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Studies on ouabain-binding to $(Na^+ + K^+)$-ATPase from Malpighian tubules of the locust, *Locusta migratoria* L.

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A study has been made on the binding of [^{3}H]ouabain to $(Na^+ + K^+)$-ATPase in microsomal preparations from Malpighian tubules of *Locusta migratoria*. The rate constants at 30°C were $1.5 \cdot 10^3 \pm 3.5 \cdot 10^2$ $M^{-1} \cdot s^{-1}$ and $3.7 \cdot 10^{-3} \pm 0.6 \cdot 10^{-3}$ s^{-1} for the association and dissociation of the ouabain and the receptor, respectively. This yielded a dissociation constant of $2.5 \cdot 10^{-6}$ M. Scatchard plots indicate heterogeneity of ouabain binding. These have been analysed on the basis that binding occurred at two classes of independent sites. High-affinity sites were characterised by a dissociation constant of 0.2 ± 0.1 μM and low capacity ($B_{max} = 11.0 \pm 1.2$ pmol/mg protein). Low-affinity sites were characterised by a dissociation constant of 4.2 ± 1.3 μM and B_{max} equal to 25.9 ± 2.5 pmol/mg protein. K_d for the low-affinity site was not significantly different from the I_{50} value of 1.12 μM, suggesting that this class of site may be associated with inhibition of $(Na^+ + K^+)$-ATPase activity. Comparison of $(Na^+ + K^+)$-ATPase activity and amount of ouabain bound indicate a turnover of 2645 ATP hydrolysed/site per min. It is estimated that there are in excess of $3.4 \cdot 10^6$ high-affinity sites and $8.1 \cdot 10^6$ low-affinity sites per cell (i.e., a total of $1.15 \cdot 10^7$ sites/cell). This total site density value, taken in conjunction with the turnover number, predicts rates of metabolic demand and cation translocation which are consistent with observed values.

Introduction

The Malpighian tubules of *Locusta migratoria,* in common with those of a number of other species which have been studied (e.g. *Calliphora* [1], *Tipula paludosa* [2] and *Schistocerca gregaria* [3]) are able to transport K^+ against a chemical gradient over a wide range of external K^+ concentrations. In addition, K^+ are transported in preference to Na^+ even when present at much lower concentrations in the bathing medium [4]. Measurements of potential difference across the tubule wall indicate that the lumen is positive with respect to the bathing medium [5–7]. On these grounds, it has been proposed that the transport of K^+ is an active process. Nevertheless, both Na^+ and K^+ are necessary for maximal fluid secretion [8,9].

A number of models have been proposed to explain ion and fluid secretion across Malpighian tubules of insects [10–14]. Almost all require that active ion transport occurs across the basal and apical cell membranes, and that K^+ and/or Na^+ are transported into the lumen by an apical electrogenic cation pump. Entry of some K^+ into the cell has been proposed to occur, at the basolateral membranes, in exchange for Na^+ [1,10,15,16]. A K^+-activated ATPase pump has been proposed for the apical surface [17], but it must be emphasised that no firm biochemical evidence exists for

Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

K^+-ATPase activity in preparations from Malpighian tubules [8].

Previous studies [8,18,19] have revealed the presence of an ATPase enzyme system in microsomal preparations from Malpighian tubules of *Locusta.* This ATPase system has two components, one activated by Mg^{2+} alone (Mg^{2+}-ATPase) and the other synergistically stimulated by the addition of Na^+ and K^+. The latter activity is inhibited by ouabain and represents the ($Na^+ + K^+$)-ATPase (EC 3.6.1.3). This enzyme activity has been characterised for a number of insect species and for a variety of different tissues [20] and exhibits properties similar to those described for vertebrate preparations [21].

($Na^+ + K^+$)-ATPase enzymes have been implicated in cation and fluid transport processes in a variety of different tissues from a number of different species [4,8,19,22–25]. However, the literature concerning the ouabain sensitivity of insect epithelia is conflicting, with some species being reported to be refractory to ouabain [26]. The sensitivity of ($Na^+ + K^+$)-ATPase to cardiac glycosides such as ouabain differs markedly from species to species [27]. Some researchers suggest that ($Na^+ + K^+$)-ATPase from different sources bind ouabain at the same rate and that differences in sensitivity are determined by differences in the rates of dissociation of the glycoside from the enzyme [27,28]. Rubin et al. [29] report that the ouabain dissociation reaction is considerably faster with microsomal preparations from brain of *Manduca sexta* than with bovine brain, accounting for the decreased sensitivity of the insect enzyme to inhibition by ouabain. Other workers suggest that the lower sensitivity of ($Na^+ + K^+$)-ATPase from certain species cannot be explained solely on the basis of the dissociation rate [30,31]. To date, with the notable exception of the study by Rubin et al. [29] referred to above, few studies report the use of [^{3}H]ouabain in insect tissue preparations. Fristrom and Kelly [32] and Jungreis and Vaughan [33] studied [^{3}H]ouabain binding to imaginal discs of *Drosophila melanogaster*, and midgut and nerve of three lepidopteran species, respectively. Unfortunately, both these studies were carried out under conditions that were inappropriate for determining maximal binding, owing to the inclusion of K^+ in the incubation medium.

The present study was undertaken to characterise ouabain binding to the ($Na^+ + K^+$)-ATPase of Malpighian tubules of *Locusta migratoria,* to determine the sensitivity of the enzyme preparation to ouabain and to assess the likely contribution of the ($Na^+ + K^+$)-ATPase 'pump' to cation translocation in this tissue.

Materials and Methods

Mature adult locusts, *Locusta migratoria* L., were used and these were taken from a population maintained under crowded conditions at 28 ± 0.5 °C and 60% relative humidity.

The methods of preparation of the membrane microsomal homogenate, of enzyme assay, and of protein determination were essentially as described previously [8,20]. In determining enzyme activity, two incubation media, having the following composition, were used:

(1) 4 mM magnesium chloride;
(2) 4 mM magnesium chloride/100 mM sodium chloride/20 mM potassium chloride.

Each medium contained 3 mM ATP (Tris salt) final concentration and 50 mM histidine-HCl to a final volume of 2 ml. All tubes were thermoequilibrated for 15 min before starting the experiment by the addition of 0.5 ml of microsomal suspension. Incubations were carried out at 30 ± 0.1 °C.

In view of suggestions that insect ($Na^+ + K^+$)-ATPases are relatively insensitive to ouabain [33], the activity of this enzyme was calculated as the difference in inorganic phosphate liberated in media containing Na^+, K^+ and Mg^{2+}, and Mg^{2+} alone; previous work with this preparation having shown that ($Na^+ + K^+$)-ATPase activity determined by this method is not significantly different from that obtained by ouabain inactivation [8].

Ouabain binding. Ouabain binding was determined by a rapid Millipore filtration procedure similar to that described elsewhere [20,34,35]. Approximately 1 mg microsomal enzyme protein was incubated in 5 mM magnesium chloride, 2 mM EDTA, 100 mM sodium chloride, 3 mM ATP in 20 mM imidazole-HCl (pH 7.2) with [^{3}H]ouabain, at 30 °C. At the end of the appropriate incubation period a sample of the medium was removed and rapidly filtered through Millipore membrane filters

(pore size 0.45 μm) by suction. Following washing with three separate 5 ml aliquots of cold (0–4°C) washing medium, whose composition was identical to that of the incubation medium but without ouabain or ATP, the filters were dissolved in scintillation cocktail and the amount of labelled ouabain determined by liquid scintillation counting in a Packard Tri-Carb 300C liquid scintillation counter. Non-specific ouabain-binding was determined by running a parallel set of incubations in which 3 mM unlabelled ouabain was also present in the incubation medium. Specific binding of ouabain was obtained by subtraction of the ouabain bound non-specifically.

Dissociation of ouabain from the enzyme preparation was determined by a chase method. The amount of membrane suspension needed was allowed to bind [^{3}H]ouabain at 30°C, as described above, for a sufficient time (45 min) for equilibrium to be attained. At this time an excess of unlabelled ouabain was added to a final concentration of 1 mM and at appropriate times aliquots were removed, filtered, washed and counted.

To determine the rate of oxygen consumption by Malpighian tubules. Animals were killed by decapitation and the Malpighian tubules were quickly dissected out under an ice-cold Ringer solution. The tubules were then placed in the incubation chamber of a YSI Model 53 Oxygen monitor containing 3 ml of air-saturated Ringer solution at 30°C. Following a 10 min equilibration period, the rate of oxygen consumption was determined polarographically. The composition of the Ringer solution was (mM): NaCl, 100/KCl, 8.6/$CaCl_2$, 2/$MgCl_2$, 8.5/NaH_2PO_4, 4/$NaHCO_3$, 4/glucose, 34/Hepes, 25/NaOH, 11 (pH 7.2) [4,6].

To determine cell size and numbers. One cell type predominates throughout the length of the tubules of *Locusta* [36] and accounts for more than 90% of the cells present (unpublished observation). On this basis, the number of cells per Malpighian tubule was estimated by counting the number of nuclei in a series of known lengths of unfixed Malpighian tubules. These segments of tubule were then weighed and the weight of 1 mm of tubule was calculated. By relating this value to the number of cells in 1 mm of tubule it is possible to obtain an estimate for the weight of a tubule cell and from the weight of the total tubule mass in a locust, the total number of Malpighian tubule cells. This value may then be used in conjunction with determinations of protein levels in microsomal preparations, from known numbers of locusts, to provide an estimation of microsomal protein yield per cell.

Cell size was estimated from measurements made on 5 μm thick serial sections through Malpighian tubules which had previously been fixed in Karnovsky's fixative [37] prior to embedding in araldite epoxy resin.

All solutions were made up in glass-distilled, deionized water. All inorganic salts were AnalaR grade or the best commercially available; histidine, ATP and ouabain were obtained from Sigma Chemical Co.; ATP (Tris salt) was made from the sodium salt by ion exchange [20]. [^{3}H]Ouabain (1.55 TBq/mmol) was obtained from Amersham International plc, Amersham, U.K.

Results

In the present study twelve separate ($Na^+ + K^+$)-ATPase preparations were used. Each was obtained from homogenates of Malpighian tubules prepared from 40 locusts. The mean specific activity of the Mg^{2+}-ATPase and the ($Na^+ + K^+$)-ATPase was 1.56 ± 0.22 and 5.86 ± 0.82 μmol inorganic phosphate liberated/mg protein per h, respectively. Thus, ($Na^+ + K^+$)-ATPase activity accounted for ca. 75% of the total ATPase activity of these preparations.

Inhibition by ouabain of ($Na^+ + K^+$)-ATPase activity

($Na^+ + K^+$)-ATPase activity was assayed in the presence of different concentrations of ouabain over the range 10^{-8}–10^{-3} M. Microsomes were preincubated for 15 min in the presence of Na^+, Mg^{2+}, ATP and ouabain before the reaction was started by the addition of K^+ in a ouabain solution. The inhibition curve for ouabain (Fig. 1) shows that as the concentration of ouabain increased so did the inhibition of the ($Na^+ + K^+$)-ATPase activity. The concentration of ouabain effecting 50% inhibition of activity (I_{50}) was 1.12 μM (95% confidence limits = 0.7–1.9 μM).

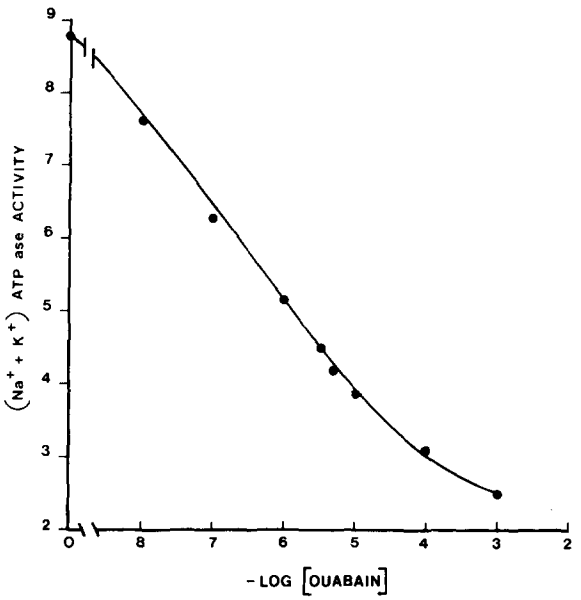

Fig. 1. Effect of different concentrations of ouabain on $(Na^+ + K^+)$-ATPase activity. $(Na^+ + K^+)$-ATPase activity was assayed as described in Materials and Methods in the presence of different concentrations of ouabain ($1 \cdot 10^{-8}$ M–$1 \cdot 10^{-3}$ M) at 30 °C. Typical experiment which is representative of four experiments. Ordinate: probits of fraction of $(Na^+ + K^+)$-ATPase activity remaining. Abscissa: negative logarithm of ouabain concentration (M).

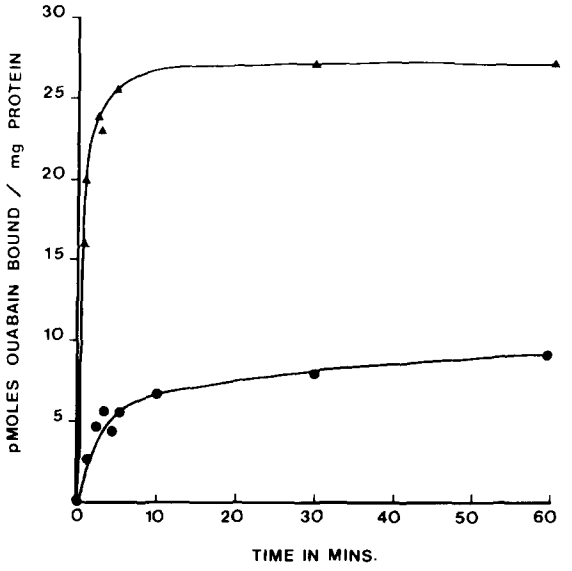

Fig. 2. Time course of specific ouabain-binding to a microsomal preparation from Malpighian tubules of *Locusta* at $3 \cdot 10^{-6}$ M (▲) and $3 \cdot 10^{-7}$ M (●) concentration. Incubation medium 5 mM Mg^{2+}/2 mM EDTA/100 mM Na^+/3 mM ATP/20 mM imidazole-HCl (pH 7.2) at 30 °C. Samples were filtered at 0–4 °C, washed and counted as described in Materials and Methods. Typical experiment representative of three experiments. Ordinate: ouabain bound (pmol/mg protein). Abscissa: time in minutes.

Ouabain binding

$(Na^+ + K^+)$-ATPase binds ouabain specifically according to the mass-law equation:

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI$$

where E is the receptor concentration, I is the ouabain concentration, EI is the ouabain-receptor complex concentration, and k_1 and k_{-1} are the association and dissociation rate constants, respectively.

The formation of [^{3}H]ouabain-enzyme complex follows second-order kinetics [38]. Thus, both the initial receptor concentration and the initial ouabain concentration need to be known before the association rate constant (k_1) can be calculated. However, Wallick et al. [27] suggest that if the concentration of ouabain is maintained in large excess of the receptor, the forward reaction becomes pseudo first-order [39]. This greatly facilitates the determination of k_1 from the equation:

$$k_1 = (k_{obs} - k_{-1})/I$$

where k_{obs} is the observed first-order approach to equilibrium [27].

Fig. 2 shows the time course of ouabain binding to a microsomal preparation from Malpighian tubules of *Locusta*. [^{3}H]Ouabain binding reached a maximum after 5–10 min and remained stable over a 1 h incubation period. Fig. 3 shows that the initial rate of binding follows pseudo first-order kinetics under the conditions used and is consistent with the findings of other researchers [27]. Such plots were used to determine k_{obs} and hence the association rate constant, k_1. The mean calculated k_1 was $1.5 \cdot 10^3$ $M^{-1} \cdot s^{-1}$ (Table I).

In the present study, the dissociation of ouabain from the enzyme was determined following incubation in the presence of $3 \cdot 10^{-6}$ M [^{3}H]ouabain

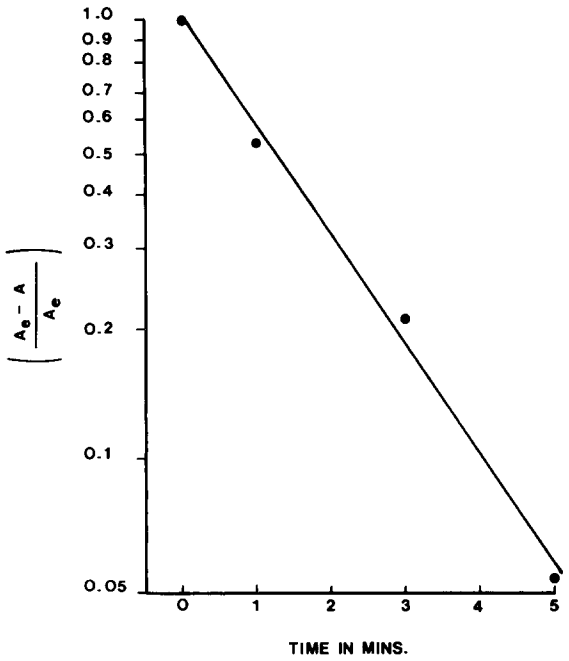

Fig. 3. Pseudo first-order binding of ouabain to a microsomal preparation of Malpighian tubules of *Locusta*. Binding was carried out in the presence of $3 \cdot 10^{-6}$ M ouabain. Experimental conditions as in Fig. 2. Typical experiment representative of three experiments. Ordinate: $(A_e - A)/A_e$ ($\log_e$ scale) where A_e and A are ouabain bound at equilibrium and at time, t, respectively. Abscissa: time in minutes.

at 30°C. The dissociation of the ouabain-receptor complex follows first-order kinetics and consequently the dissociation rate constant (k_{-1}) can be calculated from the exponential decay of ouabain binding [38] (Fig. 4). The mean calculated k_{-1} was $3.7 \cdot 10^{-3}$ s^{-1} (Table I).

The Michaelis constant or the equilibrium dissociation constant (K_d) can be calculated by the equation:

$$K_d = \frac{k_{-1}}{k_1}$$

(see Table I) or can be measured directly from the equilibrium binding of ouabain as a function of ouabain concentration. The binding of [^{3}H]ouabain to Malpighian tubule ($Na^+ + K^+$)-ATPase preparations was determined after 45 min incubation at different concentrations of ouabain and the data were plotted according to Scatchard

TABLE I

KINETIC CONSTANTS FOR OUABAIN-BINDING TO MICROSOMAL PREPARATIONS OF MALPIGHIAN TUBULES OF *Locusta*

Constants were obtained as described in Materials and Methods. n represents the number of independent experiments and a and b represent values for high- and low-affinity sites, respectively. K_d and B_{max} were calculated from Scatchard plots of data.

Parameter		n	
k_1 ($M^{-1} \cdot s^{-1}$) ± S.E.		3	$1.5 \cdot 10^3 \pm 3.5 \cdot 10^2$
k_{-1} (s^{-1}) ± S.E.		5	$3.7 \cdot 10^{-3} \pm 0.6 \cdot 10^{-3}$
k_{-1}/k_1 (M)			$2.5 \cdot 10^{-6}$
K_d (M) ± S.E.	a	3	$0.2 \cdot 10^{-6} \pm 0.1 \cdot 10^{-6}$
	b	3	$4.2 \cdot 10^{-6} \pm 1.3 \cdot 10^{-6}$
B_{max} (pmol/mg protein) ± S.E.	a	3	11.0 ± 1.2
	b	3	25.9 ± 2.5

[40] (Fig. 5). It can be seen that the resulting plot is curvilinear, suggesting either that there is binding to multiple independent binding sites or that

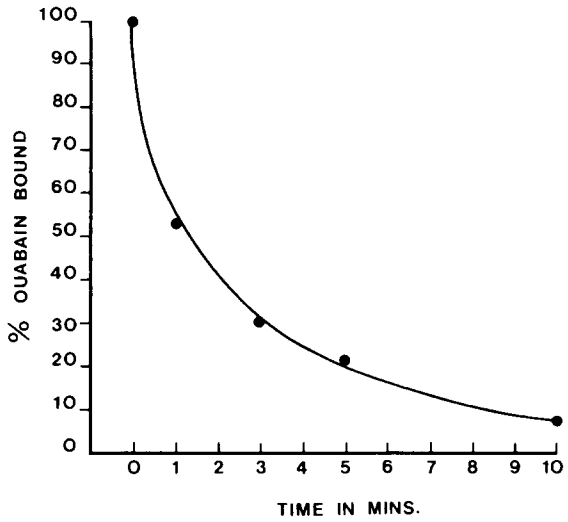

Fig. 4. Time course of ouabain dissociation from ($Na^+ + K^+$)-ATPase preparation. The enzyme-ouabain complex was formed by incubating the tissue for 45 min at 30°C in the presence of $3 \cdot 10^{-6}$ M [^{3}H]ouabain. The dissociation reaction was started by the addition of unlabelled ouabain to a final concentration of $1 \cdot 10^{-3}$ M (see Materials and Methods). Typical experiment representative of five experiments. Ordinate: percent ouabain bound. Abscissa: time in minutes after addition of excess unlabelled ouabain.

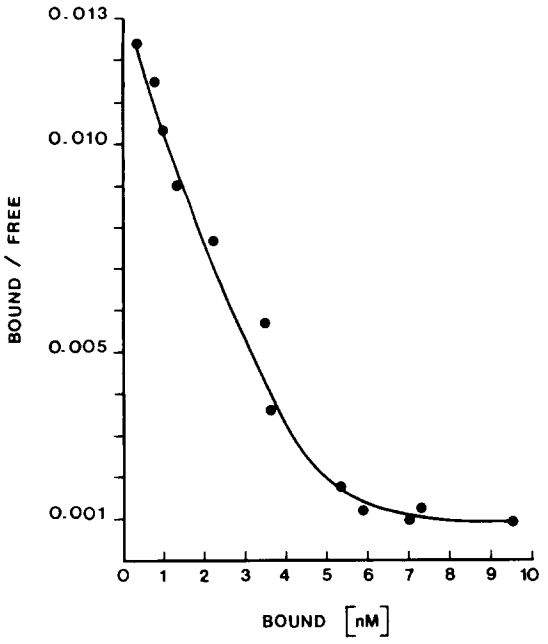

Fig. 5. Scatchard plot of ouabain binding to a microsomal preparation from Malpighian tubules of *Locusta*. The enzyme-ouabain complex was formed by incubating the preparation for 45 min at 30 °C with $3 \cdot 10^{-8}$–10^{-5} M ouabain. Experimental conditions as in Fig. 2 and Materials and Methods. Typical experiment representative of three experiments. Ordinate: ratio of bound/free ouabain. Abscissa: concentration of ouabain bound (nM).

there is negative cooperativity between binding sites [41,42]. More recently, Noel and Godfraind [43] have concluded, from their studies on rat heart, that ouabain-specific binding occurs at two classes of independent sites. On this basis, the binding capacities and affinities for high- and low-affinity sites have been determined in the present study (Table I). The results suggest that about 30% of binding sites displayed a high affinity for ouabain ($K_d = 0.2 \cdot 10^{-6}$ M), whereas 70% of binding sites were characterised by a lower affinity ($K_d = 4.2 \cdot 10^{-6}$ M) (Table I).

Estimates of cell size and numbers

It was estimated that the total cell number for the Malpighian tubules of an adult locust was about 363 000. Furthermore, approximately 189.4 $\pm$ 6.9 μg microsomal protein were extracted per insect from Malpighian tubules ($n = 5$ independent determinations, each involving 40 locusts). Thus approximately $5.2 \cdot 10^{-7}$ mg of microsomal protein are derived from each cell.

Measurements on serial sections through Malpighian tubules of *Locusta* indicate that the mean cell volume is 72430 ± 2355 μm^3 ($n = 8$) with approximate dimensions of 85 μm $\times$ 85 μm $\times$ 10 μm.

Discussion

The $(Na^+ + K^+)$-ATPase of Malpighian tubules of *Locusta* exhibits many of the properties of $(Na^+ + K^+)$-ATPases from other species [20,26]. It is maximally activated at an ATP/Mg^{2+} ratio of 1 : 1.3 and at 100 mM Na^+/20 mM K^+ [18] and is inhibited by ouabain [8,18–20]. In the present study, the concentration of ouabain which half-maximally inhibited enzymatic activity was 1.12 μM. This agrees well with I_{50} values reported for other insect preparations [20].

Many studies on the binding of cardiac glycosides to $(Na^+ + K^+)$-ATPase preparations result in saturable binding which involves a single class of binding site [42,44]. In the present study curvilinear Scatchard plots were obtained which suggests that microsomal preparations from Malpighian tubules of *Locusta* bind ouabain specifically with a dissociation constant of $0.2 \cdot 10^{-6}$ M and $4.2 \cdot 10^{-6}$ M at high- and low-affinity sites, respectively. Other workers have reported the possible existence of at least two classes of binding sites of high and low affinities with different tissues from a variety of species [41,43,45–48]. As pointed out earlier, such heterogeneity might be due to the presence of different $(Na^+ + K^+)$-ATPase conformations with different affinities for ouabain or the existence of independent binding sites. The existence of two classes of independent binding sites in rat heart has been proposed [43]. Furthermore, it is reported that K^+ increased the proportion of the high-affinity sites in microsomal fractions from guinea-pig heart [45]. However, the possibility that such curved plots are due to negative cooperativity cannot be ruled out. Noel and Godfraind [43] reported that, in rat heart, the proportion of low- and high-affinity components observed in enzyme inhibition studies was similar to that measured in [^{3}H]ouabain binding experiments. They suggested that this might be ex-

plained on the basis of two isozymes being present. Furthermore, they suggest that, since ^{86}Rb uptake by cells in intact tissue was not affected by ouabain at concentrations less than 10^{-5} M but was almost completely abolished at 10^{-4} M [49], only low-affinity sites are inhibitory in vivo. Similarly, on the basis that K_d for low-affinity sites was a value close to ouabain I_{50}, it was suggested that the low-affinity sites could be inhibitory sites in guinea-pig heart [45]. Giunta et al. [50] have proposed a regulatory model in which it is suggested that at very low levels of cardiac glycoside, binding to the high-affinity sites causes conformational changes leading to enzyme activation, whereas at higher cardiac glycoside levels, binding to the low-affinity site results in enzyme inhibition. These same workers [50,51] suggest a model for activity modulation of $(Na^+ + K^+)$-ATPase activity, in vivo, involving ouabain-like compounds, such as have been reported in a variety of animal tissues [52–56]. Such compounds inhibit enzymatic activity in the same range of concentrations as ouabain. Thus it is proposed that under physiological conditions the level of circulating ouabain-like compounds can saturate the high-affinity site, promoting enzyme activation. An increase in the concentration of such digitalis-like compounds, it is argued, leads to saturation of the lower-affinity binding site also, resulting in enzyme inhibition. In the present investigation, the value for I_{50} was not significantly different from the dissociation constant (K_d) for the low-affinity site, suggesting that here also, the low-affinity site may be responsible for enzyme inhibition. The significance of the high-affinity sites and the application of the regulative model of $(Na^+ + K^+)$-ATPase activity, described above, to insect Malpighian tubules must await further investigation.

Table II compares the kinetic constants of ouabain-binding to $(Na^+ + K^+)$-ATPase in microsomal preparations of Malpighian tubules with those obtained elsewhere, from a variety of species. It can be seen that the association rate constant (k_1), the dissociation rate constant (k_{-1}) and K_d for *Locusta* are within the range of values quoted

TABLE II

COMPARISON OF MEAN KINETIC CONSTANTS FOR OUABAIN BINDING IN PREPARATIONS FROM DIFFERENT SPECIES

a and b represent values for high- and low-affinity sites, respectively.

Tissue	k_1 ($M^{-1} \cdot s^{-1}$)	k_{-1} (s^{-1})	k_{-1}/k_1 (μM)	K_d (μM)	Ref.
Malpighian tubules of *Locusta*	$1.5 \cdot 10^3$	$3.7 \cdot 10^{-3}$	2.5	a 0.2 b 4.2	present study
Rabbit kidney	$0.5 \cdot 10^2$	$2.4 \cdot 10^{-5}$		0.5	62
Manduca sexta brain	$1.9 \cdot 10^4$	$2.2 \cdot 10^{-3}$		0.12	29
Rat intestine	$1.3 \cdot 10^3$	$3.6 \cdot 10^{-2}$	29.0	15	35
Cavia cobaya kidney				a 0.35 b 2.1	50 50
Chick cardiac cells	$7.6 \cdot 10^2$	$5.0 \cdot 10^{-3}$	6.6	a 0.03–0.05 b 2–6	46 46
Rat heart				a 0.21 b 13	43 43
Rabbit nephron	$5.0 \cdot 10^2$	$1.0 \cdot 10^{-3}$	2.0	1.8	64
Rectal gland of *Squalus*				a 0.2 b 5.0	65 65
Whole imaginal discs of *Drosophila*	$4.7 \cdot 10^2$	$8.6 \cdot 10^{-5}$		0.18	32

for a variety of tissues and different species.

There is disagreement concerning the ratio of ATP-binding and ouabain-binding sites to phosphorylation sites on the $(Na^+ + K^+)$-ATPase. Jørgensen [57,58] has shown that there is one ouabain-binding site, one ATP-binding site and one phosphorylation site per 280000 molecular weight unit. Similarly, a ratio of 1 : 1 for ATP sites to phosphorylation sites [59] and a 1 : 1 ratio for ouabain-binding sites to phosphorylation sites [28,60] has been reported elsewhere. However, $(Na^+ + K^+)$-ATPase from guinea-pig kidney binds 4 mol ouabain/mol ^{32}P-labelled phosphoprotein formed [28], whilst enzyme preparations from the electric eel organ bind only 1 ouabain per two phosphorylation sites [60].

In the Malpighian tubule preparations from *Locusta* maximum binding (B_{max}) was 11.0 pmol/mg protein at the high-affinity sites and 25.9 pmol/mg protein at the lower-affinity sites (see Table I). These data, taken in conjunction with the mean $(Na^+ + K^+)$-ATPase activity measured, indicate a value of 6.3 pmol ouabain bound/μmol inorganic phosphate liberated at both the high- and the low-affinity sites. This yields an overall turnover of 2645/min, assuming that one ouabain is bound per pump site. This turnover number for the pump is compared with those reported for a number of other tissues and different species in Table III.

It was estimated that each cell yielded approx. $5.2 \cdot 10^{-7}$ mg membrane protein, a value which compared favourably with the $2.5 \cdot 10^{-7}$ mg protein/cell quoted for vertebrate intestinal cells [35]. However, in view of the fact that, in the present study, the yield was only a fraction of the total membrane protein per cell, this value is a substantial underestimate. Nevertheless, accepting its limitations we can use this value to make certain calculations on the basis of data obtained with *Locusta*. Thus, assuming 1 ouabain bound per enzyme site, it can be calculated that there are $3.4 \cdot 10^6$ high-affinity pump sites and $8.1 \cdot 10^6$ lower-affinity pump sites per tubule cell (i.e. a total of $11.5 \cdot 10^6$ pump sites/cell). This pump site density is compared with values reported for a variety of other cell types in Table IV. Harms and Wright [35] calculated that there were approx. $1.5 \cdot 10^5$ sites per cell (estimated from maximum phosphorylation studies) in rat intestine. However, their estimate was an order of magnitude larger when determined from maximum ouabain-binding data. The somewhat higher value reported for the salt-secreting chloride cells of teleost gill (viz. $1.5 \cdot 10^8$ sites/cell) [61] is thought to be related to their larger cell volume and the membrane magnification factor [62].

TABLE III

COMPARISON OF TURNOVER VALUES FOR $(Na^+ + K^+)$-ATPase FROM VARIOUS SPECIES

Preparation	Turnover (ATP hydrolysed/site per min)	Ref.
Malpighian tubules of *Locusta migratoria*	2645	present study
Red blood cell	1400	66
Rabbit nephron	2000	64
Bovine kidney	3430	28
Rat intestine	8300	35
Guinea-pig kidney	11100	28
Bovine heart	8550	28
Bovine brain	11500	28

TABLE IV

COMPARISON OF OUABAIN BINDING SITE DENSITY FOR VARIOUS SPECIES

a and b represent values for high- and low-affinity sites, respectively. * indicates value calculated from data given by Rubin et al. [29] and assuming $5.2 \cdot 10^{-7}$ mg protein per cell. + indicates values are likely to be underestimates.

Preparation	No. of sites per cell	Ref.
Malpighian tubules *Locusta migratoria*	$^+$a $3.4 \cdot 10^6$	present study
	$^+$b $8.1 \cdot 10^6$	
Chick heart membrane	a $1.5 \cdot 10^5$	46
	b $1.4 \cdot 10^6$	46
Rat intestine	$1.5 \cdot 10^6$	35
Rabbit renal tubule	$4.1 \cdot 10^6$	62
HeLa cells	$8.2 \cdot 10^5$	67
Cultured guinea-pig kidney	$7.5 \cdot 10^5$	67
Chloride cells of teleost gill	$1.5 \cdot 10^8$	61
Human erythrocyte	228	38
Manduca sexta brain	$2.5 \cdot 10^7$ *	29
Whole imaginal discs of *Drosophila melanogaster*	$1.78 \cdot 10^4$	32

If the ($Na^+ + K^+$)-ATPase pump of Malpighian tubule cells of *Locusta* move Na^+ and K^+ with the stoichiometry of $3Na^+ : 2K^+$ per ATP hydrolysed at each site (see above) then, at the calculated turnover rate, $9.1 \cdot 10^{10}$ Na^+ could be maximally pumped out of a tubule cell per min in exchange for $6.1 \cdot 10^{10}$ K^+. If one assumes that the intracellular Na^+ concentration is approx. 13 mM as reported for *Rhodnius* [63] and that cell volume is 72430 μm^3, then each cell contains ca. $5.7 \cdot 10^{11}$ Na^+ (No. of intracellular $Na^+ = Na^+$ concentration (13 mM) × cell volume × Avagadro's number). Thus, at maximal pump rate, intracellular Na^+ would be depleted in about 9 min at 30°C. In addition, total ATP utilization, on the basis of 1 ATP hydrolysed per cycle of the pump, would be $3.0 \cdot 10^{10}$ ATP/cell per min. Thus, 363000 tubule cells (i.e., estimated tubule cell number per locust) would hydrolyse $1.1 \cdot 10^{16}$ ATP per min and if 3ATP are produced for each atom of oxygen consumed then, at maximum turnover, the total oxygen consumption necessary to sustain this pump rate would be $3.0 \cdot 10^{-9}$ mol O_2 per min. This is equivalent to 0.12 μmol O_2/g wet weight per min. In the present study, the Malpighian tubules from *Locusta* consumed oxygen at a rate of 1.5 ± 0.2 μmol/g wet weight per min. Thus, approximately 8% of total metabolic activity would appear to be necessary to sustain maximal pump turnover at 30°C. This value compares favourably with the observation that 18% of tubule oxygen consumption is inhibited by 1 mM ouabain, bearing in mind the various assumptions made in its calculation and given that the higher reported levels of inhibition [4] would include secondary effects on metabolic rate due to the run-down of ion gradients.

Finally, it has been estimated that up to 1500 cells (approx. 75% of the estimated total cell number per tubule) may be responsible for the secretion of 'urine' at a mean rate of 3.4 nl/min per tubule, by in vitro preparations such as those used by Anstee et al. [4,19]. If all the K^+ transported into the cell by the ($Na^+ + K^+$)-ATPase pump were ultimately transferred to the lumen of the tubule by an apical electrogenic pump, the K^+ concentration of the secreted 'urine' could be as high as 45 mM. Indeed, at the basal secretion rate of 2.5 nl/min reported by Morgan and Mordue [9], the K^+ concentration could be as high as 61 mM, a value which would represent a substantial component of the 140 mM K^+ concentration reported for locust 'urine' [4,7]. However, the exact values calculated in the present study should not be interpreted too precisely and whilst it is unlikely that the pump would be operating maximally at all times, it is nevertheless apparent that, since the number of 'pump' sites per cell has been underestimated, the measured turnover values are adequate to account for substantial K^+ transport in Malpighian tubules of *Locusta migratoria*.

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References

1 Berridge, M.J. (1968) J. Exp. Biol. 48, 159–174
2 Coast, G.M. (1969) J. Physiol. (Lond.) 202, 102p–103p
3 Maddrell, S.H.P. and Klunsuwan, S. (1973) J. Insect Physiol. 19, 1369–1376
4 Anstee, J.H., Bell, D.M. and Fathpour, H. (1979) J. Insect Physiol. 25, 373–380
5 Anstee, J.H., Bell, D.M. and Hyde, D. (1980) Experientia 36, 198–199
6 Fathpour, H., Anstee, J.H. and Hyde, D. (1983) J. Insect Physiol. 29, 773–778
7 Morgan, P.J. and Mordue, W. (1983) J. Comp. Physiol. 151, 175–183
8 Anstee, J.H. and Bell, D.M. (1975) J. Insect Physiol. 21, 1779–1784
9 Morgan, P.J. and Mordue, W. (1981) J. Insect Physiol. 27, 271–279
10 Berridge, M.J. and Oschman, J.L. (1969) Tissue Cell 1, 247–272
11 Maddrell, S.H.P. (1971) Adv. Insect Physiol. 8, 199–331
12 Maddrell, S.H.P. (1977) in Trasport of Ions and Water in Animals (Gupta, B.L., Moreton, R.B., Oschman, J.L. and Wall, B.J., eds.), pp. 541–569, Academic Press, London
13 Phillips, J. (1981) Am. J. Physiol. 241, R241–R257
14 O'Donnell, M.J. and Maddrell, S.H.P. (1984) J. Exp. Biol. 110, 275–290
15 Gee, J.D. (1976) J. Exp. Biol. 64, 357–368
16 Gee, J.D. (1976) J. Exp. Biol. 65, 323–332
17 Harvey, W.R., Cioffi, M., Dow, J.A.T. and Wolfersberger, M.G. (1983) J. Exp. Biol. 106, 91–117
18 Anstee, J.H. and Bell, D.M. (1978) Insect Biochem. 8, 3–9
19 Donkin, J.E. and Anstee, J.H. (1980) Experientia 36, 986–987
20 Anstee, J.H. and Bowler, K. (1984) in Measurement of Ion Transport and Metabolic Rate in Insects (Bradley, T.J. and Miller, T.A., eds.), pp. 187–220, Springer-Verlag, New York

21 Bonting, S.L. (1970) in Membranes and Ion Transport (Bittar, E.E., ed.), pp. 257–363, Wiley-Interscience, London
22 Skou, J.C. (1969) in Molecular Basis of Membrane Function (Tosteson, D.C., ed.), pp. 455–482, Prentice-Hall, Englewood Cliffs, NJ
23 Proverbio, F., Robinson, J.W.L. and Whittembury, G. (1970) Biochim. Biophys. Acta 211, 327–336
24 Whittam, R. and Wheeler, K.P. (1970) Annu. Rev. Physiol. 32, 21–60
25 Podevin, R.A. and Boumendil-Podevin, E.F. (1972) Biochim. Biophys. Acta 282, 234–249
26 Anstee, J.H. and Bowler, K. (1979) Comp. Biochem. Physiol. 62A, 763–769
27 Wallick, E.T., Pitts, B.J.R., Lane, L.K. and Schwartz, A. (1980) Arch. Biochem. Biophys. 202, 442–449
28 Erdmann, E. and Schoner, W. (1973) Biochim. Biophys. Acta 307, 386–398
29 Rubin, A.L., Clark, A.F. and Stahl, W.L. (1981) Biochim. Biophys. Acta 649, 202–210
30 Wallick, E.T., Dowd, F., Allen, J.C. and Schwartz, A. (1974) J. Pharmacol. Exp. Ther. 189, 434–444
31 Pitts, B.J.R., Wallick, E.T., Van Winkle, W.B., Allen, J.C. and Schwartz, A. (1977) Arch. Biochem. Biophys. 814, 431–440
32 Fristrom, J.W. and Kelly, L. (1976) J. Insect Physiol. 22, 1697–1707
33 Jungreis, A.M. and Vaughan, G.L. (1977) J. Insect Physiol. 23, 503–509
34 Hansen, O. (1971) Biochim. Biophys. Acta 233, 122–132
35 Harms, V. and Wright, E.M. (1980) J. Membrane Biol. 53, 119–128
36 Peacock, A.J. (1975) Ph.D. Thesis, University of Durham, Durham, U.K.
37 Karnovsky, M.J. (1965) J. Cell Biol. 27, 137A
38 Erdmann, E. and Hasse, W. (1975) J. Physiol. (Lond.) 251, 671–682
39 Hill, A.V. (1909) J. Physiol. (Lond.) 39, 361–373
40 Scatchard, G. (1949) Ann. N.Y. Acad. Sci. 51, 660–671
41 Hansen, O. (1976) Biochim. Biophys. Acta 433, 383–392
42 Wallick, E.T., Lane, L.K. and Schwartz, A. (1979) Annu. Rev. Physiol. 41, 397–411
43 Noel, F. and Godfraind, T. (1984) Biochem. Pharmacol. 33, 47–53
44 Erdmann, E. (1981) in Handbook of Experimental Pharmacology (Greeff, K., ed.), 56/1, pp. 337–380, Springer, Berlin
45 Godfraind, T., De Pover, A. and Lutete, D-N.T. (1980) Biochem. Pharmacol. 29, 1195–1199
46 Kazazoglou, T., Renaud, J.-F., Rossi, B. and Lazdunski, M. (1983) J. Biol. Chem. 258, 12163–12170
47 Fricke, U. and Klaus, W. (1977) Br. J. Pharmacol. 61, 423–428
48 Fricke, U. (1985) Br. J. Pharmacol. 85, 327–334
49 Erdmann, E., Phillipp, G. and Scholz, H. (1980) Biochem. Pharmacol. 29, 3219–3229
50 Giunta, C., De Bertoli, M., Sanchini, M. and Stacchini, A. (1985) Gen. Pharmacol. 16, 183–188
51 Giunta, C., De Bertoli, M., Stacchini, A. and Sanchini, M. (1984) Comp. Biochem. Physiol. 79B, 71–74
52 Flier, J.S., Edwards, M.W., Daly, J.W. and Myers, C.W. (1980) Science 208, 503–505
53 Lichtstein, D. and Samuelov, S. (1980) Biochem. Biophys. Res. Commun. 96, 1518–1523
54 Godfraind, T., De Pover, A., Castanede-Hernandez, G. and Fagoo, M. (1982) Archs. Int. Pharmacodyn. Ther. 258, 165–167
55 Godfraind, T., Castanede-Hernandez, G., Ghysel-Burton, J. and De Pover, A. (1983) Curr. Top. Membr. Transp. 19, 913–915
56 Schwartz, A. (1983) Curr. Top. Membr. Transp. 19, 835–841
57 Jørgensen, P.L. (1974) Biochim. Biophys. Acta 356, 53–67
58 Jørgensen, P.L. (1977) Biochim. Biophys. Acta 466, 97–108
59 Hegyvary, C. and Post, R.L. (1971) J. Biol. Chem. 246, 5235–5240
60 Albers, R.W., Koval, G.J. and Siegel, G.J. (1968) Mol. Pharmacol. 4, 324–336
61 Karnaky, K.J., Kinter, L.B., Kinter, W.B. and Stirling, C.E. (1976) J. Cell Biol. 70, 157–177
62 Shaver, J.L.F. and Stirling, C. (1978) J. Cell Biol. 76, 278–292
63 Gupta, B.L., Hall, T.A., Maddrell, S.H.P. and Moreton, R.B. (1976) Nature 264, 284–287
64 El Mernissi, G. and Doucet, A. (1984) Am. J. Physiol. 247 (Renal Fluid Electrolyte Physiol. 16), F158–F167
65 Silva, P., Epstein, J.A., Stevens, A., Spokes, K. and Epstein, F.H. (1983) J. Membrane Biol. 75, 105–114
66 Joiner, C.H. and Lauf, P.K. (1978) J. Physiol. (Lond.) 283, 155–175
67 Baker, P.F. and Willis, J.S. (1972) J. Physiol. (Lond.) 224, 441–462