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THE DIFFERENCE IN SENSITIVITY TO CARDIAC STEROIDS OF ($Na^+ + K^+$)-STIMULATED ATPase AND AMINO ACID TRANSPORT IN THE INTESTINAL MUCOSA OF THE RAT AND OTHER SPECIES

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SUMMARY

1. The effect of various cardioactive steroids on the activity of a microsomal ($Na^+ + K^+$)-activated ATPase from rat intestinal mucosa has been studied and compared with their effects on L-phenylalanine and D-galactose transport by rings of rat intestine *in vitro*. A similar comparison between the sensitivities to ouabain of microsomal ($Na^+ + K^+$)-ATPase and of phenylalanine transport in the intestines of the mouse, guinea-pig and toad has been made.

2. The rat intestinal enzyme is 50% inhibited by a concentration of 1×10^{-4} M ouabain, 1×10^{-5} M scillaren A and 4×10^{-6} M scilliroside. At concentrations which almost completely inhibit the ($Na^+ + K^+$)-ATPase activity, these steroids have no effect on the transport of phenylalanine or galactose by the rat intestine. Only at concentrations of 1×10^{-3} M are scillaren A and scilliroside able to reduce phenylalanine accumulation significantly, the same concentration of ouabain being effective only in the absence of external potassium ions. Digitoxin, 1×10^{-4} M, a comparatively apolar glycoside, had no action on phenylalanine transport in the rat intestine.

3. The effect of ouabain on the ($Na^+ + K^+$)-ATPase and phenylalanine transport system in the mouse intestine is completely analogous to its effect on these parameters in the rat.

4. A half-maximal inhibition of guinea-pig intestinal ($Na^+ + K^+$)-ATPase by ouabain occurs at an inhibitor concentration of 2×10^{-6} M, but

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phenylalanine transport by this tissue is only half-maximally reduced at a concentration of 3×10^{-5} M. Similarly, in the rabbit intestine, there appears to be a difference of an order of magnitude between the sensitivities of the two parameters.

5. In the toad, 50 % inhibition of the enzymic activity is observed at a concentration of 3×10^{-5} M ouabain, whereas a concentration of 8×10^{-4} M is required to reduce phenylalanine accumulation by one half.

6. These findings are consistent with the suggestion that an $(Na^{+} + K^{+})$-stimulated ATPase is not the only enzyme in the epithelial cells of the intestinal mucosa that is responsible for sodium extrusion (the mechanism for sodium extrusion being intimately coupled with the mechanism for active amino acid transport); therefore, a second, as yet unidentified, enzyme system must be postulated to account for bulk sodium flow through the intestine.

INTRODUCTION

An ATPase stimulated by sodium and potassium ions is widely assumed to be involved in sodium pumping across biological membranes (see reviews by Skou, 1965; Schatzmann, 1967; Gajdos, 1967; and Katz & Epstein, 1967). The possibility that sodium pumping involves a mechanism independent of ATP break-down has only rarely been considered; in these cases, the participation of cytochrome enzymes has been inferred (Siekevitz, 1965; Kessler, 1966; Kessler, Landwehr, Quintanilla, Weseley, Kaufmann, Arcila & Urbaitis, 1968). From indirect evidence, it was also proposed (Robinson, 1967) that the sodium pump mechanism of the rat and mouse intestinal mucosa which is assumed to drive the uphill transport of amino acids and monosaccharides in this tissue (Crane, 1965; Robinson & Vannotti, 1966) consists of a mechanism which does not involve a $(Na^{+} + K^{+})$-stimulated ATPase. This conclusion derived from certain properties of the transport systems of the rat and mouse intestine which did not parallel the findings in rabbit intestine, where the $(Na^{+} + K^{+})$-dependent hydrolysis of ATP is firmly believed to be associated with the sodium pump activity (Rosenberg, Coleman & Rosenberg, 1965). The decisive property which questions the participation of an ATPase in the pumping of sodium in the rat and mouse intestine is the insensitivity of sodium, amino acid and sugar transport to cardioactive steroids when studied *in vivo* (Cocco, Rosensweig & Hendrix, 1965) or *in vitro* (Ponz, 1963; Faust, 1964; Binder, Boyer, Spiro & Spencer, 1966; Dettmer, Müller & Kuhfahl, 1967; Lauterbach, 1967; Robinson, 1967).

In order to provide a firmer basis for the hypothesis that a $(Na^{+} + K^{+})$-ATPase is not principally involved in transport in the rat intestine, the activity of this enzyme was measured in homogenates of the rat intestinal

mucosa and its sensitivity to cardiac steroids was determined. Further studies were also carried out on the effects of different cardioactive steroids on phenylalanine or galactose transport under different conditions. No correlation between the sensitivity of the ATPase and of the transport systems was observed, although in other tissues from the rat in which the ATPase is supposed to provide the sodium pump mechanism, a relatively good correlation between the sensitivities of the enzyme and the pump has been demonstrated (Grobecker, Piechowski & Greeff, 1963; Repke, Est & Portius, 1965). It is therefore concluded that the $(Na^+ + K^+)$-ATPase is unlikely to constitute the active sodium pump that is coupled to amino acid transport in this tissue under normal conditions. A comparison with the intestines of other species has disclosed a marked discrepancy between the sensitivities of pump and enzyme of the same animal to ouabain, a finding which suggests that a $(Na^+ + K^+)$-stimulated, ouabain-sensitive ATPase is unlikely to represent the sole sodium pumping mechanism in any intestinal epithelia. A preliminary report of some of these results has appeared (Robinson, 1968), and during the course of the work independent confirmation of some of the findings was published from elsewhere (Dettmer *et al.* 1967).

METHODS

Materials. For transport studies, L-amino acids and D-sugars of the purest commercial form were employed. The corresponding radioactive compounds were obtained uniformly labelled from the Radiochemical Centre, Amersham, G.B. or New England Nuclear, Boston, U.S.A.

Ouabain (Strophanthin-G) was purchased from E. Merck, A.G., Darmstadt, or Sigma Chemical Company, St Louis, U.S.A.; digitoxin originated from Serva-Entwicklungslabor, Heidelberg; and scillaren A and scilliroside were graciously provided by Sandoz, A. G., Basel and Nürnberg.

To prepare aqueous solutions of the steroids of sufficiently high concentrations, a solubilizer was required for all compounds except ouabain. Methanol was used in the case of scilliroside, ethanol or glycerol for scillaren A, and ethanol for digitoxin; the final concentration of the solubilizer never exceeded 1 %.

Determination of amino acid or sugar uptake. The preparation of slices of rat, mouse and rabbit intestine was performed exactly as described previously (Robinson, 1967). The guinea-pig intestine was prepared in the same way as for the rat. For experiments on the toad (*Bufo marinus*), the material employed consisted of the duodenum of freshly captured animals which had not had time to undergo intestinal atrophy due to autophagy. The duodenum was removed from pithed toads, opened along the mesenteric border and cut into strips of wet weight 20–30 mg, which were then treated in the same manner as the slices of mammalian intestine. As incubation medium, Krebs bicarbonate buffer was used for mammalian tissue and frog–Ringer for the toad, both solutions being prepared as described by Dawson, Elliott, Elliott & Jones (1959). The slices were incubated at 37° C for 1 hr (by which time equilibrium had been reached) in a solution of [^{14}C]L-phenylalanine or [^{14}C]D-galactose in the requisite medium, 0·2 % D-glucose (w/v) being added in the former case. The steroids were also added to the incubation media when appropriate, and controls were run in the presence of solubilizer without steroid.

After the incubation, the slices were weighed and dissolved in 0·1 ml. 30% KOH (w/v) and counted in a liquid scintillation counter in a toluene/ethanol mixture, conforming to usual practice (Robinson, 1966, 1967). The total amino acid or sugar content of the tissue was calculated by comparison with the specific activity of the incubation medium, aliquots of which were counted under identical conditions. In all control experiments, the substrate was accumulated against a concentration gradient. Five slices from each animal were subjected to each treatment and each experiment was repeated three to six times with different animals. The results were analysed by means of a global analysis of variance which takes into account the variation between slices within each animal and the variation between animals (Wardlaw & Van Belle, 1964). From the error variance of the experiment can be calculated a value of the Least Significant Difference at a given value of P ($D_{p=0\cdot05}$; $D_{p=0\cdot01}$; $D_{p=0\cdot001}$) which may be used to compare differences between any of the means of the experiment, since the same number of samples is used in each experimental condition and the variances of the different means are similar.

Preparation of a ($Na^+ + K^+$)-stimulated ATPase. The mucosa from the rat intestine was scraped free from the underlying muscular layers and homogenized at 0° C in 0·25 M sucrose containing 1 mM-EDTA (10 ml. solution per g wet tissue). Tris buffer did not appear to be necessary, despite the stipulations of Dettmer *et al.* (1967) that glucose (liberated from the sucrose in the absence of Tris) inhibits the ($Na^+ + K^+$)-ATPase. The homogenate was centrifuged for 10 min at 3000 *g*, the sediment resuspended in the same volume of sucrose/EDTA and recentrifuged at the same speed. The two supernatants were then combined and recentrifuged for 1 hr at 100,000*g*. The sediment from this centrifugation was suspended in 4 ml. of the sucrose/EDTA medium and employed for the study of the ATPase activity.

This crude microsomal fraction contained, in the case of the rat, a preponderance of Mg^{2+}-ATPase which precluded accurate determination of the properties of the ($Na^+ + K^+$)-ATPase. However, it was found that on standing for several days at 0° C, a spectacular rise in the ($Na^+ + K^+$)-activated component of the rat enzyme preparation occurred. An increase of this nature has been observed, coupled with a reduction in the Mg^{2+}-ATPase activity, in ATPase preparations from other tissues (Schoner, von Ilberg, Kramer & Seubert, 1967), but no such dramatic alteration in the balance of the enzymes as that shown in Fig. 1 for the rat intestine has previously been demonstrated. In the case of the rat enzyme, a peak in the activity of the ($Na^+ + K^+$)-stimulated moiety is attained after about 8 days.

The enzyme from the mouse intestine was prepared in an identical fashion, except that tissue from six animals was pooled for the initial homogenization. The stimulation of the ($Na^+ + K^+$)-activated component of the enzyme complex on ageing takes place much more rapidly in the case of the mouse enzyme and the final inactivation occurs during the third day (as opposed to after 2 weeks in the rat). Hence no detailed time course of ageing process was plotted for this species.

The enrichment of the ($Na^+ + K^+$)-ATPase activity on ageing was not encountered with guinea-pig or toad intestinal homogenates. In these species, it was necessary to use another method to increase the yield of the ($Na^+ + K^+$)-stimulated component, namely the addition of a detergent to the original suspension medium. Deoxycholic acid, 0·2%, neutralized with Tris buffer, pH 7·2, was employed (Schoner *et al.* 1967). This method, incidentally, was ineffective in increasing the ($Na^+ + K^+$)-ATPase activity in the microsomal fraction of the rat or mouse intestine. The microsomal fraction of the guinea-pig and toad intestinal epithelia was isolated from the homogenate in the same manner as that of the rat (though better results were obtained in the toad if the initial centrifugation was performed at 5000 *g*) and was suspended in sucrose/Tris/deoxycholate/EDTA for assay. The suspension was then tested immediately.

Assay of the ATPase activity. Aliquots of the enzyme suspension were incubated for 10 min in the presence of 100 mM imidazole buffer (pH 7·2), 5 mM-$MgCl_2$ and 2 mM-Na^+-free ATP. When appropriate, 100 mM-NaCl and 10 mM-KCl were added. The cardiac steroids were also added to certain samples at concentrations that were dictated by the experiment in question. In these experiments, the enzyme was only exposed to the glycosides during the 10 min test period. Preliminary experiments showed that under these conditions, there was a linear relationship between phosphate liberation and incubation time, and between phosphate liberation and enzyme concentration. The incubation was terminated by addition of 6% perchloric acid and the liberated phosphate was determined in the deproteinized solution by the method of King (1932). The protein content of the original suspension was measured by the method of Lowry, Rosebrough, Farr & Randall (1951).

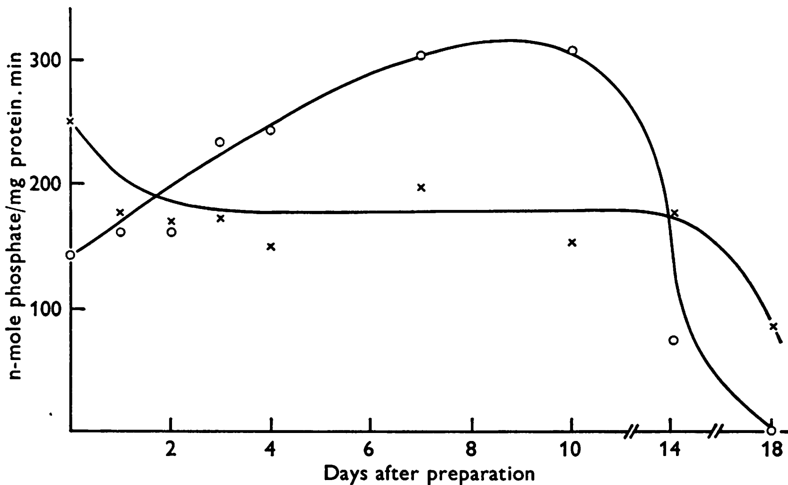

Fig. 1. Development of the ($Na^+ + K^+$)-stimulated ATPase activity during ageing of the rat intestinal microsomal preparation. The ordinate represents the enzymic activity (n-mole phosphate liberated/mg protein . minute). The enzyme was aged at 0° C. ○—○ represents the activity of the ($Na^+ + K^+$)-ATPase (total ATPase minus residual ATPase) and ×—× represents the Mg^{2+}-ATPase (residual ATPase).

RESULTS

Experiments on the rat intestine

Inhibition of the microsomal ($Na^+ + K^+$)-ATPase. The effect of ouabain, scillaren A and scilliroside on the activity of the ($Na^+ + K^+$)-stimulated component of the ATPase of the rat intestine is depicted in Fig. 2. Ouabain provokes a half-maximal inhibition at a concentration of about 100 μM, a value that confirms the findings of Berg & Szekerczes (1966), who incidentally used a fresh rather than an aged preparation of the enzyme. Scillaren A, known to be a potent inhibitor of rat erythrocyte ATPase (Hoffman, 1962), reduced the ($Na^+ + K^+$)-ATPase activity by

50% at a concentration of 10 μM, whereas scilliroside, the glycoside to which the rat is pharmacologically most sensitive (Rothlin & Schalch, 1952), caused a 50% inhibition at a concentration of 4 μM. The influence of digitoxin was not determined, but in other rat tissues, it is known to have roughly the same potency of action on the $(Na^+ + K^+)$-ATPase as ouabain (Repke *et al.* 1965).

As explained in the Methods section, a fresh preparation of rat intestinal ATPase contains only a relatively small component which is activated by sodium and potassium ions, and therefore an aged preparation, in which

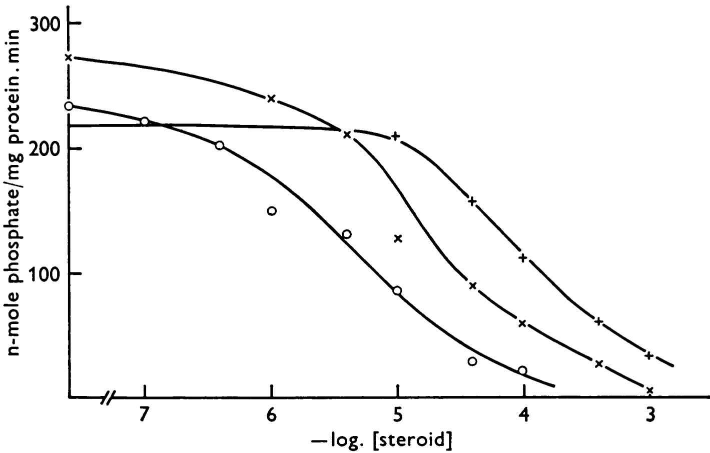

Fig. 2. Dose–response curve of the inhibition of the $(Na^+ + K^+)$-ATPase from the rat intestinal mucosa by different cardiac steroids: ○—○, scilliroside; ×—×, scillaren A; +—+, ouabain. Each experiment was carried out with a different preparation of the enzyme. The points represent the means of at least three separate determinations.

the proportion of Mg^{2+}-stimulated ATPase has been reduced, had to be used for the quantitative assessment of the sensitivity of the $(Na^+ + K^+)$-activated enzyme to cardiac steroids. It is imperative to point out, however, that the small component in the fresh preparation that is stimulated by sodium and potassium ions is always sensitive to ouabain. Typical values (expressed in μ-mole phosphate liberated/mg protein . min) for an experiment on a fresh preparation are:

Total ATPase activity in absence of Na^+ and K^+	0·238
Total ATPase activity in presence of Na^+ and K^+	0·357
Total ATPase activity in presence of Na^+, K^+ and 1 mM ouabain	0·240

This result is important, since it shows that even if the percentage of $(Na^+ + K^+)$-activated component changes on ageing, both the aged and fresh enzymes are completely inhibited by 1 mM ouabain.

Effect of cardioactive steroids on absorption mechanisms. The inability of 1 mM ouabain to inhibit the active uptake of L-phenylalanine by rat intestinal slices is confirmed by the results in Table 1. When potassium ions are omitted from the incubation medium, however, a slight inhibition of phenylalanine transport by ouabain does occur, although the control

TABLE 1. Effect of ouabain on L-phenylalanine uptake by rat intestinal rings in the presence of different potassium-ion concentrations

Potassium-ion concentration (mM)		Ouabain concentration (mM)	L-phenylalanine uptake (μ-mole/100 mg wet tissue)	Mean tissue concentration
6		—	1·663	20·8
6		1	1·642	20·5
—		—	1·732	21·7
—		1	1·405	17·6
30		—	1·331	16·6
30		1	1·291	16·1
	$D_{p=0\cdot05}$		0·252	3·15
	$D_{p=0\cdot01}$		0·342	4·28
	$D_{p=0\cdot001}$		0·457	5·71

Uptake of 5 mM-L-phenylalanine determined in the presence or absence of ouabain in Krebs buffer (6 mM-K^+) or modifications thereof. Isotonicity maintained by modifying the concentrations of Na^+ at the same time as the K^+ concentration is altered. Results are the mean of six experiments on different animals. Mean tissue concentration in mM is calculated on the basis of a dry weight of 20 % of the wet weight.

value is unaltered. Newey, Sanford & Smyth (1968) reported similar findings, which may be related to the known antagonism between cardiac steroids and potassium ions (Skou, 1965) though, in complete absence of potassium ions, an ATPase sodium pump should not function (Katz & Epstein, 1967). When the potassium content of the medium is increased, the transport of the non-electrolyte is reduced in accordance with earlier reports (Rummel & Stupp, 1960; Larralde, Bello & Fernández-Otero, 1962; Robinson, 1966), but ouabain is again without effect.

In view of the fact that ouabain originating from the Sigma Co. was found to be much more soluble in water than that of Merck, it was possible, using the former, to study the effect of 10 mM ouabain on phenylalanine transport by the rat intestine, at which concentration Newey *et al.* (1968) found all transport systems to be significantly inhibited. The results

obtained were very variable: the uptake in the absence of ouabain was $1{\cdot}807 \pm 0{\cdot}161$ and in its presence was $1{\cdot}362 \pm 0{\cdot}176$ μ-mole/100 mg wet tissue ($n = 6$ in each case). The non-significance of the difference between these two values is due to the fact that, whereas in some individual animals there was a strong inhibition (significant at the 0·1 % level), in others there was no effect at all; indeed one animal demonstrated a slight but significant stimulation in the presence of the drug!

The results in Table 2 show that ouabain has no effect on D-galactose uptake by the rat intestine at a concentration of 1 mM. A survey of the literature has shown that a few authors have reported an inhibitory effect

TABLE 2. Effect of ouabain and scillaren A on D-galactose uptake by rat intestinal rings

Drug added	Solubilizer	D-galactose uptake (μ-mole/100 mg wet tissue)
None	None	0·862
1 mM ouabain	None	0·904
1 mM scillaren A	1 % ethanol	0·408
0·25 mM scillaren A	0·25 % ethanol	0·759
None	1 % ethanol	0·714
None	0·25 % ethanol	0·838
$D_{p=0{\cdot}05}$		0·221
$D_{p=0{\cdot}01}$		0·303
$D_{p=0{\cdot}001}$		0·413

The concentration of the sugar in the incubation medium was 5 mM. The results are the means of three animals.

of ouabain on the transport of glucose by the rat intestine (Ponz, 1963; Lluch & Ponz, 1964; Newey *et al.* 1968) under certain experimental conditions, but in each case, glucose transport was determined as the amount of hexose disappearing from the incubation medium. Because of the known inhibition by high concentrations of ouabain of the catabolism of glucose by various tissues (Gordon, 1965; Clausen, 1966; Newey *et al.* 1968)—indeed these latter authors demonstrated a greater effect of the drug on intestinal metabolism than on intestinal transport—it is to be suspected that the reported inhibition of glucose transport is secondary to an inhibition of glucose metabolism. The present results demonstrate clearly that ouabain at a concentration of 1 mM does not inhibit the uptake of the non-metabolizable hexose, D-galactose.

Experiments showing the effect of other cardiac glycosides on galactose and phenylalanine uptake by the rat intestine are reported in Tables 2 to 5. Scillaren A strongly inhibits both galactose and phenylalanine transport at concentrations higher than 0·5 mM (Tables 2 and 3); the auxiliary

results in Table 3 indicate that the inhibition could not be ascribed solely to effects of the solubilizers used. As is shown in Table 4, scilliroside only very slightly lowers the phenylalanine equilibrium position at the highest concentration tested (1 mM), which is nearly 1000 times the concentration which elicits a comparable inhibition of the $(Na^{+}+K^{+})$-ATPase. The fact that scilliroside is the stronger inhibitor of the enzymic activity whereas

TABLE 3. Effect of scillaren A on L-phenylalanine uptake by the rat intestine

Scillaren A concentration (mM)	Solubilizer	L-phenylalanine uptake (µ-mole/100 mg wet tissue) Expt. 1	Expt. 2
None	None	1·542	1·570
1	1 % ethanol	0·802	0·726
1	1 % glycerol	—	0·875
0·75	0·75 % ethanol	0·973	—
0·5	0·5 % ethanol	1·326	—
0·25	0·25 % ethanol	1·554	—
0·1	0·1 % ethanol	1·692	1·607
0·1	0·1 % glycerol	—	1·625
0·05	0·05 % ethanol	1·612	—
None	1 % ethanol	—	1·380
None	1 % glycerol	—	1·441
None	0·1 % ethanol	—	1·631
None	0·1 % glycerol	—	1·469
$D_{p=0\cdot05}$		0·293	0·343
$D_{p=0\cdot01}$		0·399	0·463
$D_{p=0\cdot001}$		0·538	0·617

Medium concentration of the amino acid was 5 mM. Each experiment represents the means of four animals.

TABLE 4. Effect of scilliroside on L-phenylalanine uptake by rat intestinal rings

Scilliroside concentration (mM)	Solubilizer	L-phenylalanine uptake (µ-mole/100 mg wet tissue)
None	None	1·646
1	1 % methanol	1·201
0·1	0·1 % methanol	1·618
None	1 % methanol	1·590
None	0·1 % methanol	1·513
$D_{p=0\cdot05}$		0·309
$D_{p=0\cdot01}$		0·440
$D_{p=0\cdot001}$		0·636

Medium concentration of L-phenylalanine was 5 mM. Results are the means of experiments on three different rats.

scillaren A is the stronger inhibitor of the phenylalanine uptake mechanism leads one to suspect that the influence of these two bufodienolides on non-electrolyte transport may be a non-specific effect unrelated to their inhibition of the ATPase activity.

TABLE 5. Effect of digitoxin on L-phenylalanine uptake by the rat intestine

Digitoxin concentration	Solubilizer	L-phenylalanine uptake (μ-mole/100 mg wet tissue)
None	None	1·611
0·1	1 % ethanol	1·307
0·04	0·4 % ethanol	1·530
0·01	0·1 % ethanol	1·601
None	1 % ethanol	1·409
None	0·4 % ethanol	1·643
$D_{p=0 \cdot 05}$		0·333
$D_{p=0 \cdot 01}$		0·451
$D_{p=0 \cdot 001}$		0·604

Medium concentration of L-phenylalanine was 5 mM. Results are the means of six experiments on different rats.

TABLE 6. Influence of cardioactive steroids on L-phenylalanine uptake by mouse intestinal slices

Inhibitor added	Solubilizer	L-phenylalanine uptake (μ-mole/100 mg wet tissue)
None	None	1·719
1 mM ouabain	None	1·769
0·25 mM ouabain	None	1·835
1 mM scillaren A	1 % ethanol	0·673
0·25 mM scillaren A	0·25 % ethanol	1·606
0·1 mM scillaren A	0·1 % ethanol	1·812
None	1 % ethanol	1·343
None	0·25 % ethanol	1·690
None	0·1 % ethanol	1·684
$D_{p=0 \cdot 05}$		0·257
$D_{p=0 \cdot 01}$		0·347
$D_{p=0 \cdot 001}$		0·462

Medium concentration of L-phenylalanine was 5 mM. Results are the means of four experiments on different mice.

Finally, the relatively apolar glycoside, digitoxin, which is said to permeate the guinea-pig intestinal epithelial cells more freely (Forth & Rummel, 1967), was also tested. Its low solubility precluded use of concentrations higher than 100 μM; at this level no significant effect on phenylalanine uptake was observed (Table 5) when the influence of the solubilizer

is taken into account. This result is in accordance with the findings of Baker, Wall, Watson & Long (1968) who were unable to demonstrate any effect of this drug on the transmural potential difference across the rat intestine.

Experiments on the mouse intestine

The mouse intestine reacts to cardioactive steroids in a manner completely analogous to that of the rat intestine. The response of the microsomal ($Na^+ + K^+$)-ATPase to ouabain is very similar to that of the rat

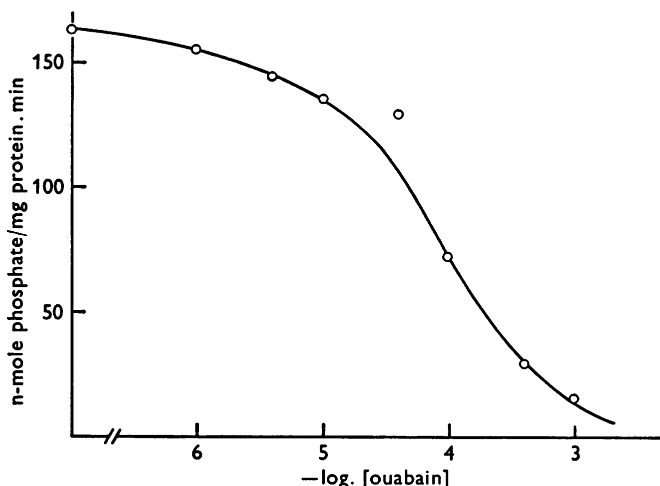

Fig. 3. Dose–response curve of the inhibition of the ($Na^+ + K^+$)-ATPase from the mouse intestinal mucosa by ouabain.

enzyme (Fig. 3), a half-maximal inhibition being observed at an inhibitor concentration of 100 μM. The inability of ouabain to inhibit phenylalanine transport by mouse intestinal slices is demonstrated in Table 6, where it is also shown that the sensitivity of this tissue to scillaren A is identical to that of the rat intestine.

Experiments on the guinea-pig and rabbit intestines

The transport of amino acids and monosaccharides by the guinea-pig intestine is known to be sensitive to ouabain (Binder *et al.* 1966) as is, of course, the ($Na^+ + K^+$)-stimulated ATPase of this tissue (Rosenberg & Rosenberg, 1968). A direct comparison of the sensitivities of these two parameters to ouabain is presented in Fig. 4, where the % inhibition of each is plotted on the ordinate (the % inhibition of the amino acid uptake is calculated by assuming that an inhibition of 100 % reduces the tissue/medium concentration ratio to unity). From the curves obtained, it can be deduced that a 50 % inhibition of the ($Na^+ + K^+$)-ATPase activity is

attained at an inhibitor concentration of 2 μM, a value which agrees closely with that published by Rosenberg & Rosenberg (1968). On the other hand, 50 % inhibition of phenylalanine transport was only provoked at a ouabain concentration of 30 μM.

The absorption of phenylalanine by rabbit intestine is very sensitive to ouabain, a half-maximal inhibition being obtained at an inhibitor concentration of about 10 μM (Fig. 5). Unfortunately it was not possible to

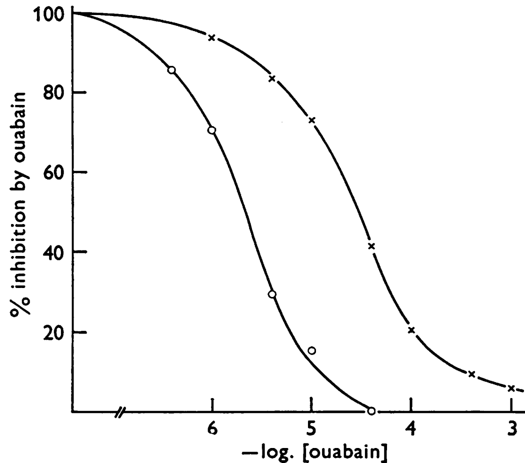

Fig. 4. Dose–response curve of the inhibition of the $(Na^{+}+K^{+})$-ATPase and of the L-phenylalanine transport system of the guinea-pig intestinal mucosa by ouabain. For the determination of the % inhibition of the phenylalanine transport system, 100 % inhibition is deemed to be effected when the tissue/medium concentration ratio for the amino acid is reduced to unity (mean tissue concentration of the amino acid calculated by assuming a wet weight/dry weight ratio of 5/1).

isolate a $(Na^{+}+K^{+})$-ATPase from the intestine of this species. However, since it is known that the sensitivity of a $(Na^{+}+K^{+})$-ATPase to cardiac steroids varies little from one tissue to another within a given species (Berg & Szekerczes, 1966), it is reasonable to suppose that the sensitivity of the intestinal enzyme varies little from the values cited in the literature for other rabbit tissues, namely a half-maximal inhibition of 0·63 μM for cardiac muscle (Auditore & Murray, 1962), 1·2 μM for lens epithelium and 1·0 μM for ciliary body (both quoted by Bonting, Caravaggio & Hawkins, 1962). Hence the ATPase would be ten times more sensitive to ouabain than the phenylalanine transport system in the rabbit as well as in the guinea-pig.

Experiments on the toad intestine

In view of the report of Repke *et al.* (1965) that the toad is even less sensitive than the rat to cardiac glycosides, it seemed of interest to study the effect of ouabain on the transport system and on the $(Na^{+}+K^{+})$-ATPase in this species. The phenylalanine transport system in the toad,

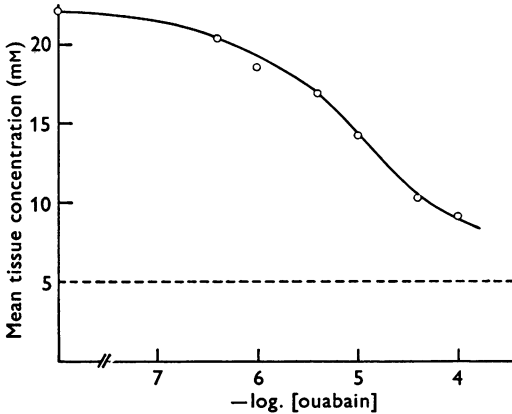

Fig. 5. Dose–response curve of the inhibition of L-phenylalanine uptake by rabbit intestinal slices by ouabain. The ordinate represents the mean tissue concentration of the amino acid after the incubation, calculated on the basis of a 5/1 wet weight/dry weight ratio. The medium concentration of the amino acid was 5 mM.

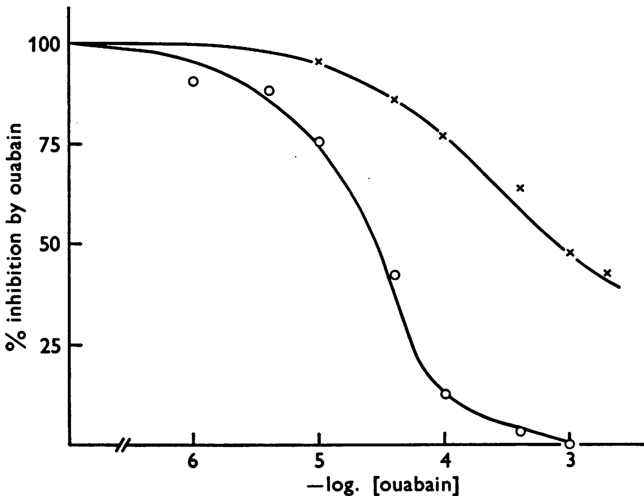

Fig. 6. Dose–response curve of the inhibition of the $(Na^{+}+K^{+})$-ATPase and of the L-phenylalanine transport system of the toad intestine by ouabain. For explanation, see legend to Fig. 4.

in contrast to the rat, is sensitive to ouabain, a significant inhibition being perceived at an inhibitor concentration of 0·1 mM and a 50 % inhibition being observed at a concentration of about 0·8 mM (Fig. 6).

Difficulties were encountered in the preparation of a $(Na^+ + K^+)$-ATPase from the toad intestine because the enzyme level seems to be particularly low in this tissue. In fact, the maximum enzymic activity obtained was only 60 n-mole phosphate liberated/mg protein.min, or approximately 20 % of the activity of the rat enzyme. Nevertheless an estimate of the sensitivity of the enzyme to ouabain has been made and a value of 30 μM for 50 % inhibition determined (Fig. 6). Repke *et al.* (1965), studying the ATPase of the heart muscle of *Bufo bufo*, also obtained a very low yield of the enzyme; they demonstrated a half-maximal inhibition of the ATPase at a concentration of 0·1 mM ouabain, the enzyme being less sensitive than that of the rat, whereas the present results show that the intestinal enzyme of *Bufo marinus* is somewhat more sensitive to this inhibitor than that of the rat. Nevertheless this difference in sensitivity of the $(Na^+ + K^+)$-ATPase is insufficient to account for the quite strong inhibition of phenylalanine transport in the toad and complete absence of inhibition in the rat in the presence of 1 mM ouabain.

DISCUSSION

The foregoing results indicate that there is a palpable discrepancy between the sensitivity of the $(Na^+ + K^+)$-stimulated ATPases studied and that of the L-phenylalanine transport systems to the various cardioactive steroids in all species, but that this divergence is greater in some animals than in others. At least three explanations can be put forward to account for these findings, namely that the gradient-coupling theory of Crane (1965) does not adequately explain intestinal amino acid transport; that the steroids are unable to gain access to the inhibitory site of the $(Na^+ + K^+)$-ATPase when studied in whole tissue slices; or that the $(Na^+ + K^+)$-stimulated, steroid-sensitive ATPase is not the only sodium pump mechanism in the intestinal epithelial cell.

According to the theory of Crane (1965), which appears to be applicable to the transport of amino acids by the rat intestine (Robinson & Vannotti, 1966), uphill transport of amino acids or hexoses occurs by an interaction between the movement of a ternary carrier/sodium ion/non-electrolyte complex across the mucosal membrane of the epithelial cell and an active sodium pump situated in the basal membrane of the same cell. Non-electrolyte entry is supposed to be driven by the downward sodium gradient across the luminal membrane which is maintained by the action of the sodium pump. One of the salient properties of intestinal sugar trans-

port which led to the development of this theory was in fact its sensitivity to agents, in particular cardiac glycosides, which are known inhibitors of the sodium pump. Dettmer *et al.* (1967) have already submitted that the lack of correlation between the sensitivity of the rat intestinal ATPase to ouabain and the sensitivity of monosaccharide transport to the same drug represents a strong argument against this theory. Conditions have also been described whereby sodium and glucose transport in the rat intestine are affected differently by changes in the environment (Rummel & Stupp, 1960), which could be construed as evidence against a tight coupling of the two processes. However, recent work by Schultz & Curran and co-workers (Schultz, Curran, Chez & Fuisz, 1967; Curran, Schultz, Chez & Fuisz, 1967) has added considerable weight to the gradient-coupling theory and also provided an explanation for some of the discrepant facts submitted by other laboratories. It is therefore this author's opinion that an explanation for the results in the current work must be found elsewhere.

The question as to whether the cardiac steroids can gain access to the $(Na^+ + K^+)$-ATPase in such a complex tissue as the intestine is almost impossible to test experimentally, but much circumstantial evidence exists to suggest that the inhibitors should be able to reach the active sites of the enzyme. Ouabain has often been shown to influence intestinal transport more effectively when administered on the serosal side of the tissue (Smith, 1964), probably because the steroid-sensitive site lies in the serosal membrane, and probably, by analogy with other cells, at its outer surface (Skou, 1965). Considerable diffusion barriers for cardioactive steroids and other large molecules have been demonstrated in the muscular layers of the rat intestine (Lauterbach, 1968), which would tend to limit access of the drugs to the enzymic sites. On the other hand, it has been demonstrated in the rat (Lauterbach, Gerber, Nitz & Prescher, 1967; Nadal, Prous & Ponz, 1967; Lauterbach, 1968) that ouabain and other glycosides are able to cross the intestinal cells *in vitro*, the movement displaying the properties of a saturable transport system. Digitoxin, with its more apolar properties, even exerts its inhibitory action on transport in the guinea-pig intestine more effectively from the mucosal slide (Forth, & Rummel, 1967), despite the fact that the pump is supposed to be serosally orientated; this indicated that it is transported across the cells more readily than it diffuses through the muscular layers. In the present work, both serosal and mucosal faces of the tissue were in constant contact for one whole hour with the incubation medium, so in view of the results quoted above it is unlikely that lack of accessibility is the only factor involved in the lack of correlation between the sensitivities of the transport systems and the $(Na^+ + K^+)$-ATPase to cardiac steroids.

The third possible explanation, and the one favoured on balance in this discussion, is that the $(Na^{+}+K^{+})$-ATPase does not represent the only sodium pump mechanism in the intestinal epithelium, and that there is a second, as yet unidentified, enzyme system which is responsible for the extrusion of sodium from these cells. In certain animal species, the $(Na^{+}+K^{+})$-ATPase may represent a component of the sodium pump activity that is allied to amino acid transport, but in the rat and the mouse, it appears to be hardly involved at all.

The existence of two different sodium pumps within the same cell has been postulated in many types of cell, such as the kidney (Whittembury, 1967, 1968), the toad bladder (Thier, 1968) and the erythrocyte (Hoffman & Kregenow, 1966). In the guinea-pig kidney cortex slice, one sodium pump is found to be dependent on external potassium ions and inhibited by ouabain, and appears to be responsible for the regulation of cellular ion content; the other is independent of extracellular potassium, refractory to ouabain and inhibited by ethacrynic acid, and appears to be responsible for the regulation of cell volume (Whittembury, 1967, 1968). Although ethacrynic acid appears to have no effect on the sodium pumping mechanisms of the rat intestine (Robinson & Förster, 1968), it is tempting to suggest that a similar dichotomy exists in the rat intestine. Such an argument would be in agreement with the analysis of Schultz *et al.* (1967) who proposed that a mechanism for sodium extrusion existed in the mucosal border of the epithelial cell and was primarily responsible for the regulation of the cellular ionic content. As a corollary to these findings, it has also been recently shown that two distinct types of $(Na^{+}+K^{+})$-dependent ATPases with different kinetic characteristics and different responses to hormonal regulators exist in the different membranes of the toad bladder epithelium (Gachelin & Bastide, 1968). A similar duplicity of ATPase types appears to exist in the goldfish intestine (Smith, Colombo & Munn, 1968).

Evidence from other laboratories has also accumulated to question the identity of the rat intestinal sodium pump with a $(Na^{+}+K^{+})$-ATPase. After fractionation of the rat intestinal mucosa, Berg & Chapman (1965) found the greatest concentration of $(Na^{+}+K^{+})$-ATPase in the brush border, not in the serosal or lateral membranes which the physiological concept of the intestinal epithelial cell would require. Histochemical studies confirmed that a $(Na^{+}+K^{+})$-ATPase was to be found in the basal membrane of the goldfish intestine (Hollands & Smith, 1964), a species whose transport systems are ouabain-sensitive (Smith, 1964), but the same authors failed to locate a corresponding enzyme in the basal membrane of the rat intestine. Earlier results from this laboratory (Robinson, 1966, 1967) have also suggested that different enzymic mechanisms are

involved in pumping sodium in the rabbit intestine on the one hand and in the rat and mouse intestines on the other. On preincubation in a sodium-free medium, rat and mouse intestinal slices rapidly lose their ability to accumulate amino acids against a concentration gradient, whereas rabbit intestinal slices are almost unaffected by this treatment. By inference, this loss of activity was attributed to an inactivation of the sodium pump in the basal membrane of the cell (Robinson, 1966, 1967). Since there seems to be a correlation among different animal species between susceptibility to the sodium-free preincubation and insensitivity to cardiac steroids, it was previously postulated that in rat and mouse intestine, the principal sodium pump mechanism does not involve the $(Na^{+}+K^{+})$-ATPase. It is interesting in this regard that amino acid and glucose transport in the rat kidney are inhibited by ouabain (Fox, Thier, Rosenberg & Segal, 1964; Ruedas & Weiss, 1967), but this tissue is not irreversibly incapacitated by a preincubation in Na^{+}-free, K^{+}-substituted medium (Fox *et al.* 1964), a fact that suggests that these findings are tissue-specific as well as species-specific.

In summary, it is suggested that sodium extrusion from the intestinal epithelial cell is a complex phenomenon which shows some variation between species. All species seem to possess a $(Na^{+}+K^{+})$-ATPase in the intestinal epithelium, but there is a great diversification in its involvement in bulk sodium transport linked to amino acid and monosaccharide accumulation. In the rat intestine, at one extreme, the enzyme appears to be primarily located in the brush border and negligibly involved in trans-cellular sodium transport; it is probably responsible for the regulation of cellular ionic content. In the other species studied (i.e. the toad and the guinea-pig), this enzyme is responsible to a greater or lesser extent for bulk sodium movement, but it is probable, unless the accessibility of the active site of the enzyme to cardiac steroids *in vitro* is much smaller than would be expected, that another enzyme system is also involved; this is the enzyme system that is responsible for almost all trans-cellular transport in the rat. This hypothesis also provides an explanation for an important qualitative difference between bulk sodium transport across epithelia and sodium extrusion from single cells, namely that epithelial sodium transport does not appear to be accompanied by potassium transport in the opposite direction. Kessler *et al.* (1968), studying bulk sodium transport in the kidney *in vivo*, have provided evidence to question the involvement of energy emanating from ATP and have postulated the direct participation of cytochrome enzymes in this process. It will be interesting to see whether this work can be extrapolated to the intestinal epithelium.

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