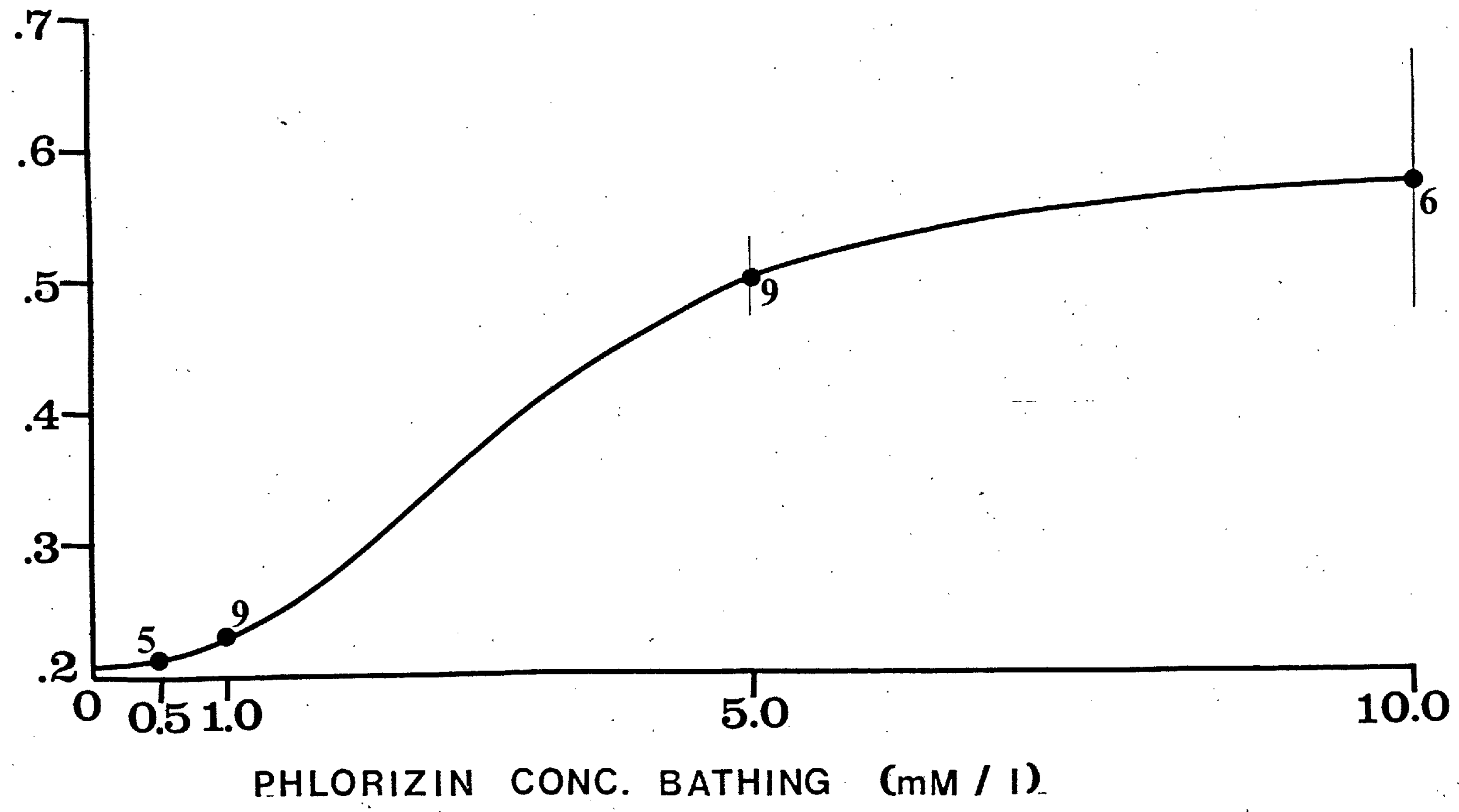
TABLE 5.2.2.1.

Calculation of initial velocities (v_o) based on the first derivative of Newton's formula.

Rate values mM/min	Δ^1	Δ^2	Δ^3	Δ^4
0.0000				
	0.0009			
0.0009		0.0224		
	0.0233		0.1942	
0.0242		0.2166		-0.6174
	0.2399		-0.4232	
0.2641		-0.2066		
	0.0333			
0.2974				

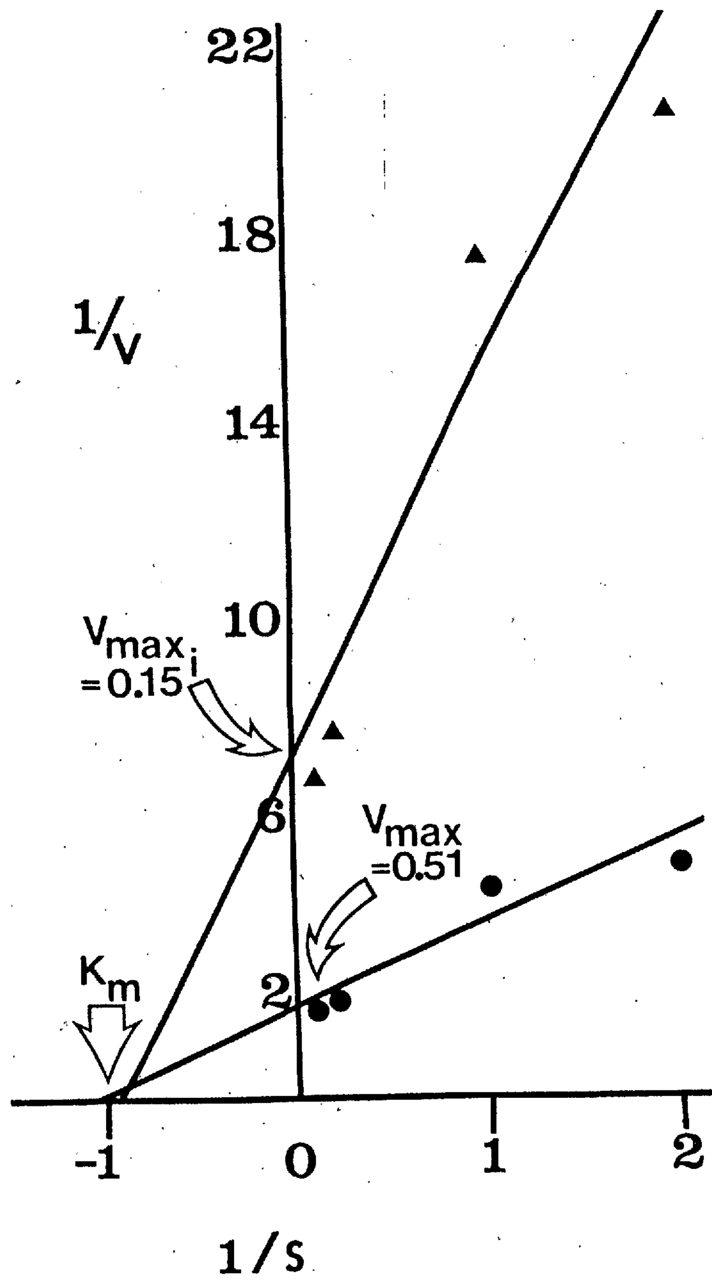
$$v_o = \Delta^1 - \Delta^2/2 + \Delta^3/3 - \Delta^4/4$$
$$= 0.0009 - 0.0224/2 + 0.1942/3 - (-0.6174/4)$$
$$= 0.0009 - 0.0112 + 0.0647 + 0.1543$$
$$= \underline{0.2087}$$

Fig. 5.2.2.2. Dependence of the rate of secretion of phlorizin on the concentration of phlorizin in the bathing medium. Vertical lines indicate extent of standard error and subscript figures indicate the number of observations.



RATE OF PHLORIZIN SECRETED (mM/min)
.7
.6
.5
.4
.3
.2
0
0.5
1.0
5.0
10.0
5
9
9
6
PHLORIZIN CONC. BATHING (mM / l)

Fig. 5.2.2.3. A Lineweaver-Burk plot of initial rate dependence on the phlorizin concentration when 50 mM/l ● and 100 mM/l ▲ glucose was present in the bathing medium showing non-competitive inhibition by glucose. Straight lines were fitted by a regression line according to the least square method.



22
18
1/v
14
10
Vmax i
=0.15
6
Vmax
=0.51
Km
2
-1
0
1
2
1/s

seen to be 0.98 mM and the value for V_{max} to be 0.51 mM/min. Similar values were obtained when using the Woolfe-Hofstee plot of v (rate) against v/s (Fig.5.2.4.2.) (K_m=1.01 mM; V_{max}=0.58 mM/min).

5.2.3. Test for any reabsorption of phlorizin.

The transport of phlorizin via the hindgut was tested using a hindgut preparation as described in Chapter 2 (2.2.4.). No significant reabsorption of phlorizin from the hindgut lumen to the haemolymph was observed (Table 5.2.3.).

Using the double droplet Malpighian tubule preparation phlorizin reabsorption by the Malpighian tubules was tested. The rate of phlorizin reabsorption was very low and insignificant (Table 5.2.3.).

5.2.4. Effect of the glucose concentration in the bathing medium.

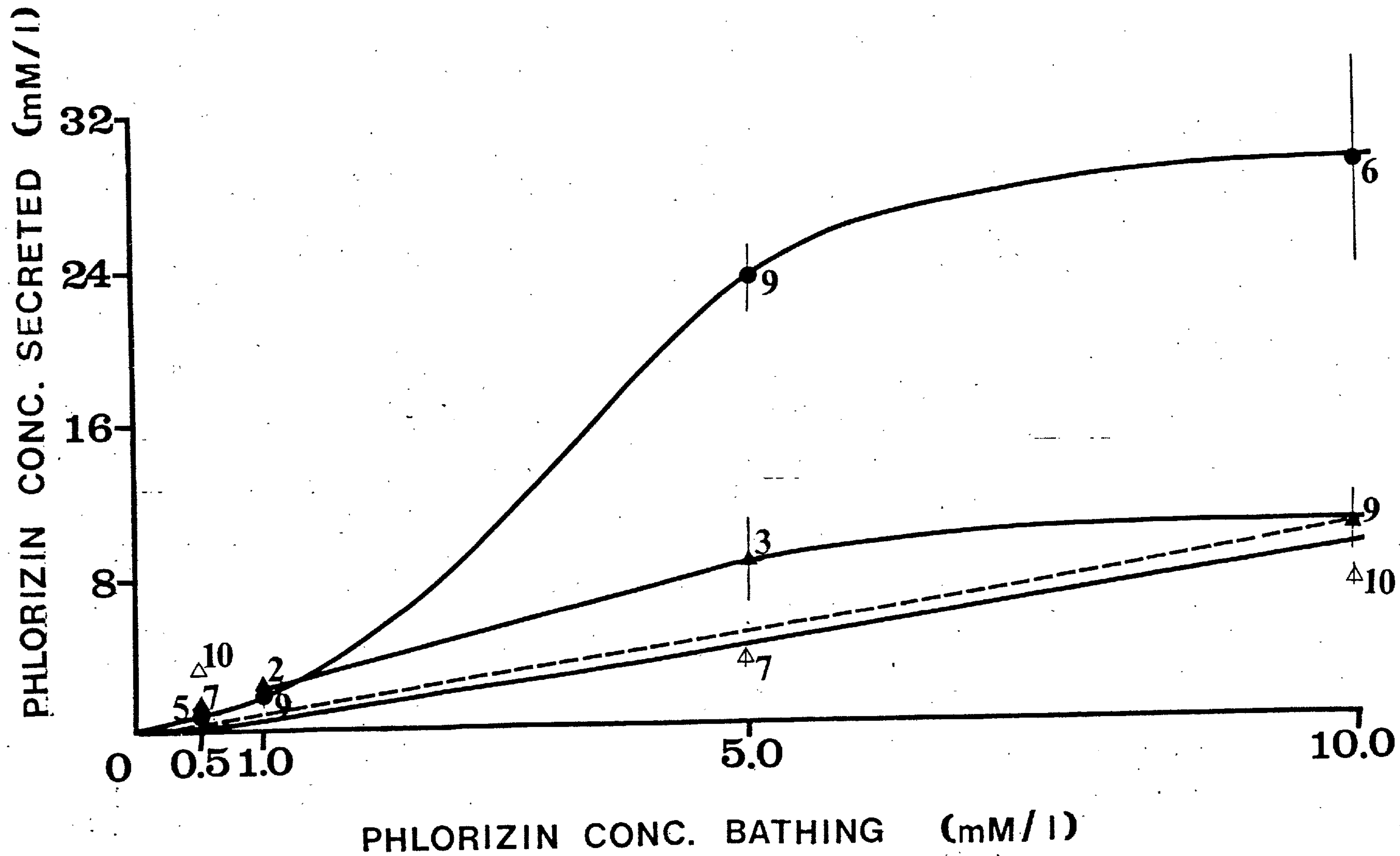
Phlorizin transport was thus far investigated using a glucose concentration in the bathing medium of 50 mM/l. The effect of increasing the glucose concentration was investigated by using 100 mM/l and 200 mM/l glucose in the bathing medium. These results are represented in Fig.5.2.4.1. and compared

TABLE 5.2.3.

Test for any reabsorption of phlorizin by the hindgut and the Malpighian tubules.

Experimental Condition	Reabsorption Ratio (M_2/M_1)	% Reabsorbed
Hindgut	7.5×10^{-5}	0.1×10^{-6}
Malpighian tubules:		
$M_1 = M_2 =$ phlorizin	0.12	0.2×10^{-3}
$M_2 =$ phlorizin	0.086	0.1×10^{-3}
$M_1 =$ phlorizin	0.004	0.1×10^{-4}

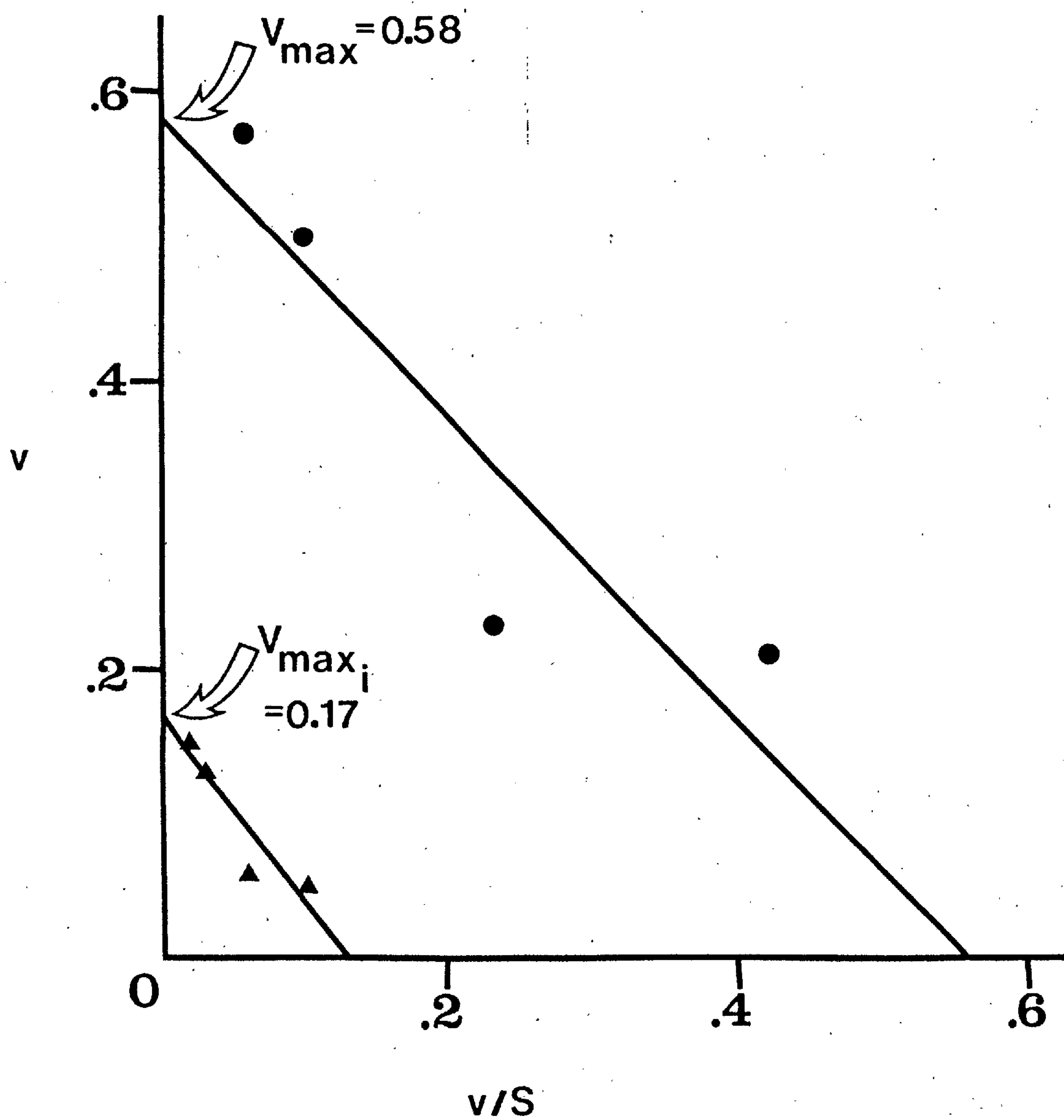
Fig. 5.2.4.1. The effect of the phlorizin concentration in the bathing medium on its concentration in the secreted fluid when 50 mM/l ●, 100 mM/l ▲ and 200 mM/l △ of glucose were present in the bathing medium. The dashed line is the relationship where both the bathing medium and the secreted fluid contain equal concentrations of phlorizin. Vertical lines show the extent of standard error and subscript figures indicate the number of observations.


PHLORIZIN CONC. SECRETED (mM/l)
32
24
16
8
0
0.5
1.0
5.0
10.0
PHLORIZIN CONC. BATHING (mM/l)

with the 50 mM/1 control (Fig.5.2.2.1.). When the glucose concentration is increased to 100 mM/1 the saturation curve lowers to a level very close to the limits of passive transport. When it is further increased to 200 mM/1 any active transport is completely abolished and a saturation curve cannot be easily obtained.

In order to determine the type of competition that occurs between the substrate phlorizin (S) and the inhibitor glucose (i) a Woolf-Hofstee plot of the above results was determined. Fig. 5.2.4.2. shows that a non-competitive inhibition by glucose occurs. Since glucose does not affect the slope of the curve and therefore the equilibrium constant (K_m), it does not compete with phlorizin for the binding sites but exerts other influences which alter the reaction kinetics and therefore the intercept on the y-axis. This is more apparent when the results are plotted according to a Lineweaver-Burk plot (Fig.5.2.2.3.) where V_{max}=0.51 and V_{max_i}=0.148 mM/min whereas K_m is not altered significantly. The inhibitor dissociation constant K_i was determined from these two plots to be i/0.78 mM, where i is the inhibitor concentration. In the case of a glucose concentration of 100 mM/1 K_i=128.21 mM (Table 5.2.4.1.).

Fig. 5.2.4.2. Analysis of the non-competitive inhibition of phlorizin transport by glucose according to Woolf and Hofstee: ● 50 mM/l glucose, ▲ 100 mM/l glucose. Regression lines were fitted by the least square method.



V_{max}=0.58
V_{max_i}
=0.17
.6
.4
.2
0
v
.2
.4
.6
v/S

TABLE 5.2.4.1.

Constant values for the kinetics of phlorizin transport.

Constant	Lineweaver-Burk	Woolf-Hofstee
K_m	0.98 mM	1.01 mM
V_{max}	0.51 mM/min	0.58 mM/min
V_{max_i}	0.148 mM/min	0.167 mM/min
K_i = i/0.78; K_{50} = 64.10 mM K_{100}=128.21 mM K_{200}=256.41 mM		

5.2.5. In vivo clearance of injected phlorizin.

Locusts were injected with 0.22 µg of ^{3}H-phlorizin and the radiolabel was followed within the different tissues. The results are shown in Table 5.2.5. where it can be seen that the haemolymph level drops by 85% within 30 minutes. The levels within the tubules, hindgut and faeces increase within 15 min after which it still rises in the rectum and faeces (to 92% after 24 hours) but drops in the tubules and hindgut. Clearly the phlorizin injected is rapidly removed from the haemolymph and accumulated in the faeces showing an ability to handle this toxin.

5.2.6. Thin -layer chromatographic investigation of the secreted fluid.

The radiolabelled secreted drops were pooled and collected for spotting on a T.L.C. plate. A sample of the bathing medium Ringer was also spotted as a control. The results are represented in a form of a profile in Fig.5.2.6. and show two regions in which the radiolabel spots: Rf 0.3 and Rf 0.5. The main peak of activity corresponds to Rf 0.5 in the secreted fluid which differs from the peak at 0.3 of the bathing medium.

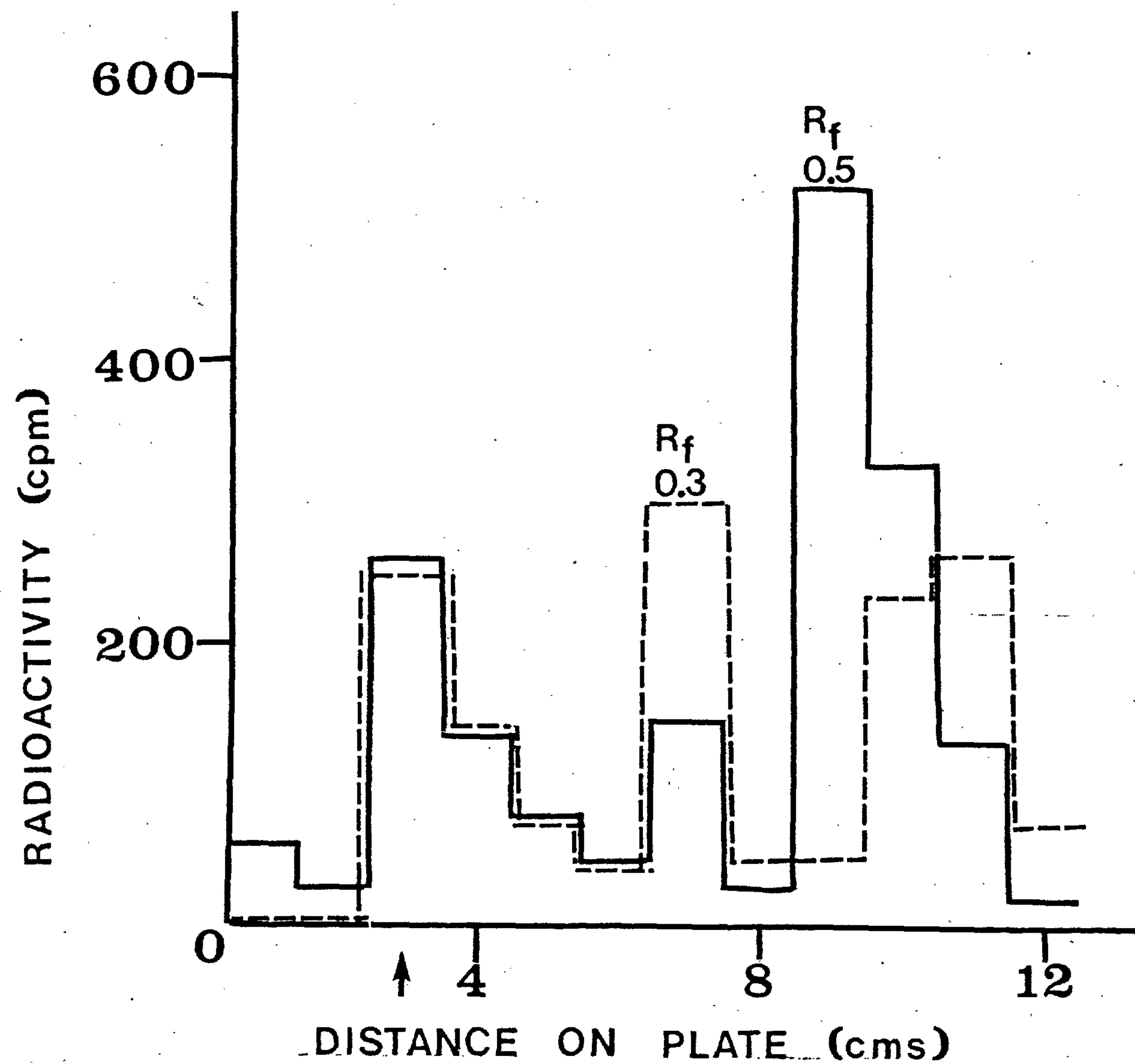
TABLE 5.2.5.

In vivo clearance of injected phlorizin (0.22 ug)

(cpm x 10^2)

Time (min)	Haemolymph	Tubules	Hindgut	Faeces
0	53	2	0	0
5	45	4	1	2
10	42	8	1	5
15	33	7	2	8
30	8	2	5	-
60	4	0	-	6
120	2	0	1	-
180	2	0	0	-
240	1	0	0	26

Fig. 5.2.6. Thin-layer chromatograph profile of radiolabelled phlorizin in the bathing medium (dashed line) and the secreted fluid (solid line). The arrow marks the initial spotting area.


600
400
200
0
RADIOACTIVITY (cpm)
R_f 0.5
R_f 0.3
4
8
12
DISTANCE ON PLATE (cms)

5.3. DISCUSSION

This investigation shows clearly that Malpighian tubules can concentrate phlorizin to levels reaching some 20 times those found in the bathing medium. Glucose has been shown to be a non-competitive inhibitor.

In the light of research on the inhibitory kinetics of glucose transport by phlorizin in vertebrate tissues it has been shown by several workers (see Chapter 6) that phlorizin is a competitive inhibitor whereas phloretin is the non-competitive inhibitor. This has been largely explained by the postulation of two closely associated binding sites for glucose: (a) a glucose site and (b) a phenol site, where phlorizin will bind simultaneously to both sites whereas phloretin will only bind to the phenol site but cause certain conformational changes which will interfere with the glucose site. In *Locusta*, investigation of the fluid secreted by the Malpighian tubules has shown that the main source of radiolabel was phloretin. It thus seems probable that phloretin itself binds to a phenol site which, as in the vertebrate tissues, is closely associated with a glucose transporting site. Glucose, in turn, interferes with the binding of phloretin in a similar manner to phloretin interference with glucose transport.

The presence of both phlorizin and phloretin in the secreted fluid poses certain questions. It may be that the tubules possess differing abilities in the hydolysis of phlorizin, or it may be that both substances are present in the secreted fluid as a result of variations in the excretory physiology of individual tubules. It has been shown previously (Chapter 2) that Locusta tubules vary greatly in the rate of fluid secretion and basal rates for each tubule had to be obtained as mean values were unreliable. Thus tubules with slow rates of secretion could allow phlorizin molecules to linger within the tubule cells and thus enable hydolysis whereas tubules with faster rates would pump phlorizin out before hydrolysis had occurred. Which is the actively transported molecule, however, cannot be established on the present data. However, it remains clear that active transport does occur which is then followed by an osmotic gradient causing an increase in fluid secretion. This fact reveals a possibility that several so called mimics of diuretic hormone may well be merely reflections of the ability of the tubules to actively secrete foreign and potentially toxic substances, rather than being directly involved in the activation of fluid secretion through activation of ionic electrogenic pumps.

The question arose as to which of these corresponded to which chemical. Phlorizin was thus hydrolysed to phloretin, the aglycone (as described in Chapter 2, 2.3.6.) and these two compounds were run as standards against the secreted fluid. The phenols were detected by spraying with Folin-Ciocalteu reagent and 20% aqueous sodium carbonate (see 2.3.6.) and showed up as blue spots. The Rf of phlorizin was 0.3, and that of phloretin 0.5. Both spots were observed in the secreted fluid showing the presence of both compounds. This corresponded with the above profile in that both compounds were present however, the intensity of the spot at Rf 0.5 was not more intense than that at Rf 0.3 and thus did not indicate an accumulation as was shown by the radioisotope T.L.C. A quantitative analysis of the staining sensitivity was done by spotting different amounts of phloretin. This showed that a difference of intensity could only be detected between amounts as high as 1.00 and 0.25 µg spots. Amounts encountered in the secreted fluid would be in the region of 0.0008 µg/µl (1.93 mM/l). Pooling of the secreted fluid provides amounts in the region of 5 µl and thus detection of a 0.004 µg spot would be difficult with this method.

6. TRANSPORT OF GLUCOSE & THE ROLE OF Na^+

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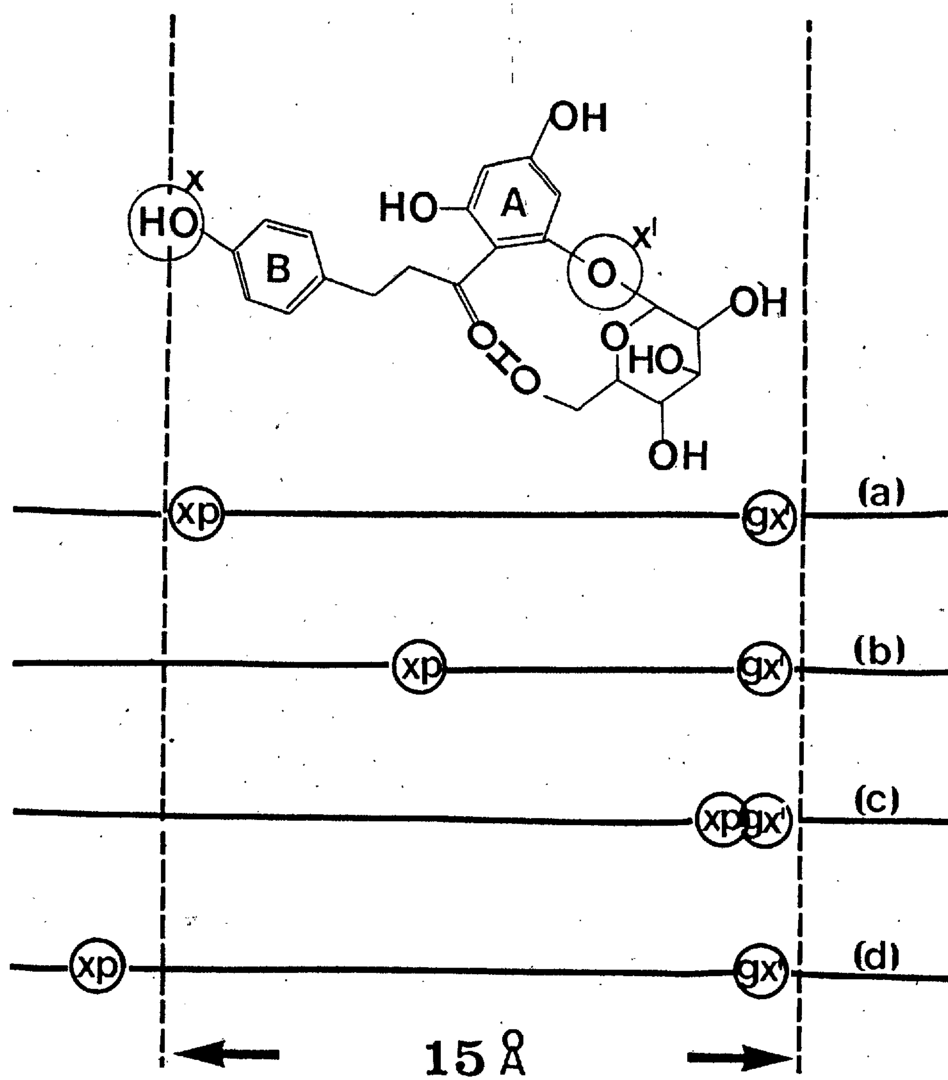
6.1. INTRODUCTION

The transport of glucose in vertebrate tissues has been investigated in some detail. On the other hand very little is known on glucose transport in invertebrate tissues. Transport studies have been facilitated by the findings that glucose is inhibited by the plant glycoside phlorizin. It has been generally accepted that phlorizin competes with glucose for a common binding site on a component of the brush border membranes of the renal tubule (Diedrich, 1966, 1968) responsible for the reabsorption of glucose, and the intestinal mucosa responsible for the absorption of glucose and galactose (Alvarado & Crane, 1962; Kinter & Wilson, 1965; Alvarado, 1967; Diedrich & Stringham, 1970 a,b; Bode *et al.*, 1972). Phlorizin (fig. 6.1.) is a phenolic β-D-glucoside which can be hydrolyzed to phloretin and glucose by a phlorizin hydrolase. This enzyme, which has been isolated in the intestinal mucosa of hamster and rat but was absent in the renal brush border (Diedrich, 1972), splits phlorizin β-glucosidic bonds.

A number of phlorizin-like glycosides and phloretin have been tested for their ability to inhibit glucose transport by several workers (Diedrich, 1966; Diedrich

Fig. 6.1. Model proposed to explain the possible interactions of the phlorizin molecule on the cell surface. (After Alvarado, 1967). For explanations of hypothetical cases (a) - (d) see text.



OH
HO
A
HO
x
B
x'
O
OH
O
HO
O
O
OH
xp
gx'
(a)
xp
gx'
(b)
xp
gx'
(c)
xp
gx'
(d)
15 Å

& Stringham, 1970a,b; Batt & Schachter, 1971; Bode *et al*, 1972). As a result of these investigations a topography of the active centres of the glucose receptor has been proposed (Diedrich & Stringham, 1970b). The receptor possesses two coplanar phenolic rings with the optical distance between the terminal hydroxyl O atoms being 12-14 Å.

The inhibitory kinetics of glucose transport indicate a competitive inhibition of phlorizin but a non-competitive inhibition of the aglycone phloretin. Diedrich & Stringham (1970b) suggest that the interaction of phlorizin and the glucose-receptor site is primarily due to the glucose moiety. The attached phlorizin could in turn cause reciprocal conformational changes which aid the remainder of the phlorizin molecule in the attachement to an aglycone receptor. This double binding causes a higher affinity of phlorizin to the sugar-carrier of 3-4 times higher than that of glucose. Thus, phlorizin is visualized as binding simultaneously to two different but closely associated receptors; a glucose or sugar and a phenol binding site (Alvarado, 1967).

Binding studies of phlorizin (Bode *et al*., 1972) and glucose (Chesney et al., 1973) indicated the presence

of two distinct receptor sites in isolated brush border membranes of rat kidney. One system has a high affinity to glucose, low affinity to phlorizin and another a high affinity to phlorizin and low affinity to glucose.

The glucose moiety of phlorizin is not involved in the inhibition of the glucose passage in the red blood cells (LeFevre, 1959; Crane, 1960) and the guinea pig intestine (Sahagian, 1965) as inhibition is as effective or more effective with phloretin. LeFevre also showed inhibition by several artificial estrogens which resemble phloretin. Here too, it was found that the structure of the phenol group was important for inhibition but the lack of structural relationship to sugars posed certain questions. LeFevre suggested that certain groups on the red cell membrane are capable of reversibly binding to phenolic groups which are also involved in the sugar transport system. Alvarado (1967) explained the differences among tissues in their response to phlorizin and phloretin to be due to differences in the spatial relationships between the sugar site and the phenol site (fig. 6.1. (a), (b), (c), (d)). This figure depicts a model proposed by Alvarado of the interactions of the phlorizin molecule on the cell surface. Two separate regions of the membrane

show the glucose binding site (g) and the phenol binding site (p) separated by a fixed distance (15 Å) which corresponds to the separation of the glucose and phloretin moieties of the phlorizin molecule. The main points of interaction are suggested to involve the -OH at C-2 of glucose and the oxygen at the B ring of phloretin. The possibilities of (b), (c), and (d) show differences in spacing between these binding sites which may occur in other cells or organisms. As the distance gets closer together kinetically phloretin would appear as a fully competitive inhibitor of glucose transport (b) & (c). This could be the case with the guinea pig intestine and the red blood cells. In the hypothetical case (d) where the distance between the sites g & p are above a certain critical limit, no effect of phloretin on glucose transport should occur.

Much evidence exists that the transport of glucose is coupled to Na^+ transport (Chez _et al_., 1967; Bihler, 1968; Schultz & Curran, 1970; Dettmer _et al_., 1972; Schafer, 1972; Kimmich & Randles, 1973). Ouabain inhibition of glucose transport was thus explained by an indirect effect on glucose transport by affecting intracellular Na^+ concentrations due to its inhibition of Na-K-ATPase.

Phlorizin has also been shown to affect renal excretion of glucose in crustaceans (Binns, 1969) and molluscs (Potts, 1967). Knowles (1975a) working on blowfly isolated tubules showed that phlorizin increases both the rate of fluid secretion by the tubules and the secretion of glucose. It seemed that the overall effect of phlorizin was to increase the permeability of the tubules to glucose resulting in an increase in the excretion of glucose. However, if an increase in the permeability constant is expected the rate of fluid secretion would be expected to decrease. This is not the case with phlorizin as it increases fluid rate (see Chapter 3). This point has led Knowles to argue that phlorizin primarily reduces the glucose reabsorption rather than increasing the glucose permeability constant. Reabsorption is also apparent as the activity ratios of D-glucose are lower than those of other sugars of equivalent molecular weight (e.g. sorbose). Knowles found significant differences between the reabsorption of glucose and sorbose but failed to show any effect on glucose reabsorption by phlorizin directly.

This chapter describes the investigation made on the transport of glucose and other sugars by the tubules of *Locusta*, the effect of phlorizin and other active chemicals on these transporting processes whereby more direct evidence of phlorizin's inhibition on glucose reabsorption is given and the possible interaction of Na^+.

6.2. RESULTS

6.2.1. Permeability of the tubules to sugars:

6.2.1.1. Effect of phlorizin and phloretin on glucose transport.

The presence of phlorizin at a concentration of 1mM/l (10^{-3}M) was tested on the transport of glucose by the tubules. After normal secretion was established the bathing medium of the test tubules was changed to a medium containing glucose (^{14}C) at a concentration of 50 mM/l plus phlorizin (1 mM/l). Similar controls were set up containing the same concentration of glucose ^{14}C without the addition of phlorizin. Samples of secreted fluid were collected at 20 min intervals and were analyzed for radioactivity. The results of these experiments are shown in fig. 6.2.1.1.1. It can be seen that the normal transport of glucose reaches a lower level than when phlorizin is present, activity ratios becoming increasingly higher in the presence of phlorizin. These results are comparable to those found by Knowles (1975a) on the tubules of *Calliphora vomitoria*.

Similar experiments were set up with different concentrations of glucose and the effect of phlorizin is seen to be more pronounced reaching significantly higher levels at higher concentrations of glucose (fig. 6.2.1.1.2. and 6.2.1.1.3.). In all these

Fig. 6.2.1.1.1. The effect of 1 mM/l phlorizin ◆ on the transport of 50 mM/l glucose. Vertical lines show extent of standard error and subscript figures indicate the number of observations, ● is the glucose control.

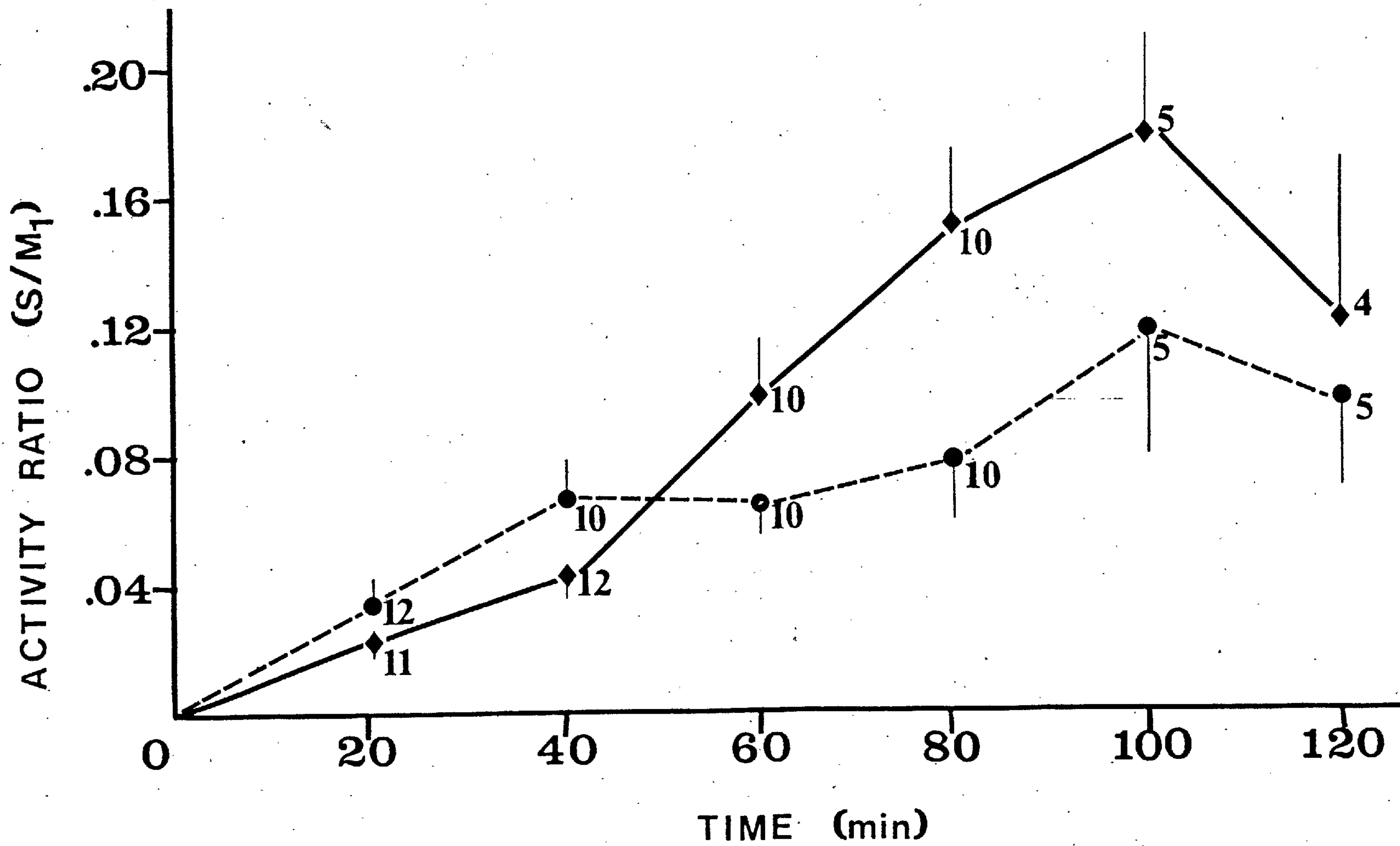

ACTIVITY RATIO (S/M1)
.20
.16
.12
.08
.04
0
20
40
60
80
100
120
TIME (min)
12
11
10
12
10
10
10
10
5
5
4
5

Fig. 6.2.1.1.2. The effect of 1 mM/l phlorizin ◆ on the activity ratios of different concentrations of glucose. Vertical lines show extent of standard error and subscript figures indicate the number of observations, ● is the glucose control.

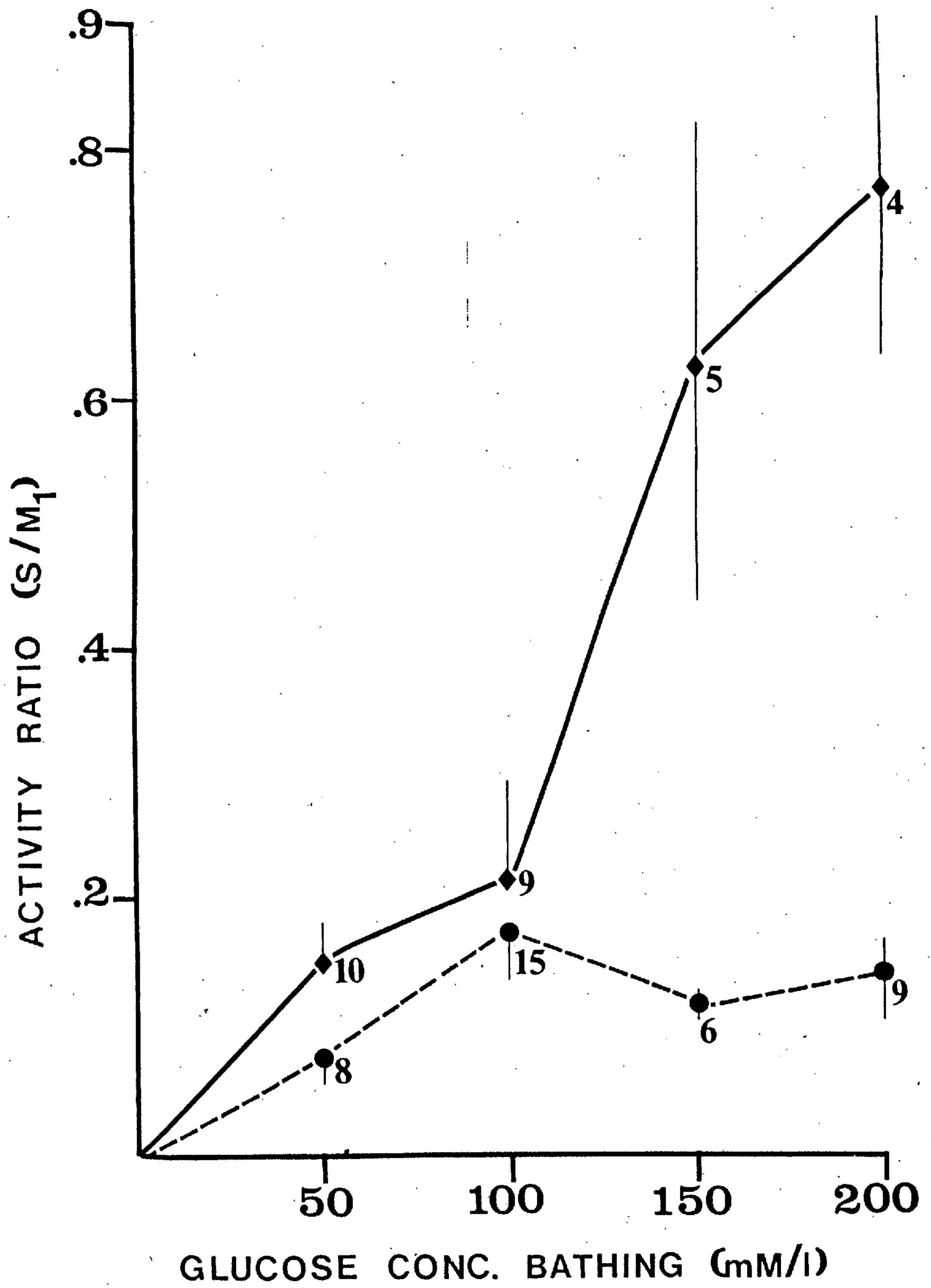
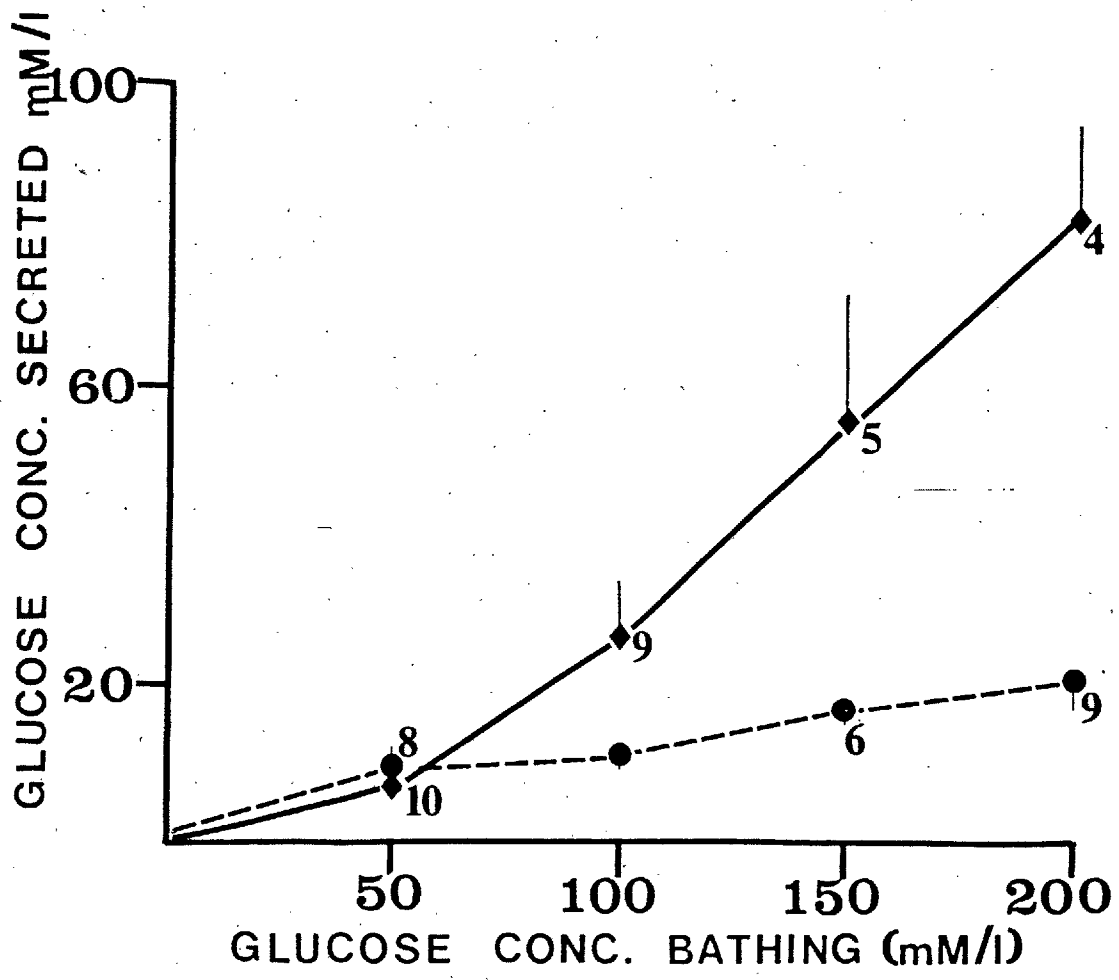

.9
.8
.6
.4
.2
ACTIVITY RATIO (S/M1)
4
5
9
10
15
6
9
8
50
100
150
200
GLUCOSE CONC. BATHING (mM/l)

Fig. 6.2.1.1.3. The effect of 1 mM/l phlorizin ◆ on the glucose concentration secreted by the tubules at different concentrations of glucose in the bathing medium. Vertical lines show extent of standard error and subscript figures indicate the number of observations, ● is the glucose control.


GLUCOSE CONC. SECRETED mM/l
100
60
20
50
100
150
200
GLUCOSE CONC. BATHING (mM/l)
8
10
9
5
4
6
9

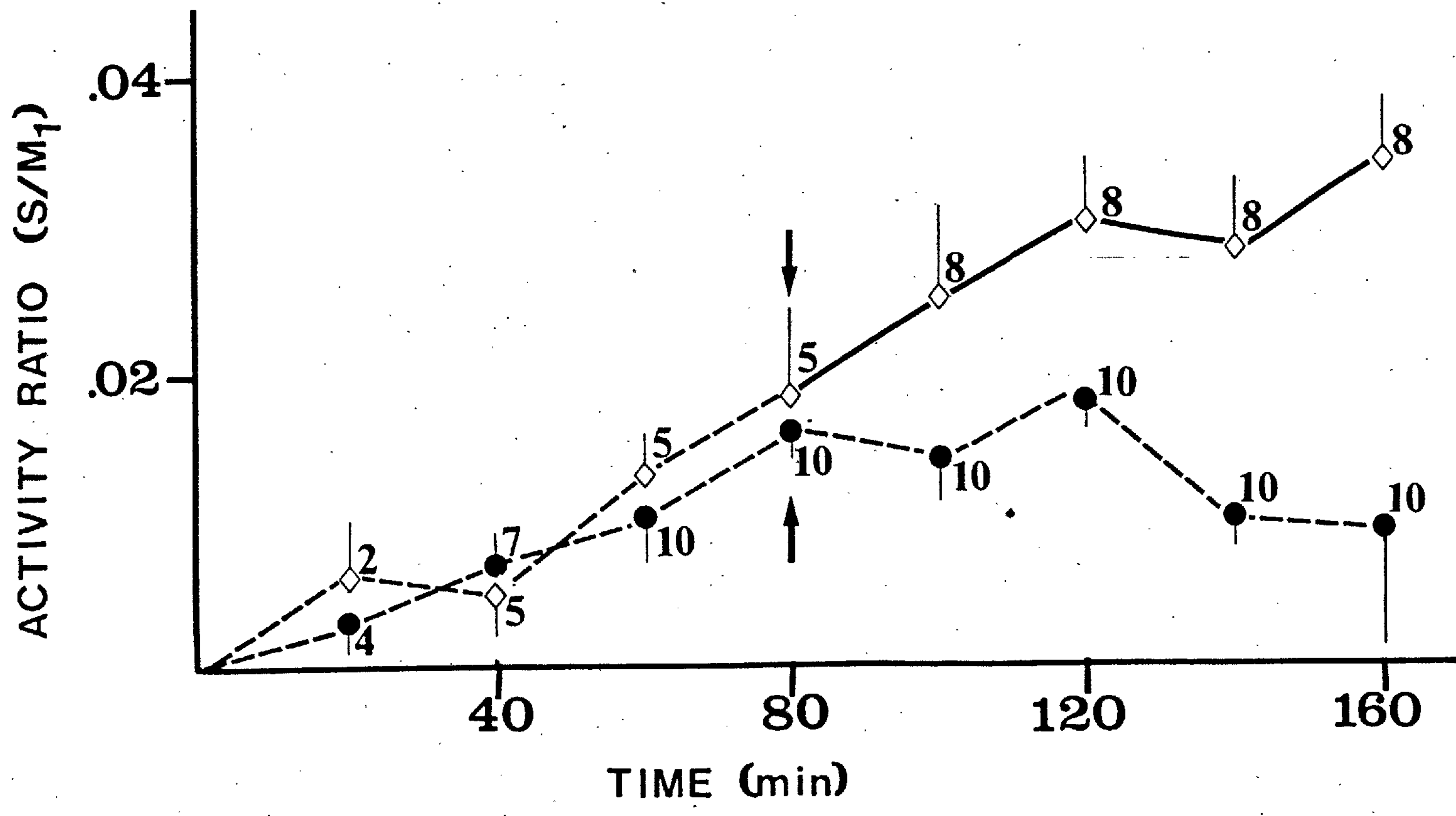
experiments the activity ratios achieved do not exceed 1 indicating a transport system which follows the concentration gradient.

In the light of the results obtained in Chapter 5 on phlorizin permeability, the presence of phloretin, the aglycone, in the secreted fluid made it necessary to test the effect of phloretin on glucose transport. Using the same source of phloretin (hydolysed phlorizin, see Chapter 2) its effect on the glucose permeability was tested after normal secretion was obtained. Due to its lower solubility in water a concentration of 10^{-4}M (0.1 mM/1) was used. An identical control was set up containing the same glucose ^{14}C concentration (10 mM/1) without the addition of phloretin. The results of these experiments are depicted in fig. 6.2.1.1.4. Phloretin, even at the lower concentration, produces a similar effect of increasing glucose transport (278% increase).

6.2.1.2. Effect of phlorizin on the transport of other sugars.

The presence of phlorizin at the same concentration was also tested on the transport of sorbose, fructose, N-acetyl glucosamine, trehalose and maltose (all ^{14}C). Similar experiments were thus set up containing 50 mM/1 of the above sugars in the presence and absence of

Fig. 6.2.1.1.4. The effect of 0.1 mM/l phloretin ◇ on the transport of 10 mM/l glucose. Vertical lines show extent of standard error and subscript figures indicate the number of observations. Arrows indicate bathing medium changing time, ● is the glucose control.



ACTIVITY RATIO (S/M1)
.04
.02
TIME (min)
40
80
120
160
2
4
7
5
5
10
5
10
8
10
8
10
8
10
8
10

phlorizin. The results of these experiments are shown in fig. 6.2.1.2.1. and 6.2.1.2.2. In the absence of phlorizin the normal permeability of the tubules to these sugars was obtained. These are shown in fig. 6.2.1.2.1. This figure shows that as the molecular weight of the sugar increases the activity ratio and therefore the permeability of these sugars decreases. In addition, when these values are compared to the activity ratios of glucose and trehalose it can be seen that these two sugars show different permeability characteristics. Clearly some mechanism is involved in reducing the permeability of the tubules to both glucose and trehalose. On addition of phlorizin (fig. 6.2.1.2.2.) the activity ratios of both glucose and trehalose increases and approaches the curve of sugar passive leak. In contrast, phlorizin reduces the activity ratios of sorbose, fructose, N-acetyl glucosamine and maltose.

6.2.1.3. Effect of other sugars on glucose transport.

The activity ratios of glucose transport were determined in the presence of sorbose, N-acetyl glucosamine, fructose, trehalose, maltose and mannose. The results (fig. 6.2.1.3.1.) show only a significant increase in glucose permeability in

Fig. 6.2.1.2.1. Permeability of the tubules to various sugars showing the reduced permeability of the tubules to glucose and trehalose.

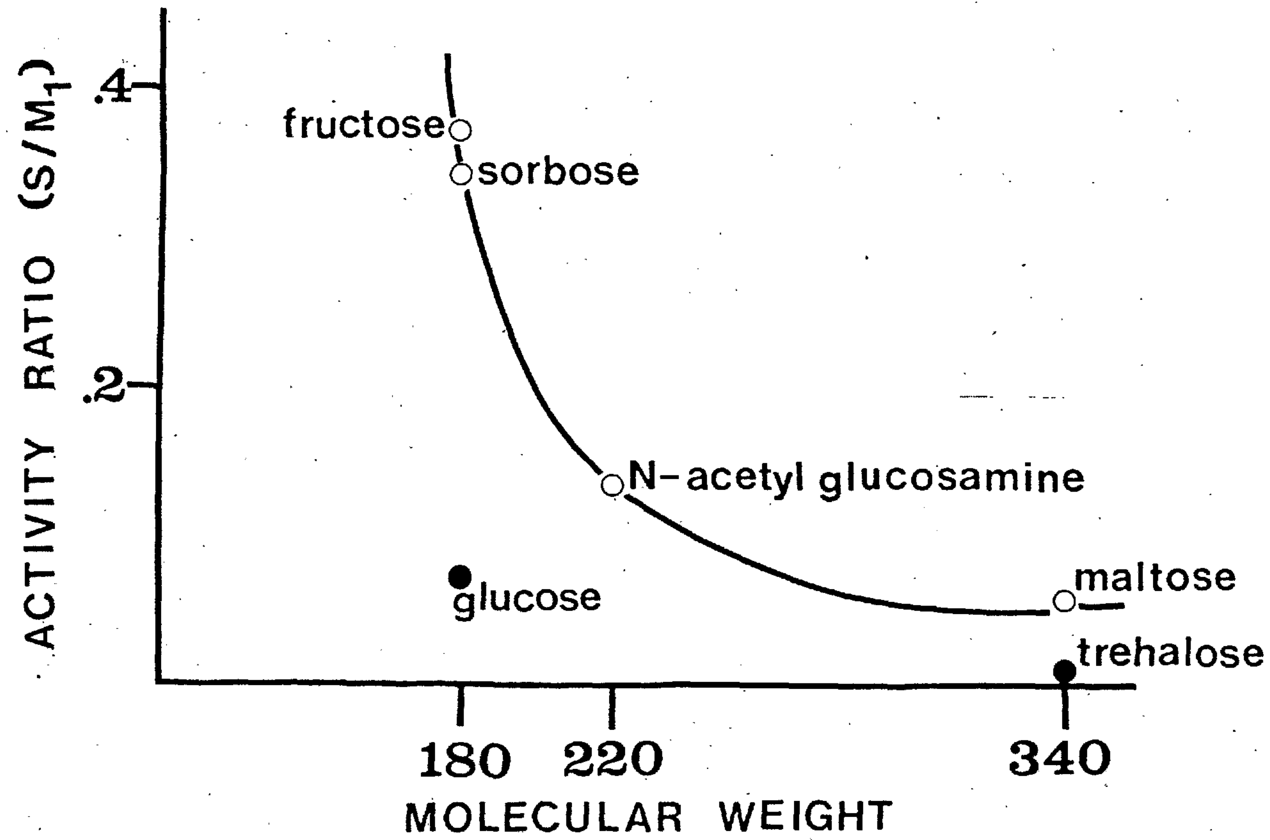
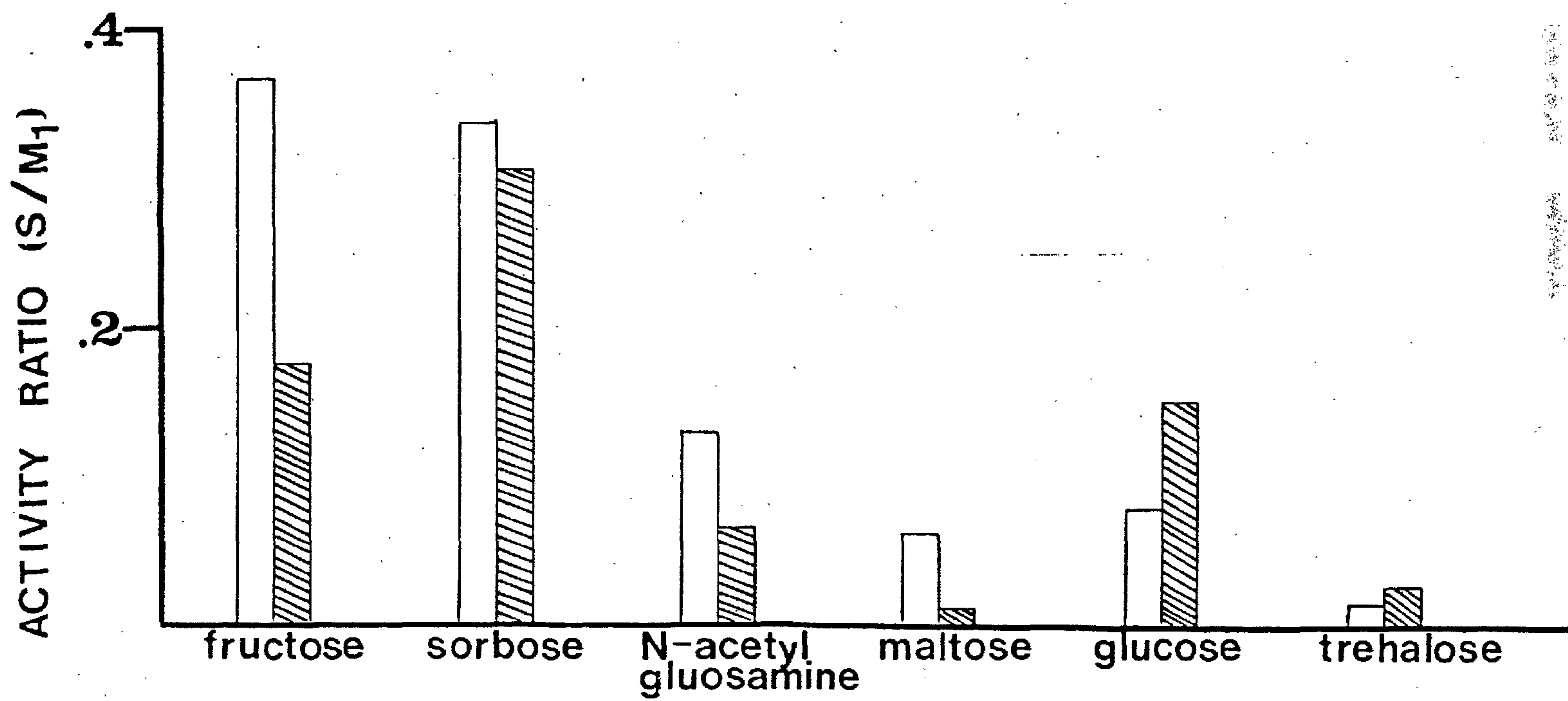
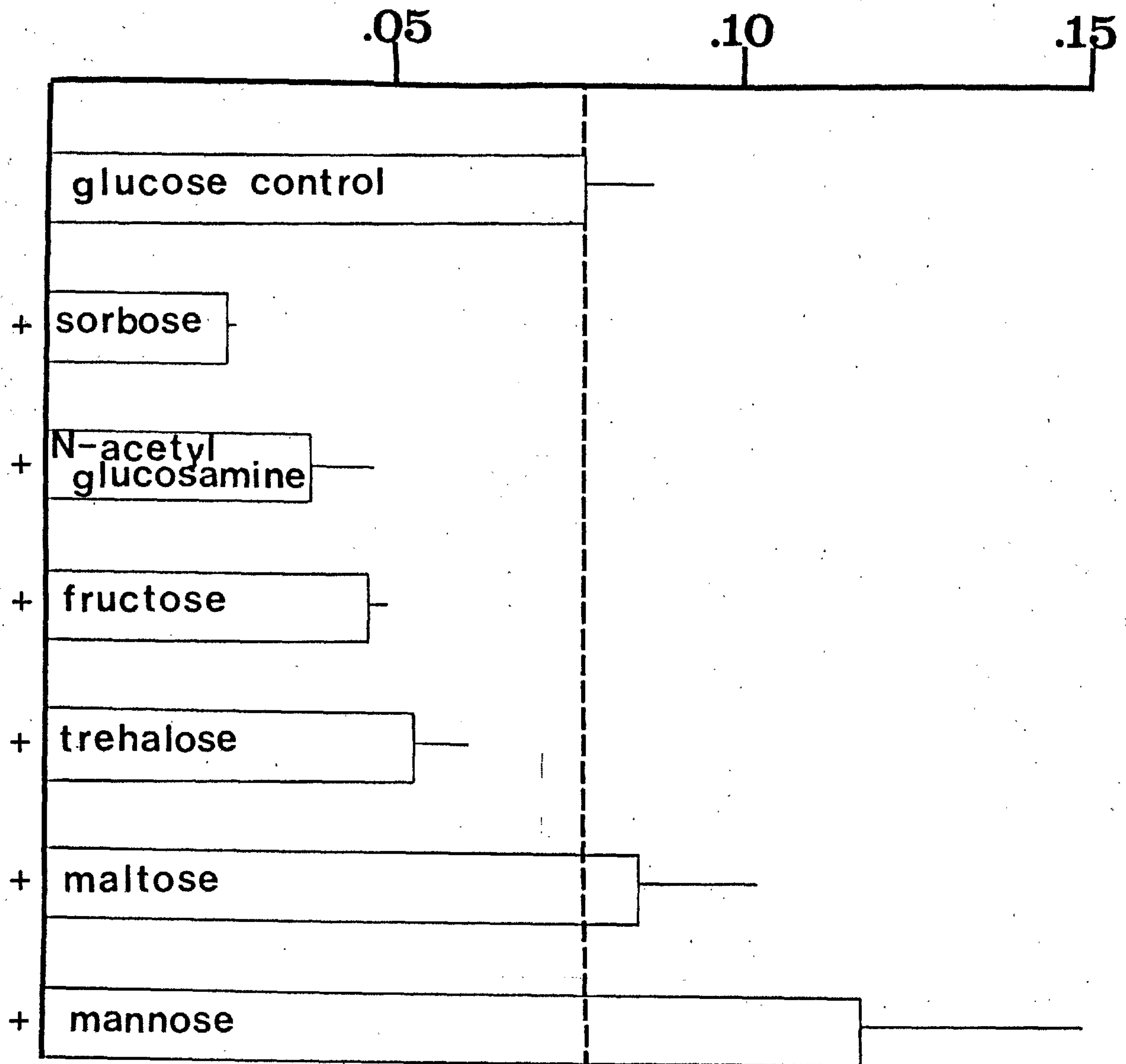

ACTIVITY RATIO (S/M1)
.4
.2
fructose
sorbose
N-acetyl glucosamine
glucose
maltose
trehalose
180
220
340
MOLECULAR WEIGHT

Fig. 6.2.1.2.2. The effect of phlorizin on the permeability of the tubules to various sugars. Open columns, phlorizin absent; crosshatched columns, phlorizin present.



.4
.2
ACTIVITY RATIO (S/M1)
fructose
sorbose
N-acetyl
gluosamine
maltose
glucose
trehalose

Fig. 6.2.1.3.1. Effect of various sugars on the transport of glucose. Vertical lines show extent of standard error and subscript figures indicate the number of observations. The dashed line indicates the normal transport of glucose in the absence of any other sugars.



ACTIVITY RATIO (S/M1) OF 14C-GLUCOSE
.05
.10
.15
glucose control
+
sorbose
+
N-acetyl glucosamine
+
fructose
+
trehalose
+
maltose
+
mannose

the presence of mannose, which indicates a possible competition by mannose for the reabsorption of glucose. When the structure of mannose is compared to glucose (fig. 6.2.1.3.2.) the similarity of the two molecules may be the explanation of the above results. The presence of the other sugars on the other hand show decreases in the glucose transport into the lumen indicating a competition for the passive leak through the membrane pores rather than the reabsorptive capacity of the tubule membrane (fig. 6.2.1.3.3.).

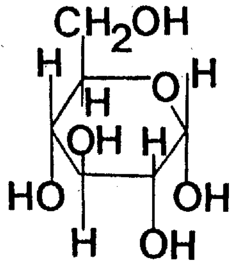
6.2.1.4. Effect of insect peptide hormones on glucose transport.

The effect of adding adipokinetic hormone, hyperglycaemic hormone and storage gland extracts of locust corpora cardiaca is shown in figs. 6.2.1.4.1. and 6.2.1.4.2. It can be seen that no significant difference occurs on the addition of any of these insect hormones on the glucose activity ratios.

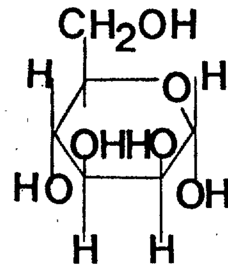
6.2.1.5. Reabsorption of sugars from the tubules.

The reduced permeability of glucose has been suggested to be due to the reabsorptive capacity of the tubule, thus the effective concentration appearing in the

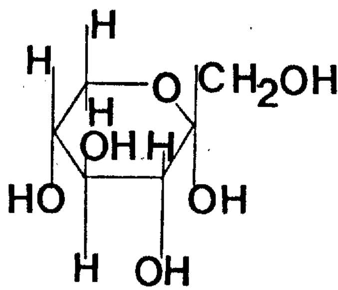
Fig. 6.2.1.3.2. Structures of the various sugars used.



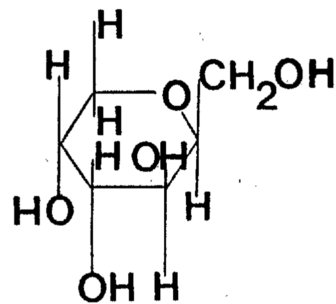
D-glucose



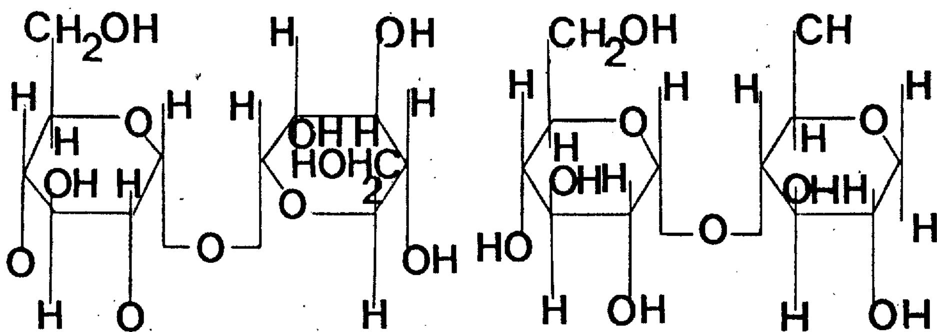
D-mannose



L- sorbose

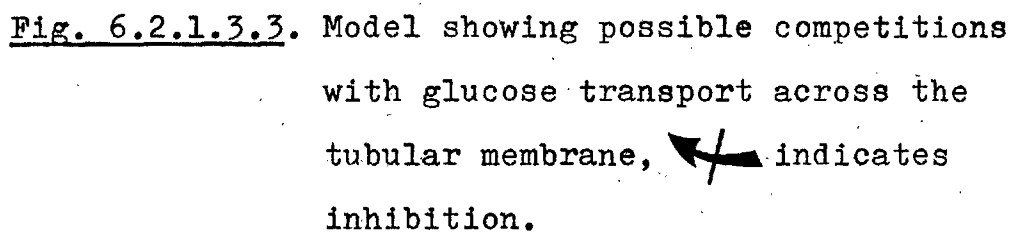


D- fructose



trehalose

maltose



<u>Fig. 6.2.1.3.3</u>. Model showing possible competitions with glucose transport across the tubular membrane, indicates inhibition.

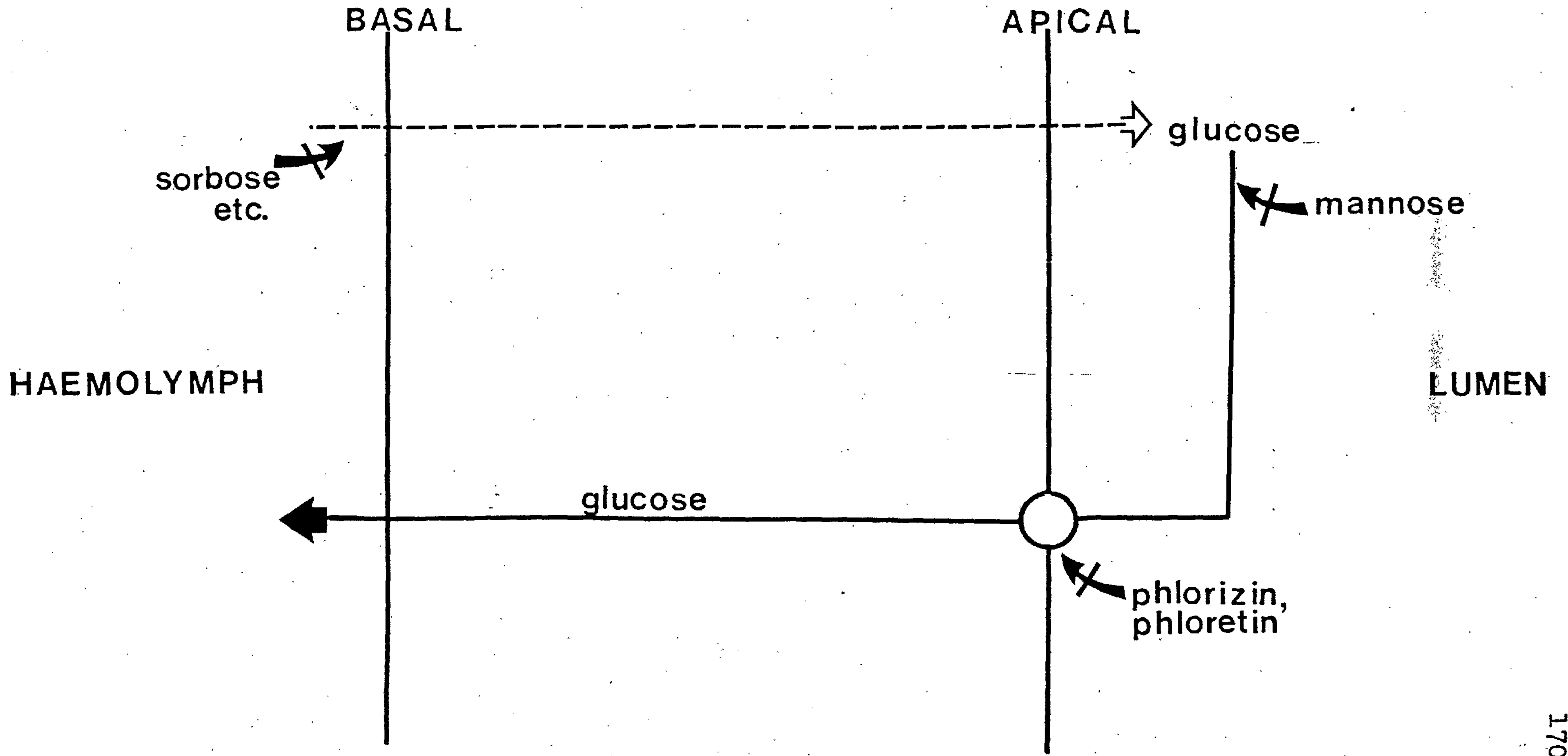

BASAL
APICAL
glucose
sorbose
etc.
mannose
HAEMOLYMPH
LUMEN
glucose
phlorizin,
phloretin

Fig. 6.2.1.4.1. Glucose transport in the presence of diuretic hormone ■ , and AKH □, ● is the glucose control. Vertical lines show extent of standard error and subscript figures indicate the number of observations.

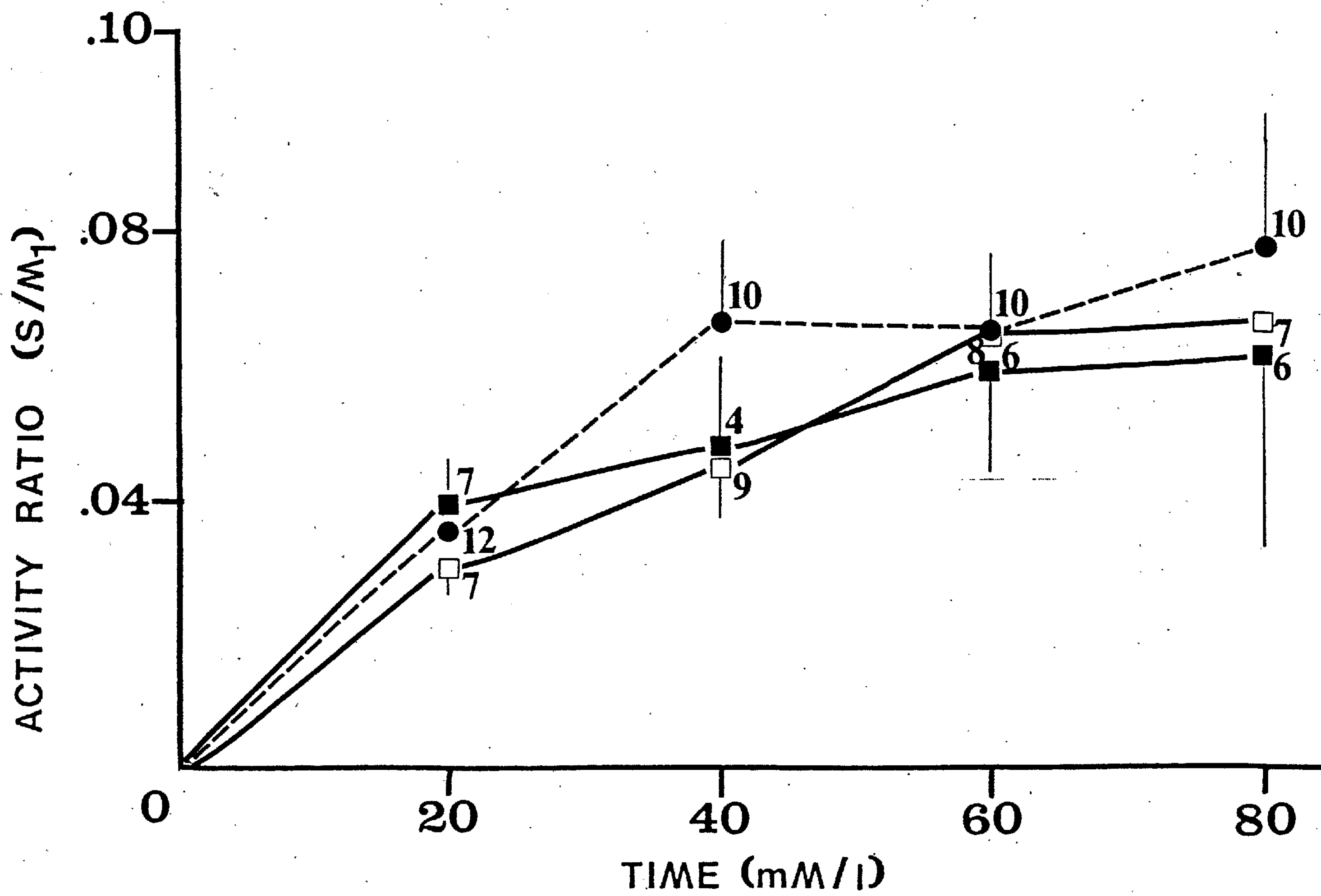
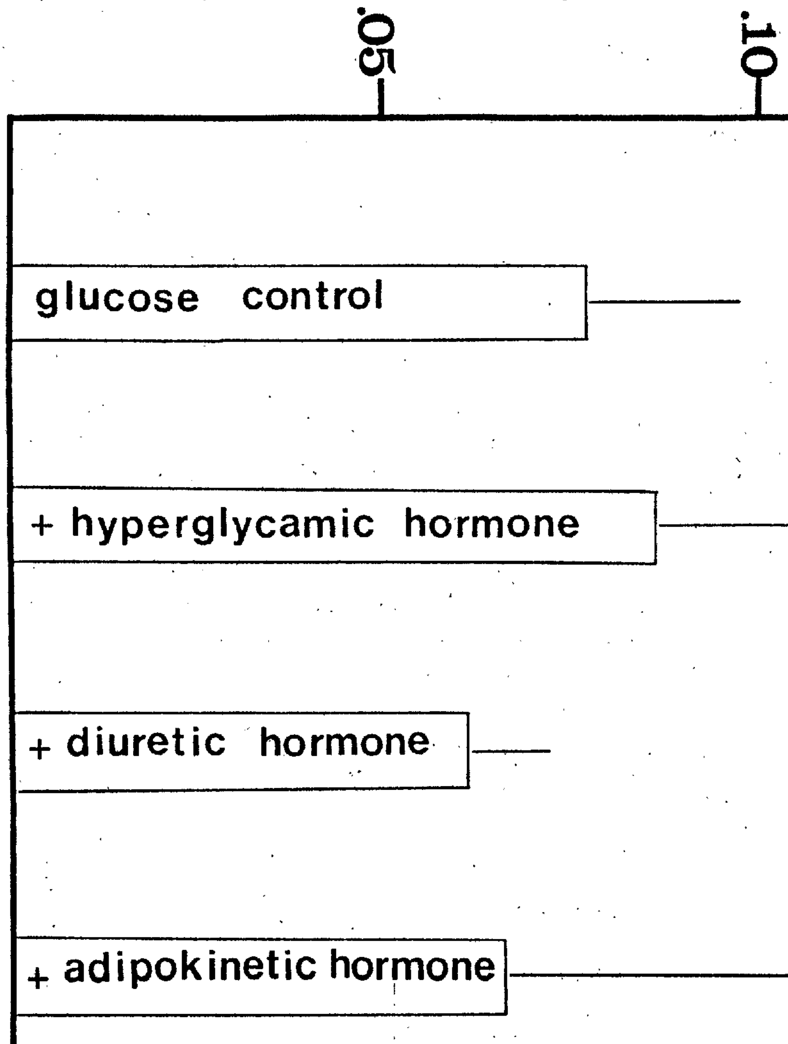

.10
.08
.04
ACTIVITY RATIO (S/M1)
0
20
40
60
80
TIME (mM/l)

Fig. 6.2.1.4.2. The effect of various insect peptide hormones on glucose transport.



ACTIVITY RATIO (S/M1)
.05
.10
glucose control
+ hyperglycamic hormone
+ diuretic hormone
+ adipokinetic hormone

lumen is lower than expected. This was tested by passing tubules through a second bathing medium droplet (M_2) (see Chapter 2) and monitoring the glucose concentration in this medium. This indicated any backflow of glucose, sorbose and trehalose. The presence of phlorizin was also tested while monitoring the sugar reabsorption. The results of these experiments are shown in Table 6.2.1.5. and fig. 6.2.1.5. Glucose reabsorption ratios when compared with sorbose reabsorption ratios is significantly higher. When phlorizin is present this reabsorption ratio is diminished to levels comparable with the original sorbose levels and with those obtained for sorbose in the presence of phlorizin. Trehalose reabsorption however, is not apparent on the present data.

6.2.2. The role of Na^+.

6.2.2.1. Effect of ouabain and ethacrynic acid on glucose transport.

The possibility that Na^+ might play a role in glucose transport was tested by examining the effect of ouabain on the glucose permeability. A dramatic increase was found when ouabain alone (316%) and ouabain and phlorizin (783%) were present in the bathing medium (figs. 6.2.2.1.1. and 6.2.2.1.2.). When ethacrynic acid, a Na^+ pump inhibitor in vertebrate kidneys, was added however, no significant change in activity ratios was observed (fig. 6.2.2.1.3.).

TABLE 6.2.1.5.

The effect of phlorizin on the reabsorption ratios of trehalose, sorbose and glucose.

	REABSORPTION RATIO (M_2/M_1)	
	Control means ± S.E.	+Phlorizin means ± S.E.
D-glucose	1.24 ± 0.12*	0.74 ± 0.11**
L-sorbose	0.82 ± 0.02	0.79 ± 0.03
Trehalose	0.31 ± 0.01	0.33 ± 0.02

*Significantly different ($P < .001$) to D-glucose + & significantly different ($P < .02$) to L-sorbose

**Not significantly different ($P > .1$) to L-sorbose & L-sorbose +

Fig. 6.2.1.5. The effect of phlorizin on the reabsorption ratios of trehalose, sorbose and glucose showing a significant reabsorption of glucose which is significantly reduced in the presence of phlorizin (crosshatched columns). Vertical lines show extent of standard error.

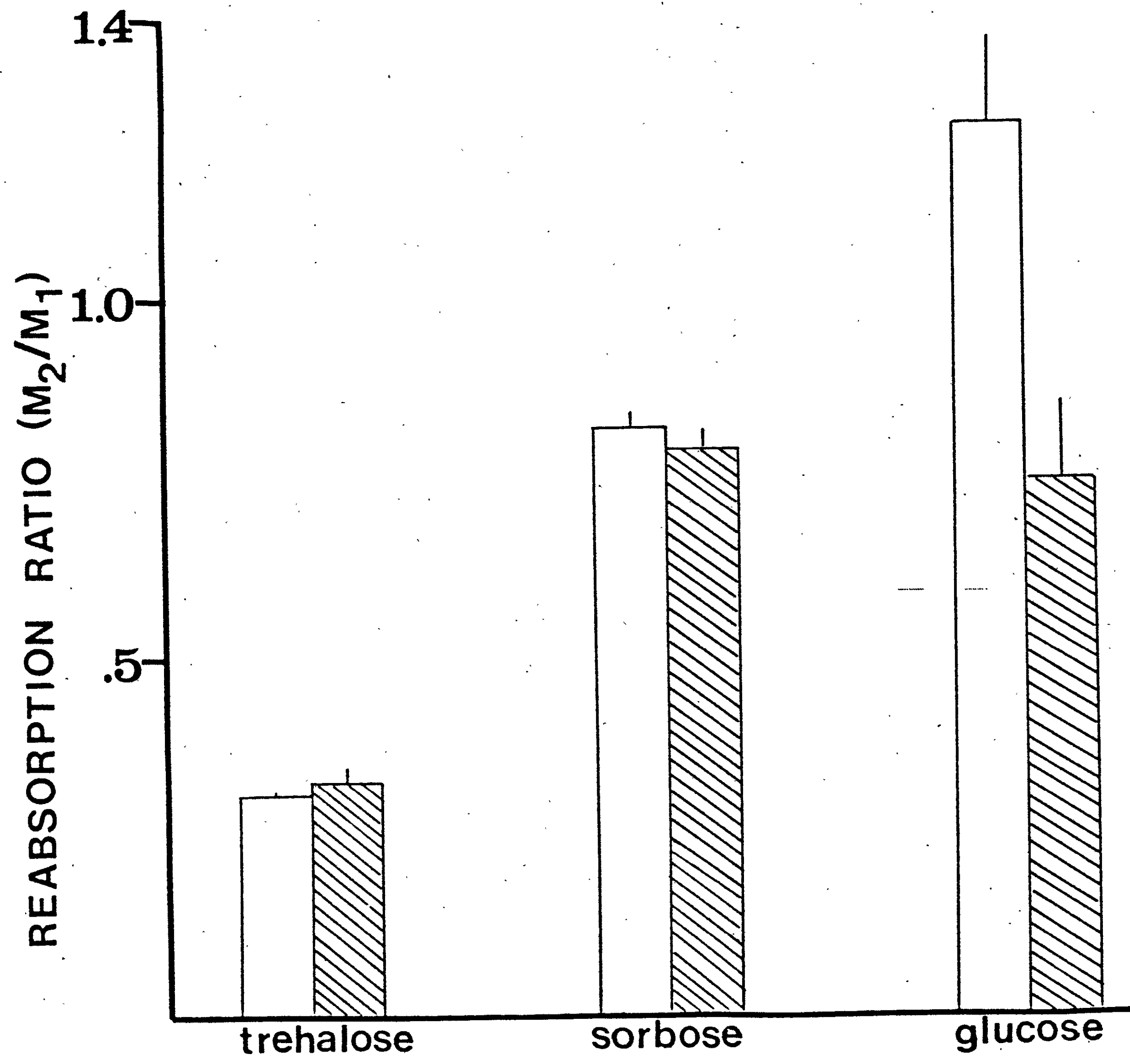

1.4
1.0
.5
REABSORPTION RATIO (M_2/M_1)
trehalose
sorbose
glucose

Fig. 6.2.2.1.1. The effect of ouabain and phlorizin on the transport of glucose, ● glucose control, ◆ (1 mM/l) phlorizin present, ▲ (1 mM/l) ouabain present, △ both phlorizin (1 mM/l) and ouabain (1 mM/l) present. Vertical lines show extent of standard error and subscript figures indicate the number of observations.

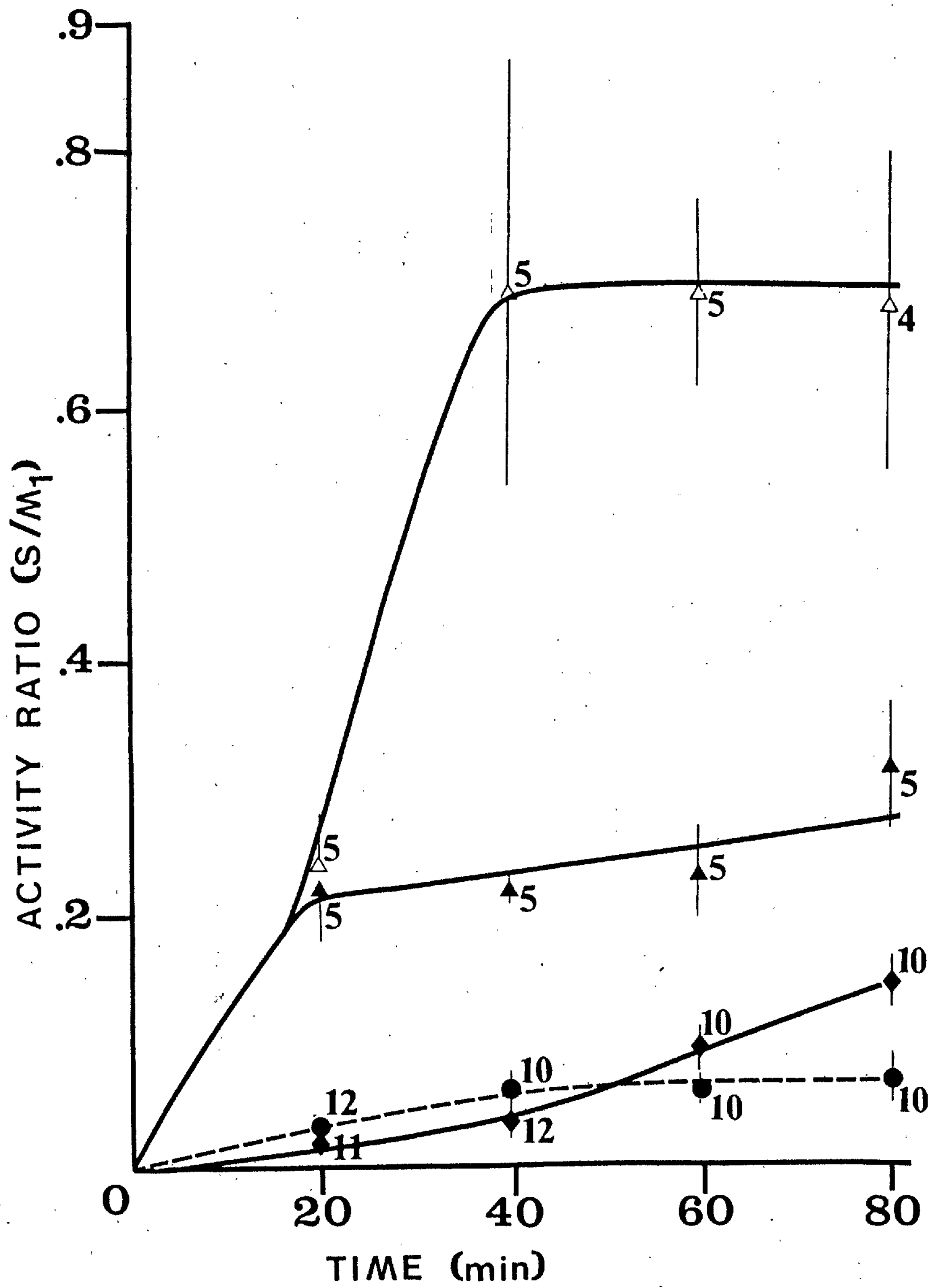

.9
.8
.6
.4
.2
0
20
40
60
80
TIME (min)
ACTIVITY RATIO (S/M1)
5
5
4
5
5
5
5
5
10
10
10
12
11
12
10
10

Fig. 6.2.2.1.2. Increases in glucose activity ratios in the presence of phlorizin and ouabain. The dashed line indicates normal levels of glucose transport. Vertical lines indicate extent of standard error & percentage figures indicate extent of increase in activity ratios.

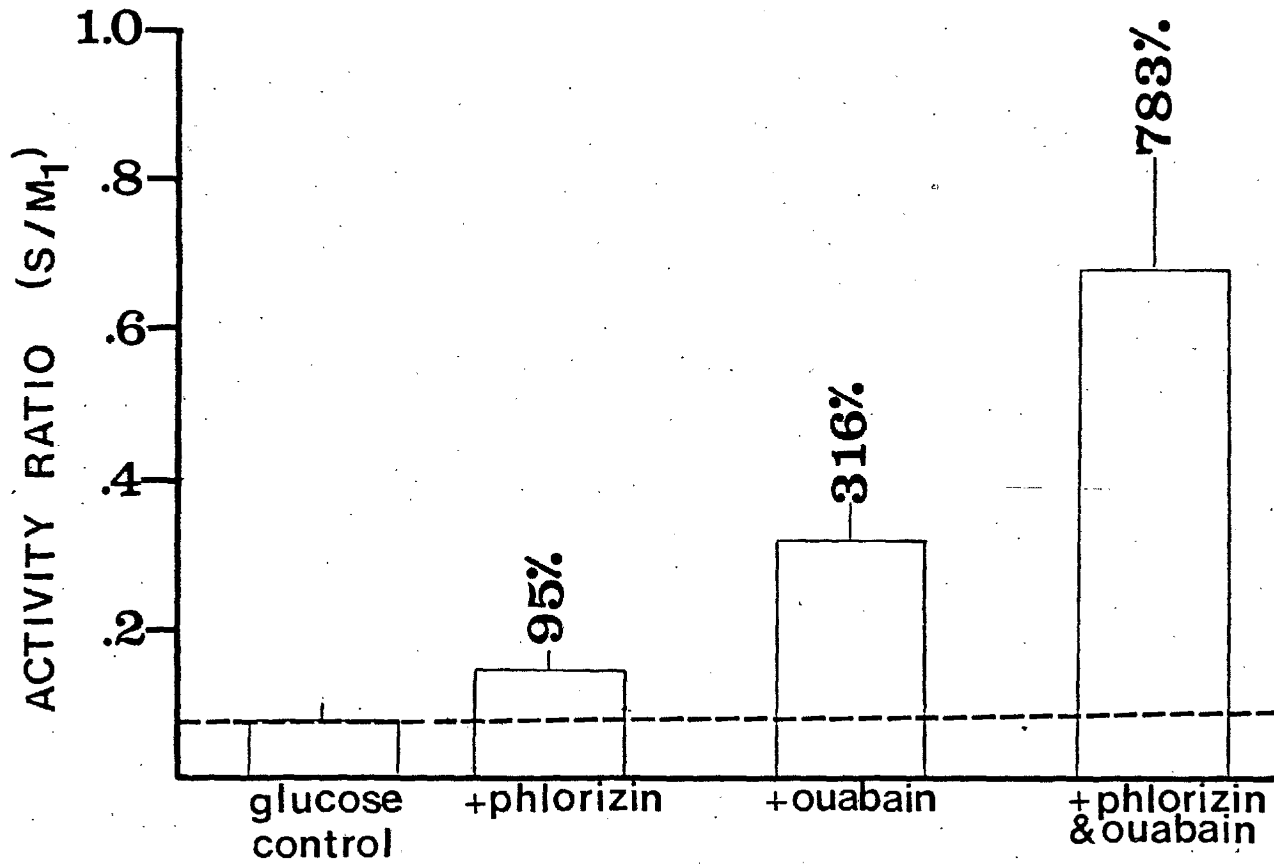
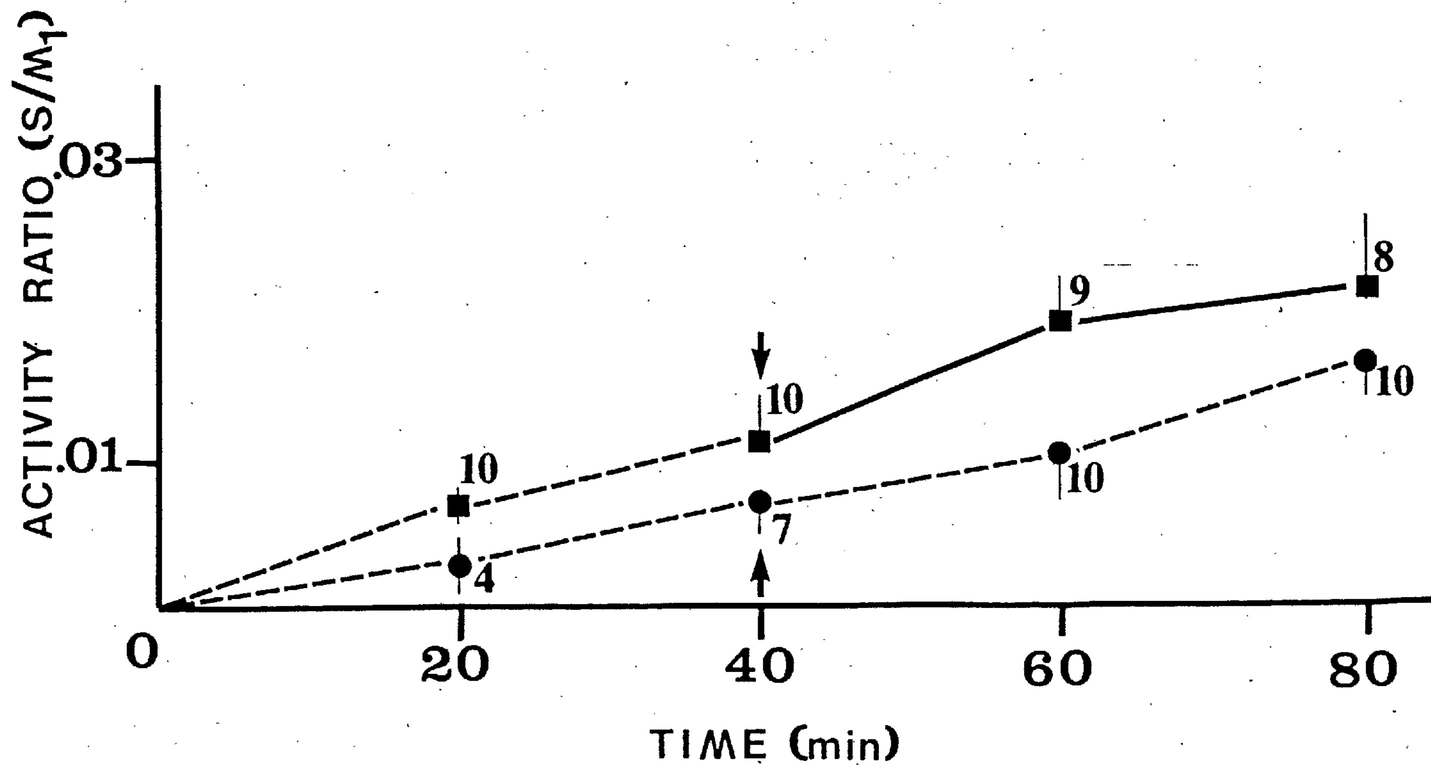

1.0
.8
.6
.4
.2
ACTIVITY RATIO (S/M1)
95%
316%
783%
glucose
control
+phlorizin
+ouabain
+phlorizin
&ouabain

Fig. 6.2.2.1.3. The effect of 1 mM/l ethacrynic acid on the activity ratio of 10 mM/l glucose, ● glucose control, ■ ethacrynic acid present. Vertical lines show extent of standard error and subscript figures indicate the number of observations.


ACTIVITY RATIO (S/M1)
.03
.01
0
20
40
60
80
TIME (min)
10
10
9
8
4
7
10
10

6.2.2.2. Effect of different Na^+ concentrations in the bathing medium.

In an attempt to elucidate the nature of Na^+ involvement in glucose transport the Na^+ concentrations in the bathing medium were altered. Changing the Na^+ concentrations in the bathing medium however, does not significantly change the activity ratio of glucose in the secreted fluid (Table 6.2.2.2.). This would tend to eliminate the involvement of the basal side of the tubule membrane in the reabsorption of glucose. Glucose passive leakage into the lumen is therefore sodium independent.

6.2.2.3. Factors affecting Na^+ transport.

The role of Na^+ in glucose transport was further investigated by investigations of Na^+ transport itself. The presence of phlorizin was tested on the transport of $^{22}Na^+$ by the tubules. After normal secretion was established the bathing medium of the test tubules was changed to a medium containing $^{22}Na^+$ at the Ringer concentration of 142 mM/l (normal Ringer) plus phlorizin (1 mM/l). Similar controls were set up containing the same concentration of $^{22}Na^+$ without the addition of phlorizin. Samples of the secreted fluid were collected at 20 min intervals and were analyzed for radioactivity. Similar experiments were set up using phloretin (0.1 mM/l) and ouabain (1 mM/l) in the bathing medium.

TABLE 6.2.2.2.

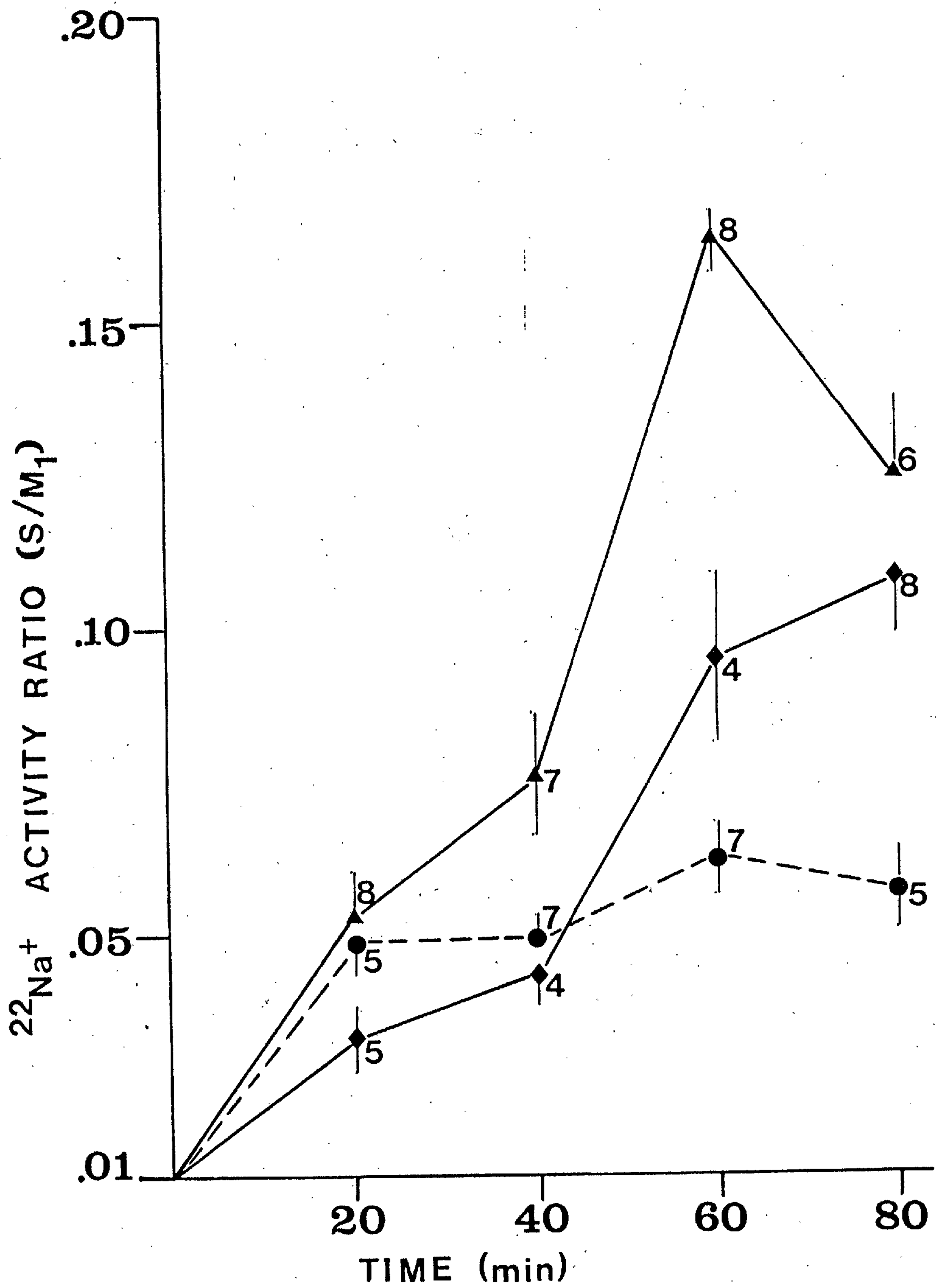
The effect of changing the Na^+ concentrations in the bathing medium on the glucose activity ratios.

$[Na^+]$ outside (mM/l)	ACTIVITY RATIOS* OF $GLUCOSE_{t=80}$
2	0.061 ± 0.008
25	0.059 ± 0.006
50	0.068 ± 0.018
142	0.077 ± 0.020
200	0.089 ± 0.008

* no significant differences in activity ratios.

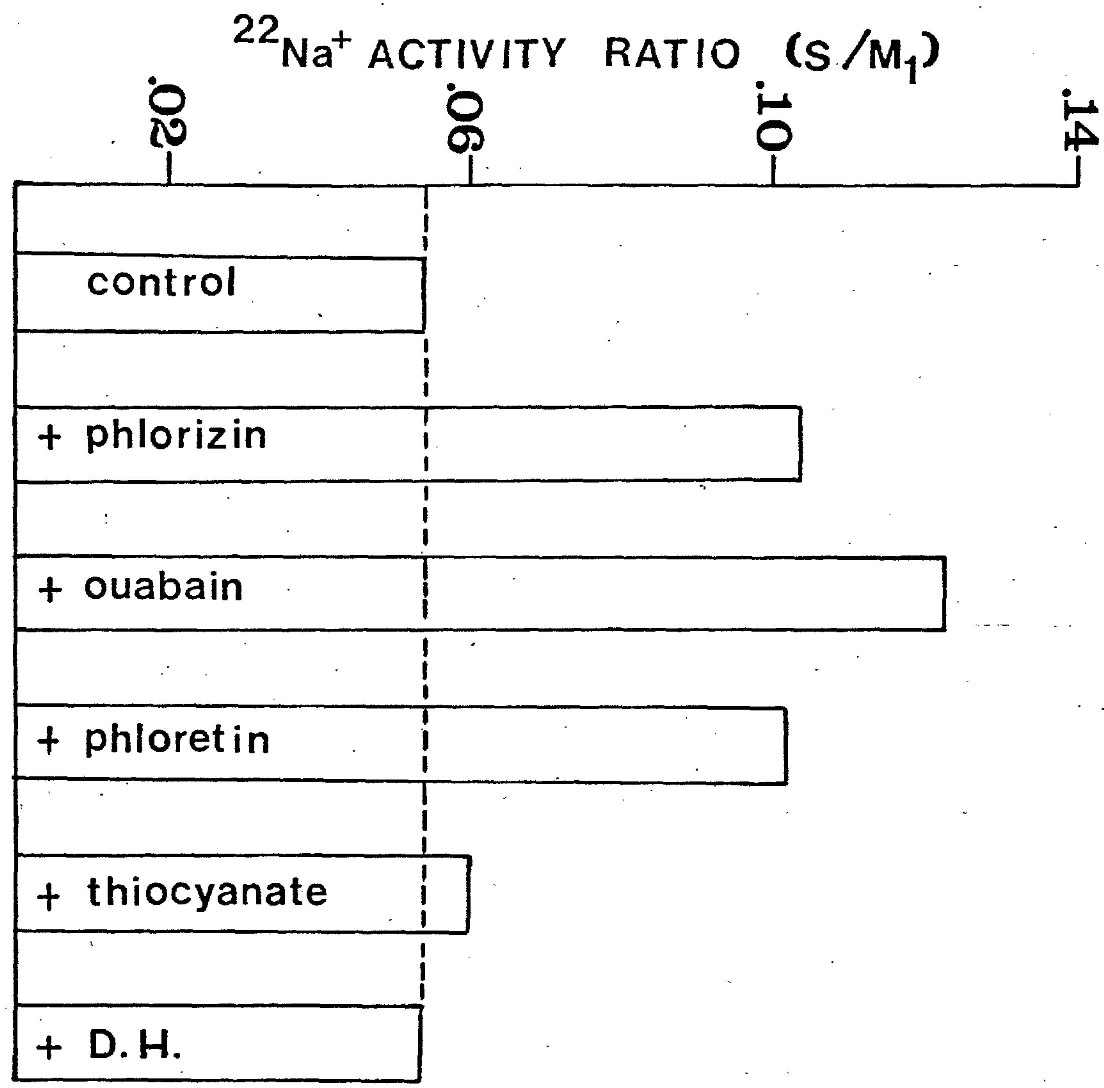
The results of these experiments are shown in figs. 6.2.2.3.1. and 6.2.2.3.2. It can be seen that all three compounds: phlorizin, phloretin and ouabain produce a significant increase in the activity ratios of Na^+. Further experiments were set up with the addition of thiocyanate (a Na^+ dependent proton pump inhibitor of oxyntic cells) and diuretic hormone. The samples of secreted fluid were collected after 80 min of secretion and analyzed for radioactivity. No significant effect was observed with these compounds on the activity ratios of Na^+ (fig. 6.2.2.3.2.). On the other hand phlorizin produced an 87.7% increase, phloretin an 82.5% increase and ouabain a 119.3% increase. These increases can be compared to the increases produced by these compounds on ^{14}C-glucose activity ratios (fig. 6.2.2.3.3.).

Fig. 6.2.2.3.1. The effect of phlorizin ◆ and ouabain ▲ on the transport of Na^+, ● Na^+ control. Vertical lines show extent of standard error and subscript figures indicate the number of observations.



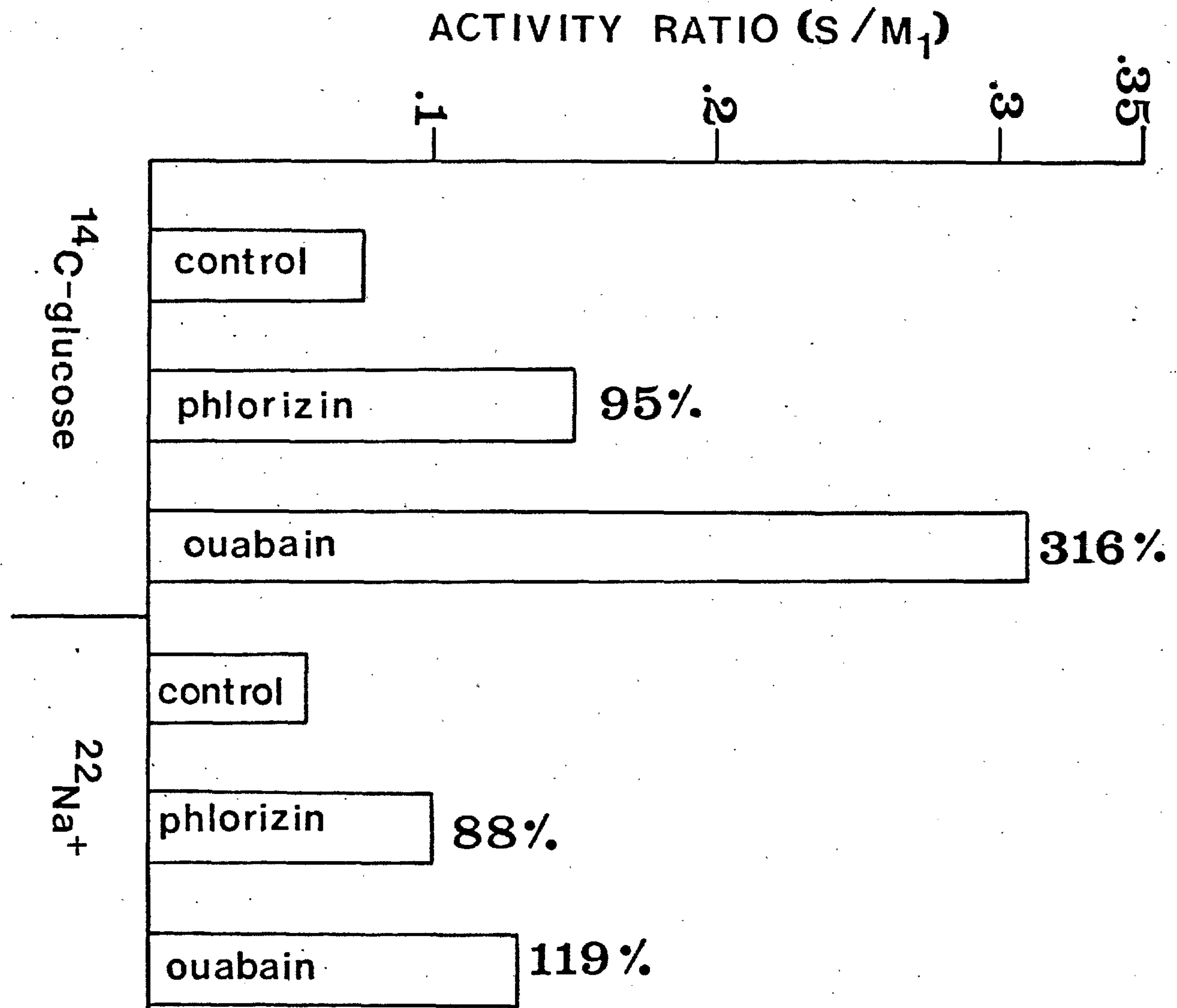
.20
.15
.10
.05
.01
$^{22}Na^+$ ACTIVITY RATIO (S/M_1)
20
40
60
80
TIME (min)
8
7
8
6
8
4
7
5
5
7
4
5

Fig. 6.2.2.3.2. Effects of phlorizin, ouabain, phloretin, thiocyanate and DH on the activity ratios of Na^+. The dashed line indicates the normal levels of Na^+ transport.



$^{22}Na^+$ ACTIVITY RATIO (S/M_1)
.02
.06
.10
.14
control
+ phlorizin
+ ouabain
+ phloretin
+ thiocyanate
+ D.H.

Fig. 6.2.2.3.3. A comparison of the increases in activity ratios of Na^+ and glucose in the presence of phlorizin and ouabain. Percentage figures indicate percentage increases in activity ratios.



ACTIVITY RATIO (S / M1)
.1
.2
.3
.35
14C-glucose
control
phlorizin
95%
ouabain
316%
22Na+
control
phlorizin
88%
ouabain
119%

6.3. DISCUSSION

The results in this Chapter have plainly shown a significant reabsorption of glucose by the Malpighian tubules of *Locusta*, which is comparable to those of *Calliphora vomitoria* (Knowles, 1975a). This is apparent indirectly by the amounts of glucose found in the lumen which do not correspond to the expected values for its size. This reabsorption is significantly altered by phlorizin, phloretin and ouabain and the values found in the lumen begin to increase in the presence of these compounds. Moreover, when both phlorizin and ouabain are present there is an enhanced increase, Dettmer *et al.* (1972) have shown that the effect of phlorizin is dependent on the Na^+: K^+ ratio and on the Na^+ and K^+ concentrations. The observed enhancement may therefore be attributed to changes in the Na^+ concentrations brought about by ouabain and thus increasing the inhibitory effect of phlorizin on reabsorption. The association of Na^+ in this reabsorption of glucose was indicated by the inhibitory effect of ouabain and by the similar effects on Na^+ activity ratios in the presence of phlorizin, phloretin and ouabain.

Phlorizin affects only one other sugar that was tested, trehalose. But, this effect is small and is not apparent in the measurements of M_2 bathing media monitoring

reabsorption back into the haemolymph (bathing medium). There is a possibility, because of the high levels of trehalase in the Malpighian tubules (Mordue, 1969b), that some trehalose is metabolized to glucose and only glucose passes back to the blood. This would explain the lower activity ratios without the concomitant high reabsorption ratio.

All the sugars tested except mannose have had no significant effect on the reabsorption of glucose but rather indicate a possible competition for the passive leak into the lumen as they cause decreases in activity ratios of glucose. Mannose, however, appears to compete for the receptor site responsible in the reabsorption of glucose as its presence causes increases in glucose activity ratios, similar to the effects observed with phlorizin, phloretin and ouabain (see summarizing Table 6.3.). All these compounds thus appear to interfere in some way with the reabsorption of glucose resulting in a disruption of glucose movement back into the haemolymph and thus an increase of glucose movement into the tubule lumen. Although similar effects are caused by these compounds it may be due to either a direct or indirect effect on the reabsorption mechanism. Certainly, for mannose the close similarity in structure to glucose may explain its effect.

TABLE 6.3.

Summary of stimulatory effects on glucose transport.

COMPOUND PRESENT	% INCREASE IN GLUCOSE ACTIVITY RATIOS
Mannose (50 mM/l)	52%
Phlorizin (1 mM/l)	95%
Phloretin (0.1 mM/l)	278%
Ouabain (1 mM/l)	316%
Phlorizin & Ouabain (both 1 mM/l)	783%

On the basis of the results obtained a hypothetical model (fig. 6.3.1.) is proposed to explain these effects. Several assumptions are made in proposing this model and it is not intended as a rigid explanation of the events occurring in the tubule cell but merely as a working model which will form the basis for further invetigations. This model is based on that proposed by Schultz & Zalusky (1963) for events occurring in the rabbit ileum (fig. 6.3.2.).

As Na^+ concentrations on the basal side of the membrane have been shown to have no effect on glucose transport it is proposed that a Na^+-K^+-ATPase system is to be found only on the basal side of the membrane. As this pump is active in maintaining a low Na^+ and high K^+ concentration inside the cell any addition of Na^+ outside the cell will be kept out by this pump and therefore not increase the availability of Na^+ for a cotransport with glucose. This assumption is supported further by evidence from vertebrate work in the findings that ouabain is most effective on the serosal (=basal) surface of the rabbit ileum whereas phlorizin is most effective on the mucosal (=apical) surface (Schultz & Curran, 1970). Parsons & Pritchard (1968) also reported that the membrane transport process directed into the cell is "vigorous" at the luminal (=mucosal, apical) face and passive permeability is relatively low, conversely the membrane transport

Fig. 6.3.1. Model proposed for the interaction of Na^+ and glucose in the isolated Malpighian tubule of *Locusta*.

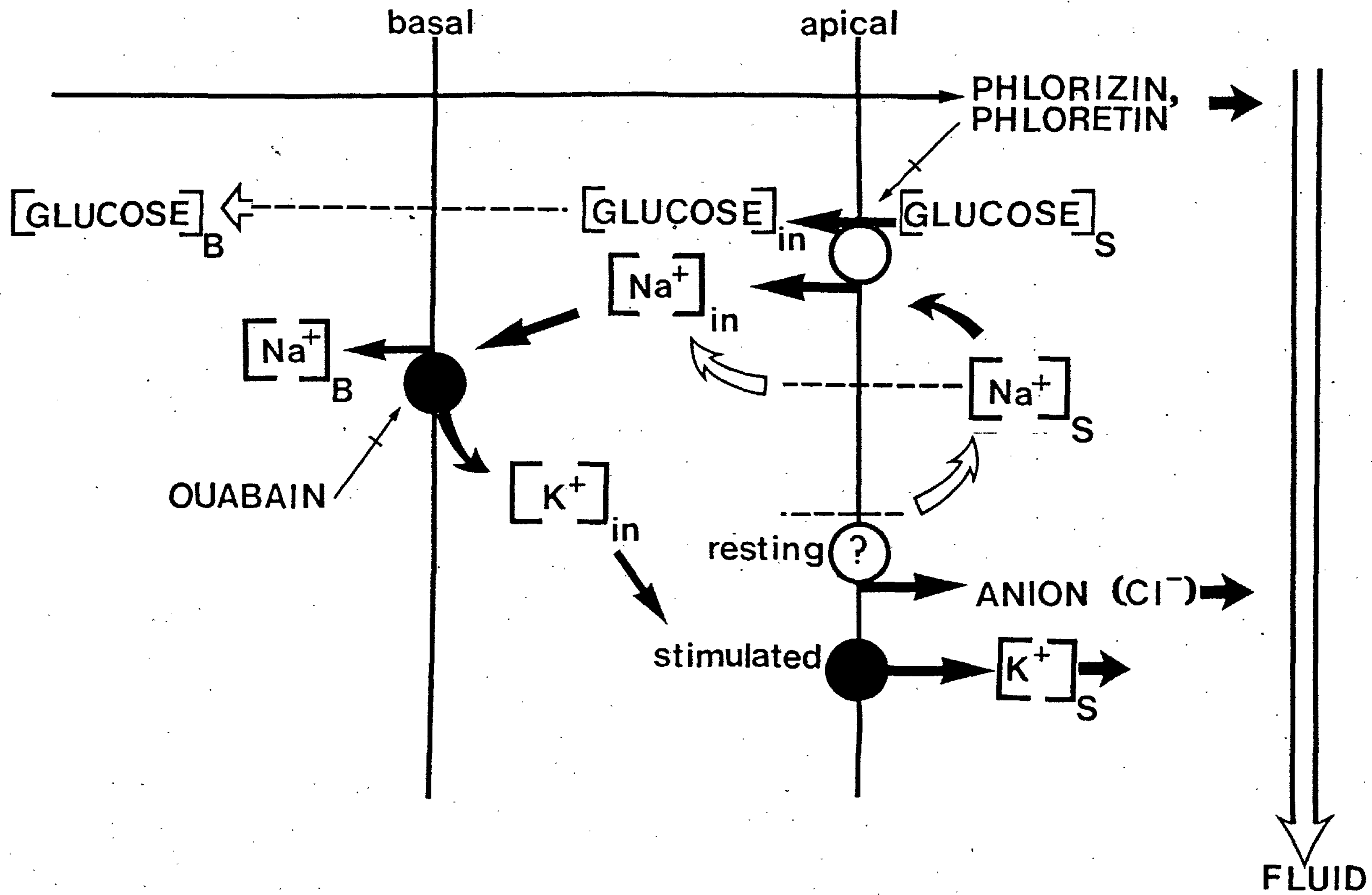
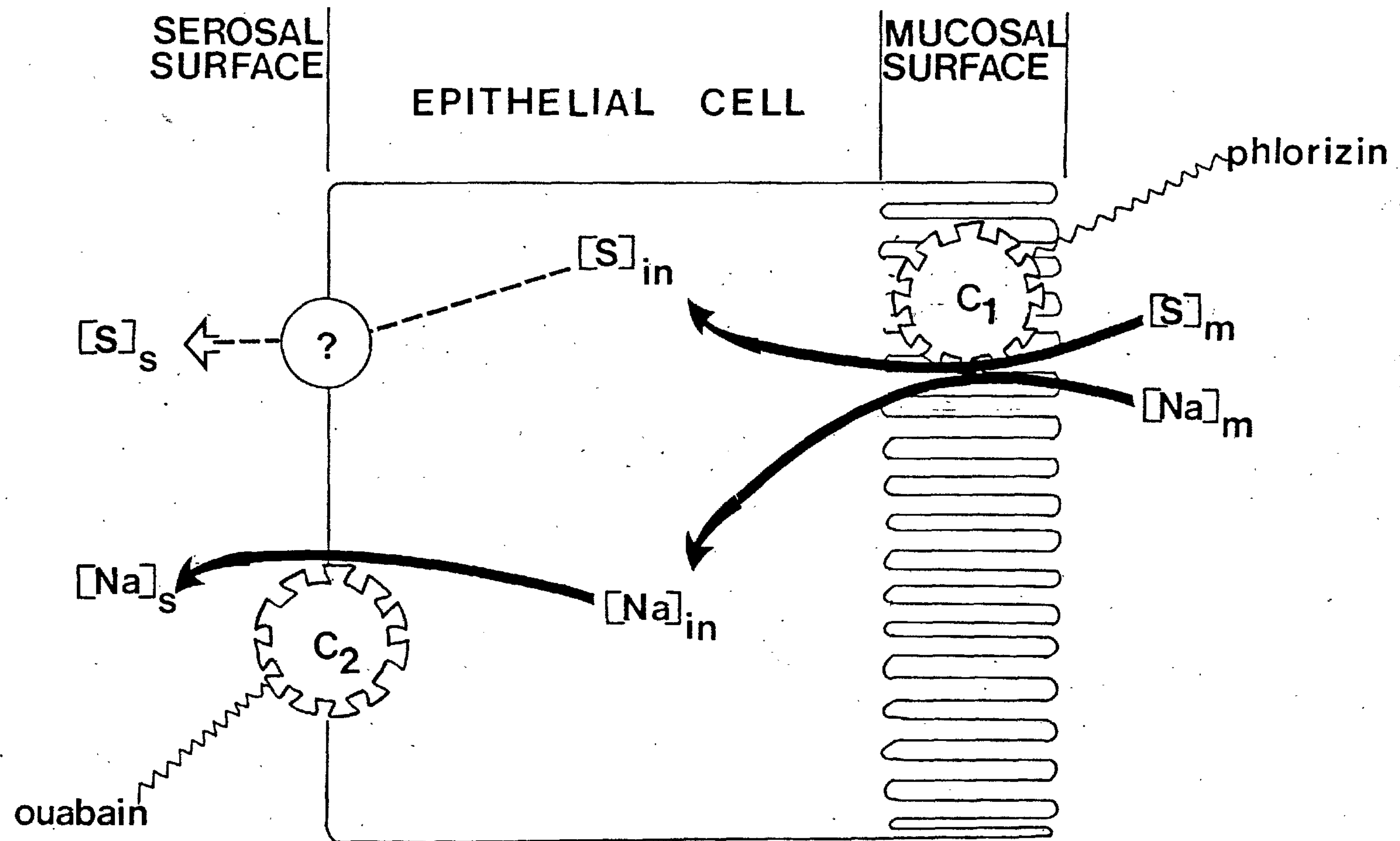

basal
apical
PHLORIZIN,
PHLORETIN
[GLUCOSE]B
[GLUCOSE]in
[GLUCOSE]S
[Na+]in
[Na+]B
[Na+]S
OUABAIN
[K+]in
resting
?
ANION (Cl−)
stimulated
[K+]S
FLUID

Fig. 6.3.2. Model for interaction of transport of Na^+ and sugars by isolated rabbit ileum as proposed by Scultz & Zalusky (1963).


SEROSAL SURFACE
EPITHELIAL CELL
MUCOSAL SURFACE
phlorizin
[S]in
C1
[S]m
[Na]m
[S]s
?
[Na]s
C2
[Na]in
ouabain

process at the serosal face is "feeble and non existant" whereas there is a relatively high passive permeability. Kinne (1976) working on vertebrate kidney showed that the basal-lateral membrane contained a high specific activity of Na^+-K^+-ATPase whereas in the brush border microvilli it was low (=luminal side). Thus, the glucose transporting pumping mechanism is present on the apical side whereas a ouabain sensitive pump, maintaining the ionic battery of the cell, will occur at the basal side. Further support, in the case of Malpighian tubules, for such an assumption is given by the models proposed by Berridge (1968) for a Na^+-K^+-ATPase exchange pump at the basal side and a K^+ electrogenic pump at the apical side. In this study it has been shown (Chapter 3) that ouabain does not affect the rate of fluid secretion. This is further support for the assumption that the Na^+-K^+-ATPase system is not involved in the main activity of the tubule cell in fluid secretion and thus its existence on the basal side is favoured. A coupling system of Na^+ and glucose, if it does exist, must therefore occur on the apical side of the membrane.

A low Na^+ concentration is maintained inside the cell by the Na^+-K^+-ATPase system and thus Na^+ will tend to move into the cell from both apical and basal sides.

Any glucose which has passively moved across into the lumen will couple to the Na^+ moving into the cell simply because intracellular Na^+ concentrations are lower than that in the surrounding medium. Thus the metabolic energy invested in the active extrusion of Na^+ at the basal end serves as a transducer for other transport processes at the apical end of the cell. The presence of Na^+ in the secreted fluid has been demonstrated (Maddrell & Klunsuwan, 1973) and indeed a gradient (low lumen, high haemolymph) for Na^+ exists across the Malpighian tubule cell of Calliphora (Gupta, 1976). The possibility exists that an anionic pump (Cl?) might occur at the apical end to which Na^+ movement may be coupled. The existence of such a pump is indicated by evidence that the lumen of the tubule is negative with respect to the bathing medium in the unstimulated locust (Coast, unpublished results). In this case Na^+ will follow this pump to maintain electric neutrality. As locust tubules continue to secrete even when unstimulated or in cauterized animals (Mordue, unpublished results) a system providing for fluid secretion in the resting tubule must occur. An anionic pump may well be the explanation to the existence of urine flow even in the unstimulated tubule. Joyner (1970) showed two cell types in Schistocerca gregaria which corresponded to the primary and stellate cells described for Calliphora (Berridge & Oschman, 1969; see Chapter 1).

The stellate cell could thus be involved in the reabsorptive function, whereas the leakage of Na^+ into the lumen may be as a result of the presence of the primary (secretory) cells.

When ouabain is present the Na^+-K^+-ATPase pump is inhibited and therefore Na^+ will start to diffuse into the cell and increase the intracellular Na^+ concentrations. This will in turn decrease the movement of Na^+ at the apical end as the concentration gradient is reduced. Therefore less coupling can occur between Na^+ and glucose and the glucose activity ratio in the lumen will increase. Thus the increase in glucose excretion is largely due to an indirect effect of ouabain on Na^+ movement. On the other hand, phlorizin and phloretin will inhibit the actual coupling of Na^+ to glucose at the apical end by competing with glucose. This is feasible as both phlorizin and phloretin have been shown (Chapter 5) to be pumped against a concentration gradient into the lumen of the tubule and therefore can affect the apical side of the membrane. Mannose would also thus compete with glucose at this end.

SUMMARY

The functioning of the Malpighian tubules of *Locusta* and *Zonocerus* has been studied using an *in vitro* preparation. Reabsorption by the tubules was tested using a modification of the above *in vitro* technique in which tubules were passed through a second bathing medium (M_2). The rate of fluid secreted by the tubules was computed from measurements of the volume of the secreted drop and the time interval during which the drop was produced. Radioactivity was measured, using a liquid scintillation counter, either by the channels ratio method which provided a value for the actual concentration of the secreted chemical, or by comparing the cpm/µl of the secreted fluid with the cpm/µl of the bathing medium which provided a value for activity ratios. Similarly, reabsorption ratios were determined by comparing the values for cpm/µl in M_2 and cpm/µl in M_1. Thin layer chromatography was used in some cases to identify the secreted fluid.

The effect of corpora cardiaca extracts on the rate of fluid secretion by the Malpighian tubules was tested. Saline extracts of storage lobes were found to be unstable at room temperatures and more than 75% of the activity was lost within 3 hours. The diuretic activity was stable in methanol extracts. Locust tubules were stimulated by diuretic hormones from *Rhodnius*, *Glossina* and *Periplaneta*. Several compounds were found to mimic the action of the diuretic hormone: cyclic-AMP, dibutyryl cyclic-AMP, adrenalin, histamine, ecdysterone, cholesterol, aldosterone, phlorizin and phloretin produced marked increases in urine secretion. No effect was obtained with 5-hydroxytryptamine or ouabain. Known inhibitors of some stimulants, such as phentolamine and ethacrynic acid, inhibited the effects of adrenalin and aldosterone respectively, but failed to inhibit the action of either locust diuretic hormone or cyclic-AMP.

The ability of the tubules to handle toxic chemicals was investigated. The transport of ouabain was investigated in Zonocerus which is predisposed to feeding on toxic plants and compared with Locusta tubules. Adult Locusta were found to have a poor survival rate when ouabain was injected into their haemolymph, whereas Zonocerus adults were capable of handling 10^5 times the concentrations that killed Locusta adults. Not only were Zonocerus tubules capable of secreting ouabain passively at a higher rate than Locusta but the presence of ouabain in the haemolymph, whether injected or originating in the diet of the insect, induced the tubules to secrete even higher levels eventually reaching levels in the secretions which were greater than those found in the bathing medium. This indicated that exposure to ouabain over periods of 12 days induced a pump in the Malpighian tubules which was capable of secreting ouabain against a concentration gradient.

As mentioned previously, phlorizin and its aglycone phloretin were found to stimulate diuretic activity in Locusta tubules. Moreover, a second dose of phlorizin produced an additional, though smaller, increase in fluid rate. The possibilities existed that the increase in the rate of fluid secretion maybe due to interferance with the mechanism of fluid secretion, or be a consequence of increased osmotic flow associated with the transportation of phlorizin against a concentration gradient. The permeability of the tubules to phlorizin was investigated in order to throw further light onto its mode of action in stimulating fluid secretion. ^{3}H-phlorizin was found to accumulate in the secreted fluid against a concentration gradient, reaching 20 times the concentration found in the bathing medium. In addition, glucose was found to be a non-competitive inhibitor of this pump. Samples of secreted fluid and bathing medium

were analyzed by thin layer chromatography. Two radio-labelled areas at Rf 0.3 and 0.5 were found in the secreted fluid, the main peak of activity was at Rf 0.5 which corresponded to the Rf of phloretin. The Rf of phlorizin was 0.3. Thus both phlorizin and phloretin were found in the secreted fluid.

The presence of phlorizin, phloretin and ouabain was found to increase the activity ratios of glucose in the secreted fluid. The glucose levels under normal conditions were lower than those expected for its molecular size. This indicated, indirectly, that a significant reabsorption of glucose back into the bathing medium (haemolymph) occurred, and that this reabsorption was significantly inhibited by phlorizin, phloretin and ouabain. More direct evidence of this reabsorption was obtained using the modified in vitro technique whereby M_2 values were monitored. Although an effect was found in the presence of ouabain, changes in the concentrations of Na^+ in the bathing medium did not significantly alter glucose levels in the secreted fluid. However, the secretion of Na^+ was found to be similarly affected by ouabain, phlorizin and phloretin: levels of Na^+ in the secreted fluid increased in the presence of these compounds. These effects were investigated further using different sugars.

This investigation has shown that the basal rate of fluid secretion in Locusta tubules can be increased not only by natural diuretic hormones, but also by several seemingly unrelated compounds. However, it has been shown that the possibility exists that many of these compounds merely cause (certainly in the case of phlorizin and phloretin) an osmotic flow associated with their passage against a concentration gradient. Thus it is important to consider this point

when attempting to explain the mode of action of the diuretic hormone. The presence of a Na^+-K^+-ATPase in tubules has been shown by various workers but its exact function has not been elucidated. Ouabain, the Na^+-K^+-ATPase pump inhibitor, has been shown by several workers as well as in this investigation to have no effect on the secretion of urine by the tubules. However, this study has shown that ouabain significantly alters both glucose and Na^+ levels in the secreted fluid. The presence of Na^+-K^+-ATPase can now be explained. It is not involved in the function of fluid secretion by the tubules but, as in other cells, it maintains an ionic battery from which energy invested in the active extrusion of Na^+ serves as a transducer for the transport of other substances such as glucose. This reveals a greater similarity in function between the Malpighian tubules of insects and renal tubules in vertebrates both of which have the important function of reabsorbing useful substances as well as excreting several toxic, harmful and unwanted substances.

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