J. Physiol. (1957) 136, 148–173

THE ACTION OF CARDIAC GLYCOSIDES ON SODIUM AND POTASSIUM MOVEMENTS IN HUMAN RED CELLS

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(*Received* 27 *October* 1956)

This paper describes the effects of a number of cardiac glycosides on the movements of sodium and potassium across the human red cell membrane. A preliminary account of some of the experiments reported here has already been published (Glynn, 1955). The aim of the work was, first, to decide whether the glycosides acted directly on the transport mechanism, and, secondly, to see if the effects of the glycosides could be made to yield any information about the molecular basis of cation transport.

Many substances affect the movements of sodium and potassium but practically all of them act indirectly. Some, like the lysins, make the membrane leaky and so increase 'downhill' fluxes; others, like iodoacetate, inhibit energy metabolism and prevent active transport. In 1953 Schatzmann found that strophanthin, an active principle from *Strophanthus gratus*, prevented the uptake of potassium and elimination of sodium that normally occur when cold-stored red cells are incubated with glucose at 37° C. Other cardiac glycosides have been found to give similar results (Joyce & Weatherall, 1955; Kahn & Acheson, 1955). Schatzmann, in his original paper, noted that concentrations of glycosides sufficient to affect ion movements had no effect on oxygen consumption or on lactic acid formation, and he concluded that the action of the drugs lay beyond the stage of energy production. It follows that the glycosides must act either by preventing energy released during glycolysis from reaching the ionic pump, or by interfering with the carrier mechanism itself. These two theories lead to different predictions, and experiments will be described which show that it is probably the second that is correct. The cardiac glycosides thus emerge as the only substances—apart of course from the metallic cations—which there is, at present, reason to believe act on the carrier system.

It is of interest to know how much of the structure of the glycoside molecule is necessary for the action on the ion fluxes, and an attempt has been made

to define the essential features by testing the effects of several substances related chemically to the cardiac aglycones. This work has been held up owing to difficulty in procuring compounds with the required molecular structure, but some tentative conclusions are possible.

Since the cardiac glycosides produce their effects on ion fluxes even in concentrations at which relatively few molecules of drug are present, it seemed worth while to try to assess the number of ion-transporting sites from the effects of a known small amount of inhibitor. Such an estimate depends on certain assumptions, but detailed study of the inhibitory action of the glycoside scillaren A has suggested that these assumptions are justified and has provided data from which a maximum figure for the number of sites has been obtained. The plausibility of this figure will be discussed.

METHODS

The methods used in handling the red cells and making the flux measurements have been described in detail in an earlier paper (Glynn, 1956).

Preparation of the solutions containing the cardiac glycosides. The glycosides were all rather insoluble in water, so concentrated alcoholic solutions were prepared with 80% ethanol, A.R. To prepare the test solutions a small quantity of alcoholic solution was added to the suspending medium. In only one experiment was the concentration of alcohol greater than 0·5% (v/v) and in all experiments an equal quantity of alcohol was added to the controls.

The glycosides used were:

(i) Digoxin	Burroughs Wellcome 'pure crystalline digoxin B.P.'
(ii) Emicymarin (iii) Allo-emicymarin	The gift of Professor T. Reichstein of Basle.
(iv) Scillaren A (v) Hexahydro-scillaren A	The gift of Professor A. Stoll of Basle.

Solutions of α-angelica lactone were prepared in the same way. The sample used was supplied by Messrs Light.

Preparation of solutions containing the steroids. A small quantity of the material (aldosterone or deoxycorticosterone) was weighed out on a micro-balance and dissolved in sufficient 80% ethanol A.R. to give a concentration of 2×10^{-3} g/ml. As soon as the material had dissolved (about 10 min) a measured quantity of the alcoholic solution was added to sufficient Ringer's solution to make the final concentration of the steroid 10^{-5} g/ml. The solutions were prepared as required. The aldosterone was a gift from the Ciba Foundation.

RESULTS

Site of action of the cardiac glycosides

Since the cardiac glycosides inhibit the cation fluxes without affecting oxygen utilization or the production of acid anaerobically (Schatzmann, 1953), their action must lie beyond the stages of respiration or glycolysis. They might act either on the mechanism by which metabolic energy is transferred to the pump—on the reactions of ATP perhaps—or on the carrier system itself. If they act by disconnecting the pump from the energy supply their effects should be restricted to those fluxes which depend on the presence of glucose;

if, however, they act on the carrier system one might expect an effect on those fluxes, or those components of fluxes, which are quite insensitive to glucose deprivation. The effects of a number of cardiac glycosides on the influx and efflux of sodium and potassium under a variety of experimental conditions can therefore be used to decide between the two possible sites of action.

The drug chosen for most of the experiments, since it was commercially available in pure form, was digoxin, a glycoside from the yellow foxglove *Digitalis lanata*: but similar results were also obtained with scillaren A, from the fleshy bulb of the squill *Urginea maritima*, and with emicymarin, a glycoside found in *Strophanthus emini*.

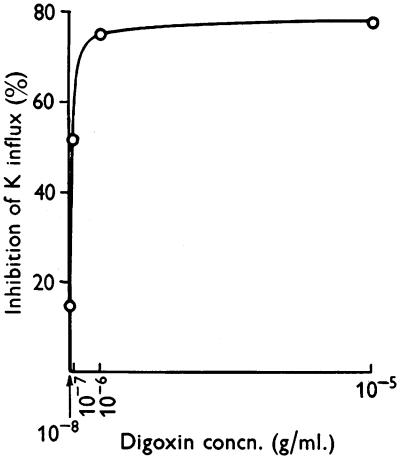


Fig. 1

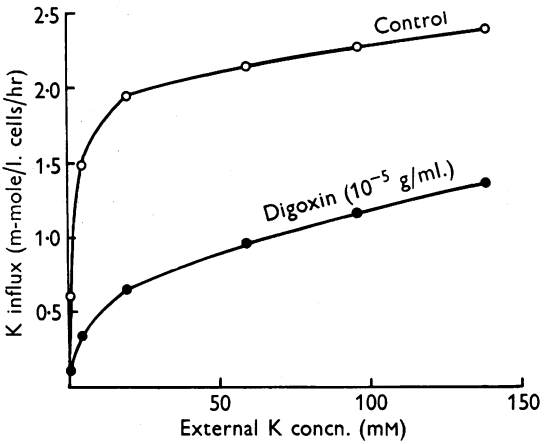


Fig. 2

Fig. 1. The effect of digoxin concentration on potassium influx during the first hour of exposure to digoxin. Expt. no. AAS. 2. External K concn. 5 mM.

Fig. 2. The effect of digoxin on potassium influx at different external potassium concentrations. Expt. no. AAS. 2. Influx measured over first hour of exposure to digoxin.

The effect of cardiac glycosides on potassium influx. The first experiments were designed to test the effect of cardiac glycosides on potassium influx at different external potassium concentrations. Influx was measured by observing the uptake of ^{42}K from labelled suspension media over a period of 1 hr. Two sets of flasks were used, one set containing, say, digoxin and the other serving as a control. A quantity of ethanol equal to that present in the digoxin flasks was always added to the controls. Preliminary trials (Fig. 1) showed that under the conditions of the experiment a digoxin concentration of 10^{-5} g/ml. had a maximal effect, and this was the concentration subsequently used. The results obtained with digoxin are shown in Fig. 2; those with scillaren A were similar. In earlier papers (Shaw, 1955; Glynn, 1956) potassium influx has been shown to consist of two components, of which one is pro-

portional to external potassium concentration, and the other—called for convenience the Michaelis component—increases with external potassium concentration at low concentrations but levels off as the external potassium concentration is further increased. The Michaelis component alone is sensitive to removal of glucose. Fig. 2 shows that digoxin greatly reduces the size of the Michaelis component but affects the linear component little if at all. (The slight convergence of the linear parts of the two curves is significant but will be discussed later.) Comparison of Fig. 2 with Fig. 3, which is taken

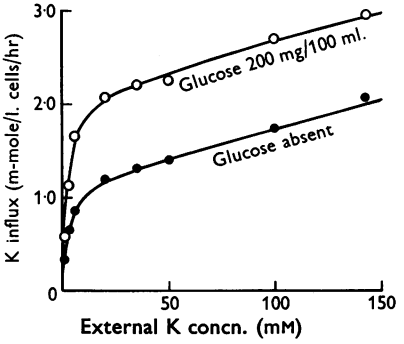


Fig. 3. The effect of glucose deprivation on potassium influx at different external potassium concentrations. Reproduced from Glynn (1956).

from Glynn (1956), shows that the effect of digoxin is much greater than the effect of depriving the cells of glucose—the difference being most obvious at low potassium concentrations, where the Michaelis component accounts for the major part of the influx. For example, with 1 mM-K outside, removal of glucose reduces potassium influx by 43%, digoxin reduces it by 82%. This difference might be explained by supposing that digoxin cuts off the energy supply more effectively than does removal of glucose, but the results are also compatible with the idea that digoxin acts directly on the ion carrier mechanism responsible for the Michaelis part of the influx.

Effect of digoxin and of glucose deprivation on potassium efflux. Potassium efflux was measured by following the appearance of activity in the medium surrounding cells loaded with ^{42}K. Cells were washed repeatedly to get rid of glucose, loaded with ^{42}K by 5 hr incubation in a labelled Ringer's solution, washed again and added to three groups of flasks whose contents were arranged as follows:

	(i)	(ii)	(iii)
Balanced salt solution	+	+	+
Glucose, 150 mg/100 ml.	+	−	+
Digoxin, 10^{-5} g/ml.	−	−	+
Ethanol, 0·5%	+	+	+

The efflux from the cells under the three sets of conditions is shown in Table 1. In the presence of digoxin efflux was reduced by about 25%, and this was not

associated with any significant reduction in haemolysis. Haemolysis was in any case very slight—always less than 0·1%—and necessitated a correction of not more than 5% in the efflux figures. The efflux from the glucose-deficient cells was not significantly less than that from the cells of the controls ($P < 0·2$). If depriving the cells of glucose really has no effect, then interference with the energy supply cannot be the explanation of the action of digoxin; if, on the other hand, glucose deprivation has a small but undetected effect, then one way of explaining the action of digoxin would be to suppose that the energy supply is cut off more effectively by digoxin than by removal of glucose. Because of the uncertainty about the effects of glucose deprivation, the results give only rather equivocal support to the theory of direct action on the carrier mechanism.

TABLE 1. The effect of glucose and of digoxin on potassium efflux from cells loaded with ^{42}K. Expt. no. FWC. 2. Duration 1 hr. The figures for efflux each represent the mean of three estimations ±S.E.

Additions to medium	K efflux (m-mole/l. cells/hr)
None	2·70±0·03
Glucose 150 mg/100 ml.	2·83±0·06
Glucose 150 mg/100 ml. + digoxin 10^{-5} g/ml.	2·17±0·03

TABLE 2. The effect of digoxin on sodium efflux at different external potassium concentrations. Glucose present. Duration 22 min. Expt. no. SD. 2. The figures at zero potassium concentration are each the mean of three determinations, ±S.E.; the other figures are each the mean of two

External K concn. (mM)	Na efflux (m-mole/l. cells/hr)	
	Digoxin absent	Digoxin present (10^{-5} g/ml.)
0·00–0·03	1·37±0·02	0·607±0·002
2·94	2·38±0·02	0·703±0·003
9·80	2·51±0·01	0·80±0·04
19·6	2·60±0·00	0·88±0·02

Effect of cardiac glycosides on sodium efflux. Experiments on the sodium fluxes were more decisive. The effect of digoxin on sodium efflux was investigated over a range of external potassium concentrations. Cells loaded with ^{24}Na by exposure to labelled Ringer's solution were washed and added to flasks containing glucose-Ringer's solutions with different potassium contents. After a timed period of incubation the suspension media were separated from the cells, and the activities of the media were measured. The period of incubation was about 20 min and was kept short to minimize changes in cell sodium content and specific activity (see section on Methods in Glynn, 1956). The results with digoxin are shown in Table 2. Similar though less detailed results were obtained with scillaren A and emicymarin.

Digoxin clearly caused a large reduction in sodium efflux at all external potassium concentrations, but the significant point is that even with minimal potassium present outside the cells—actually $[K_o] < 0·03$ mM—efflux was reduced by about 50%. In an earlier paper (Glynn, 1956) sodium efflux was

shown to consist of active and passive components, and evidence was presented suggesting that the efflux in the absence of external potassium was entirely passive. Very briefly, the evidence was first, that in the absence of potassium sodium efflux was unaffected by the presence or absence of glucose, and secondly that, in the absence of potassium, cells never showed net movements of sodium against the electrochemical potential gradient. If sodium efflux in the absence of potassium is entirely passive and yet is reduced to about half by digoxin, then digoxin must be inhibiting a passive flux—a result which is not explained by the theory that its action is to disconnect the energy supply.

Effect of digoxin on sodium influx. Cells were washed three times with potassium-free Ringer's solution and added to four sets of flasks containing warmed test solutions labelled with ^{24}Na, arranged as follows:

	(i)	(ii)	(iii)	(iv)
K-free glucose Ringer	+	+	+	+
K (10 mM)	−	−	+	+
Digoxin (10^{-5} g/ml.)	−	+	−	+
Ethanol (0·5%)	+	+	+	+

After a measured period of incubation the cells were spun down, washed three times with ice-cold Ringer's solution and analysed for ^{24}Na.

The results of two experiments of this sort are summarized in Table 3. The figures in the fifth column are the amounts of ^{24}Na present in the cells at the end of the experiment. To obtain the influx from these figures it is necessary to correct for efflux, and this has been done using data from an experiment in which uptake of ^{24}Na and net sodium movements were followed over a period of 2 hr. (This experiment is described in detail in Glynn, 1956,

TABLE 3. The effect of digoxin on sodium influx in the presence and absence of external potassium

Expt. no.	Duration (min)	External K concn. (mM)	Digoxin	Na uptake (m-mole/l. cells)	Na influx (m-mole/l. cells/hr)
AAS. 1	48·5	0	−	2·24	3·10
	62·5	0	−	2·86	3·07
	48·5	0	+	1·35	1·83
	62·5	0	+	1·71	1·81
	48·5	10	−	1·35	2·10
	62·5	10	−	1·71	2·15
	48·5	10	+	1·53	2·06
	62·5	10	+	1·99	2·10
IWFH. 1	31·0	0	−	1·52	3·25
	31·0	0	−	1·55	3·32
	31·0	0	−	1·53	3·38
	31·0	0	+	0·941	1·90
	31·0	0	+	0·954	1·93
	31·0	0	+	0·940	1·90
	31·0	13	−	1·05	2·35
	31·0	13	−	1·03	2·31
	31·0	13	−	1·04	2·34
	31·0	13	+	1·09	2·20
	31·0	13	+	1·04	2·10
	31·0	13	+	1·11	2·24

where, in figure 10, the time curves for ^{24}Na uptake and for net sodium movements are shown.) In the experiment of 31 min duration the largest correction for efflux is only about 10%, so the error in the final result due to error in the correction is not likely to be more than a few per cent. In the experiment lasting 62 min the correction is larger, but this experiment was done on the same batch of cells and at the same time as the experiment to determine the time curves, so that the data taken from that experiment and used in the correction are data obtained from the same batch of cells under the same conditions. It follows that, in this case also, the final figures for influx should be correct within a few per cent.

The most striking conclusion from these experiments is that in the absence of external potassium, digoxin reduces sodium influx by something like 40%—a little more than the reduction produced by potassium. This effect of digoxin can be seen even in the uncorrected figures. A second conclusion is that, in the presence of about 10 mM-K, digoxin has little or no effect on the sodium influx—a small decrease is suggested by the figures for the 31 min experiment, but because of the uncertainty in the efflux correction the difference is not significant. Thirdly, potassium and digoxin acting together seem to have a smaller effect than digoxin acting alone.

Of these conclusions the first is the most certain and the most important. Removal of glucose has no effect on sodium influx whether or not potassium is present (Glynn, 1956). The fact that digoxin reduces by 40% a flux which is not only downhill but is also unaffected by removal of glucose cannot be explained by supposing that the drug acts on the energy supply to the pump; an action on the mechanism by which the ions penetrate the membrane is indicated.

Further evidence that the cardiac glycosides act on the carrier mechanism is given later.

The molecular requirements for activity

Introduction. In view of the findings just described, and especially since no other substances are known to interfere with the carrier mechanism, it seemed desirable to determine what molecular structure was necessary for a substance to have effects on the ion fluxes similar to those of the cardiac glycosides. The structural features common to the cardiac glycosides are an unsaturated lactone ring, a cyclo-pentano-phenanthrene nucleus, and a mono-, di-, tri- or tetrasaccharide sugar. The unsaturated ring is usually five-membered, as in digoxin and emicymarin, but is six-membered in the squill glycosides. It is attached in the α-configuration to the C_{17} of the cyclo-pentano-phenanthrene nucleus. The sugar is attached by a glycosidic link, usually to the 3 position of the steroid nucleus in the *trans* configuration.

Schatzmann (1953) showed that the aglycones strophanthidin and digitoxigenin were effective in preventing the reaccumulation of potassium by red

cells, so the sugar portion is not essential for activity. As it was not possible to test more than a few substances, the scope of the investigation was limited by trying to answer only three questions:

(i) Is an unsaturated lactone ring active when unattached to a steroid nucleus?

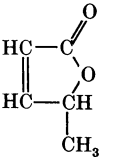
(ii) Is the configuration at C_{17} important?

(iii) Does saturation of the ring affect the activity?

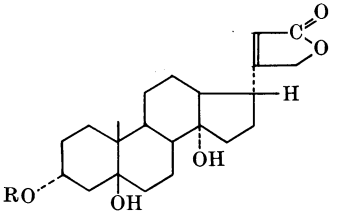
The substances tested were:

		Molecular weight			Molecular weight
(*a*)	α-Angelica lactone	98	(*d*)	Scillaren A	692
(*b*)	Emicymarin	553	(*e*)	Hexahydro-scillaren A	698
(*c*)	Allo-emicymarin	553	(*f*)	Digoxin	777

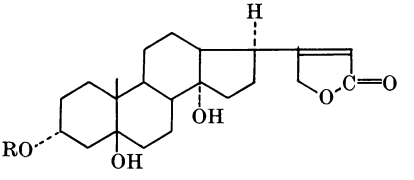
α-Angelica lactone consists of an α:β unsaturated, five-membered lactone ring, similar to that found in digoxin and many other cardiac glycosides but with a methyl group on the γ carbon instead of a steroid nucleus on the β carbon.



Allo-emicymarin differs from the glycoside found in *Strophanthus emini* by having the β configuration at C_{17}.



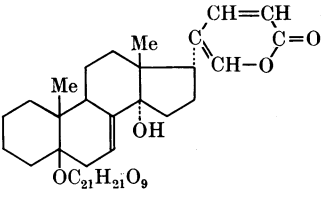
Emicymarin



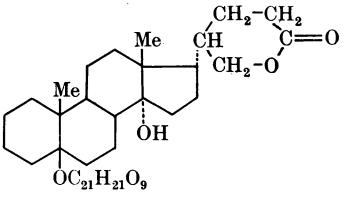
Allo-emicymarin

R = digitalose

Hexahydro-scillaren A differs from the natural scillaren A in that the two double bonds in the lactone ring and the double bond in the steroid nucleus are saturated.



Scillaren A



Hexahydro-scillaren A

Effect of α-angelica lactone, emicymarin and allo-emicymarin on sodium efflux. Table 4 shows the results of an experiment to test the effects of α-angelica lactone, emicymarin and allo-emicymarin on sodium efflux. Washed cells loaded with ^{24}Na were added to glucose-Ringer's solutions containing the substances to be tested, and the activity appearing in the media during 20 min incubation was measured. The emicymarin and allo-emicymarin were used in a concentration of 10^{-5} g/ml., and the angelica lactone at a concentration of 2×10^{-6} g/ml. which, because of the smaller molecular weight of the lactone, gave roughly the same molar concentration.

TABLE 4. Effect of α-angelica lactone, emicymarin and allo-emicymarin on sodium efflux. Expt. no. BRL. 1. Duration 22 min

Inhibitor	Concn. (g/ml.)	Na efflux (m-mole/l. cells/hr)	Inhibition (%)
None		2·60 2·52	—
α-Angelica lactone	2×10^{-6}*	2·50 2·62	0
Emicymarin	1×10^{-5}	0·545 0·548	79
Allo-emicymarin	1×10^{-5}	1·93 1·96	24

* Because of the lower molecular weight of α-angelica lactone the molar concentration was about the same as that of the glycosides.

Comparison of the efflux figures for α-angelica lactone with the efflux in the control flasks, containing glucose-Ringer's solution, shows that the lactone was completely without effect. (Since these experiments were performed Kahn & Acheson (1955) have found that α-angelica lactone does have an effect when present at a concentration 1000 times greater than that used here.) Emicymarin caused severe inhibition—about 79%—and allo-emicymarin was very much less potent, causing about 24% inhibition.

This sort of experiment, in which all the inhibitors are used at the same fairly high molar concentration and the relative amounts of inhibition produced are compared, is good enough to distinguish between active and completely inactive substances, for example between emicymarin and α-angelica lactone. For the comparison of substances of different potency, like emicymarin and allo-emicymarin, the method is only satisfactory if, for each substance, the degree of inhibition produced is proportional to the concentration of inhibitor. This is not true for the cardiac glycosides under the experimental conditions used—see, for example, Fig. 1, which shows the degree of inhibition of potassium influx when cells are incubated for 1 hr in glucose-Ringer's solutions containing different concentrations of digoxin. Ideally, to compare potencies the 'dose effect curve' for each substance to be tested should be found, and these curves should be compared. Such a

procedure would be laborious and would give rather more information than is required, so instead a compromise was adopted. The 'dose effect curve' for scillaren A under certain fixed experimental conditions was determined (Fig. 4). At the same time, with the same conditions, and with cells from the same batch, the inhibition produced by the other substances at one or two moderate concentrations was measured. It was then possible to compare the activities of the test substances with the activity of scillaren A by estimating the relative concentrations of each necessary to produce the same degree of inhibition.

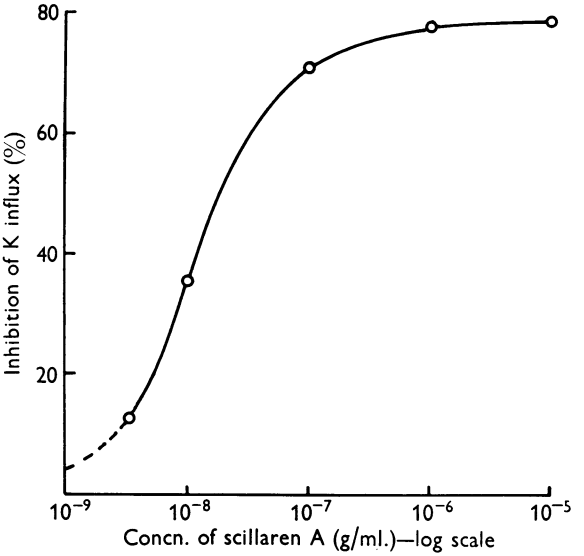


Fig. 4. 'Dose effect curve' for the action of scillaren A on potassium influx under standard conditions. Expt. no. RBK. 4. Influx was measured over first hour of exposure to scillaren. External potassium concn. 6 mM. Haematocrit *ca.* 5%. The curve is drawn by eye.

The results obtained by this method are shown in Table 5. Measurements were made of the uptake of ^{42}K by cells incubated for one hour with glucose-Ringer's solutions containing the test substances. The degree of inhibition was found by comparing the uptake of ^{42}K from a solution containing a given substance with uptake from the control solution. It must be emphasized, however, that the amount of inhibition depends on such factors as duration and haematocrit as well as on the nature of the inhibitor; comparison of the figures for different substances is only justified because these other factors were kept constant. The results would have been more reliable if a more closely spaced range of scillaren concentrations had been used, but even as they stand certain conclusions are possible. Scillaren A, emicymarin and digoxin are all extremely potent, scillaren being the most powerful with an

activity $2\frac{1}{2}$ times that of emicymarin and 7 times that of digoxin. Under the conditions of the experiment the saturated hexahydro-scillaren A has about one-hundredth of the activity of the unsaturated glycoside. Allo-emicymarin with the 'wrong' configuration at C_{17} has rather less than one-hundredth of the activity of the isomeric emicymarin.

TABLE 5. The effect of some cardiac glycosides and related substances on potassium influx. External potassium concentration 6 mM. Duration 1 hr. Expt. no. RBK. 4

Inhibitor	Concn. (g/ml.)	Influx (m-mole/l. cells/hr)	Inhibition (%)	Concn. of scillaren with same effect	Activity relative to scillaren A (approx.)
None		2·86		—	—
		2·83			
Scillaren A	10^{-5}	0·609	78·6	—	—
	10^{-6}	0·626	78·0		
	10^{-7}	0·825	71·0		
	10^{-8}	1·76	38·2		
	$3·2 \times 10^{-9}$	2·49	12·5		
Hexahydro-scillaren A	10^{-6}	1·74	37·5	1×10^{-8}	1/100
		1·82			
	10^{-7}	2·62	8·2	*	
		2·59			
Digoxin	10^{-6}	0·797	73·0	$1·4 \times 10^{-7}$	1/7
		0·740			
Emicymarin	10^{-6}	0·605	78·8	*	
		0·509			
	10^{-7}	1·07	62·4	4×10^{-8}	2/5
		1·07			
Allo-emicymarin	10^{-6}	2·46	12·1	$3·2 \times 10^{-9}$	1/300
		2·54			
	10^{-7}	2·82	2·7	*	
		2·72			

* No reliable estimate.

It is possible that some of the differences in activity between the substances tested are related to differences in the rate of uptake of inhibitor (see p. 161).

The small amount of activity possessed by samples of hexahydro-scillaren A and allo-emicymarin could conceivably be due to traces of scillaren A and emicymarin. To exclude the presence of such traces would require very refined analytical techniques and the task has not been attempted.

Taken together these results suggest that: (i) an unsaturated lactone ring is not active unless linked to an appropriate group; (ii) the configuration at C_{17} of the steroid nucleus is important; (iii) saturation of the lactone ring greatly reduces activity. The third conclusion is the least certain because it is just possible that the relative inactivity of hexahydro-scillaren is the result of the nuclear saturation. The significance of these findings for an understanding of the action of cardiac glycosides on heart muscle will be discussed later.

The action of the mineralocorticoids on the red cell fluxes

There is a fairly close chemical relationship between the cardiac glycosides and the mineralocorticoids, β oxidation of the aglycones yielding compounds

resembling the corticoids (Reichstein & Shoppee, 1943). This structural similarity, together with the fact that the mineralocorticoids affect sodium and potassium fluxes in kidney, salivary glands, sweat glands, and mammalian muscle (Flückiger & Verzar, 1954), suggested that the actions of the two groups of substances might be connected. It seemed possible that the cardiac glycosides were simulating mineralocorticoids and either reproducing their actions, or, perhaps, acting as competitive inhibitors.

Previous workers who have tested the action of adrenal steroids on red cells obtained rather discouraging results. Streeten & Solomon (1953–4) tried cortisone and ACTH *in vivo*, and cortisone and hydrocortisone *in vitro*, and found remarkably small effects. Schatzmann (1954) obtained greater effects with deoxycorticosterone acetate on rat cells but found it necessary to have the steroid present in a concentration of 1 mg/ml.

In spite of these earlier results it seemed worth while to test aldosterone—the most potent mineralocorticoid and that responsible for most of the activity of adrenal vein blood—and unesterified deoxycorticosterone. Both drugs were used at a concentration of 10^{-5} g/ml. To test the possibility of interaction between the cardiac glycosides and the steroids, cells were exposed to aldosterone (or deoxycorticosterone) and digoxin separately and together. In these experiments digoxin was present at a molar concentration 20–100 times less than the concentration of the adrenal steroid. The results with aldosterone are summarized in Tables 6 and 7, and with deoxycorticosterone in Table 8. Neither drug appeared to have any effect acting alone, and neither appeared to interfere with the action of digoxin.

The number of carrier sites

Since the cardiac glycosides appeared to act on the carrier mechanism even at dilutions at which relatively few molecules of drug were present, it looked as though an estimate of the number of sites carrying ions might be derived from measurements of the inhibition produced by small amounts of glycoside. To argue from the number of molecules of inhibitor to the number of sites inhibited is justifiable, provided that each molecule of inhibitor can inhibit only one site; an alternative possibility is that each inhibitor molecule can pass from site to site inactivating irreversibly each site visited. There are two ways of deciding between these possibilities.

(i) Reversibility: the 'site to site' theory depends on the supposition that the carrier sites remain inactive after the inhibitor molecules have left them. If the inhibition is reversible this theory can be excluded. Irreversibility is compatible with either theory.

(ii) The effect of very small amounts of inhibitor: on the 'site to site' theory, any amount of inhibitor, no matter how small, should cause maximal inhibition given sufficient time and assuming that the inhibitor is not destroyed.

In practical terms this means that as long as any inhibitor is present, inhibition should go on increasing until it becomes maximal, i.e. so great that inhibition is not increased by adding more inhibitor. On the 'one molecule, one site' theory it should be possible to choose an amount of inhibitor which gives submaximal inhibition no matter how long it is allowed to act. Experiments will now be described which were designed to test each of these points.

TABLE 6. Effect of aldosterone (electro-cortin) on potassium influx. Expt. no. TV. 3. Duration 1 hr

Additions to medium (concn. of steroids in g/ml.)	K influx (m-mole/l. cells/hr)
0·4% (v/v) ethanol	2·69
	2·75
	2·76
10^{-5} aldosterone + 0·4% ethanol	2·74
	2·72
	2·72
$5{\cdot}7 \times 10^{-7}$ digoxin + 0·8% ethanol	1·06
	1·03
	1·05
10^{-5} aldosterone + $5{\cdot}7 \times 10^{-7}$ digoxin + 0·8% ethanol	1·01
	1·04
	1·03

TABLE 7. The effect of aldosterone (electro-cortin) on sodium efflux. Expt. no. DB. 1. Duration 27 min

Additions to medium (concn. of steroids in g/ml.)	Na efflux (m-mole/l. cells/hr)
0·1% (v/v) ethanol	3·02
	3·23
	3·05
10^{-6} aldosterone + 0·1% ethanol	—*
	3·02
	3·03
1% ethanol	3·35
	3·26
	3·34
10^{-5} aldosterone + 1% ethanol	3·28
	3·41
	3·14

* Flask broken.

TABLE 8. The effect of deoxycorticosterone on potassium influx. Expt. no. GCH. 1. Duration 1 hr. 0·5% v/v ethanol present in all flasks

Additions to medium (g/ml.)	K influx (m-mole/l. cells/hr)
None	2·22
	2·24
10^{-5} deoxycorticosterone	2·22
	2·16
10^{-6} digoxin	0·666
	0·618
10^{-5} deoxycorticosterone + 10^{-6} digoxin	0·574
	0·574

Reversibility. Experiments were carried out to see if the inhibition of potassium influx in cells exposed to scillaren A could be reversed by washing. It was found that several washes in rapid succession caused very little reversal, but the same number of washes alternated with periods of incubation in the wash fluid led to a considerable decrease in inhibition—see Table 9. The slow recovery might have been caused by the gradual re-formation of some substance destroyed or inactivated by the inhibitor, or, more simply, it might have resulted from a slow loss of inhibitor from the cells. Slow loss is perhaps the more likely explanation since, as will be shown later, uptake of cardiac glycosides by the cells also seems to be a slow process.

TABLE 9. Slow recovery after exposure to scillaren A. Expt. no. TV. 3

Pre-treatment of cells	K influx (m-mole/l. cells/hr.)	Inhibition (%)
None	3·12 3·01	—
1 hr with equal vol. soln. containing 4×10^{-8} g/ml. scillaren A	1·05 1·04	66
1 hr with scillaren A, then six 20 min washes each with 3 vol. of glucose-Ringer at 37°C	1·79 1·66	43

The effect of very small amounts of inhibitor. The effect of low concentrations of scillaren A on potassium influx was measured. Samples of washed cells were incubated with equal volumes of glucose-Ringer's solution containing $3{\cdot}3 \times 10^{-9}$ g/ml. of scillaren A, for periods of from 0 to 4 hr. The cells were then washed rapidly twice and incubated for 1 hr with glucose-Ringer's solution labelled with ^{42}K. Cells that had not been treated with scillaren were incubated at the same time as a control. To determine maximal inhibition, a third lot of cells was incubated for $\frac{1}{2}$ hr with 10^{-5} g/ml. scillaren, and then for 1 hr with a solution labelled with ^{42}K and containing 10^{-5} g/ml. scillaren. In fact, with this concentration of scillaren maximal inhibition commences practically at once and the pre-treatment was probably unnecessary. To show that inhibitor was still present in the original incubating solution at the end of 4 hr, a sample of the supernatant was kept and its scillaren content assayed by a method to be described in the next section.

The results of this experiment are summarized in Fig. 5. They show that, with the low concentration of inhibitor used, inhibition increases with time at first, but then becomes constant at a level far below that produced by excess of inhibitor. Since this levelling off is not due to the disappearance of the inhibitor, it indicates that a given concentration of inhibitor can cause only a limited amount of inhibition, even given an unlimited time in which to act.

The time course of inhibition. In the experiment described in the last section inhibition developed quite rapidly in the first hour and then levelled off

during the next hour or two. It appeared possible that the explanation of this gradual onset lay in the slow uptake of inhibitor from the solution containing it. To test this, samples of cells were incubated with equal volumes of glucose-Ringer's solution containing scillaren for periods of time ranging from 10 min to several hours. At the end of the periods of incubation, which were so arranged that they all finished at the same time, the suspensions were centrifuged and the cells and supernatants separated. The cells were quickly

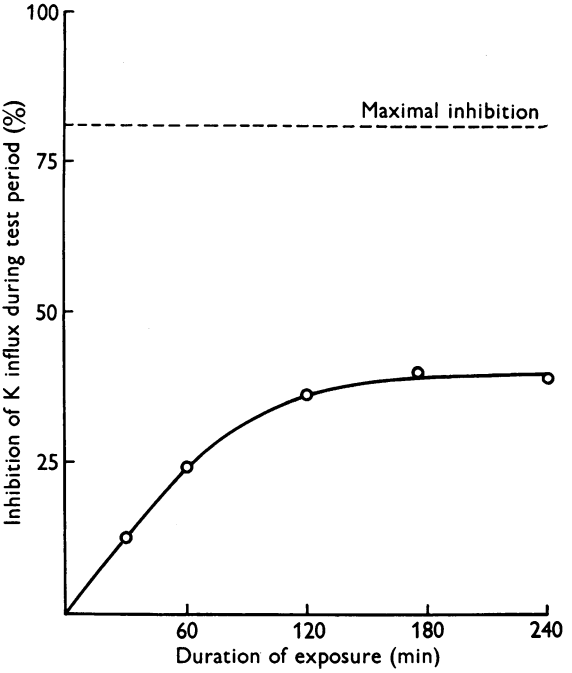


Fig. 5. The inhibition of potassium influx during a 1 hr test period following exposure to $3{\cdot}3 \times 10^{-9}$ g/ml. scillaren A for the time shown on the abscissa. Expt. no. KMH. 1. Haematocrit during period of exposure of inhibitor *ca.* 50%. Assay of the solution containing scillaren A at the end of 240 min showed that *ca.* 46% of the drug was still present.

washed, and the degree of inhibition in the cells of each batch was measured as described in the last section. The supernatants were analysed for potassium by flame photometry, and calculated amounts of ^{42}K-labelled glucose-Ringer's solution were added, so that the final concentration of potassium was the same in all the flasks and the specific activity in each flask was known. Small fixed volumes of fresh cells were now added to these solutions at 37° C, and also to a series of flasks containing ^{42}K-labelled glucose-Ringer's solution with scillaren concentrations ranging from zero to the concentration in the solution used in the first part of the experiment. The uptake of ^{42}K during 1 hr was measured and the potassium influx calculated. From the potassium

influx in the cells incubated with known concentrations of scillaren, a curve was drawn from which, knowing the potassium influx, the scillaren concentrations in the other solutions could be estimated. Working backwards, it was possible to calculate the concentration in the supernatants before the addition of ^{42}K Ringer's solution, and hence the amount of scillaren taken up by the original cells. The results are shown in Fig. 6. It is clear that the uptake of scillaren from the solution is slow, and that this could account for the gradual onset of inhibition.

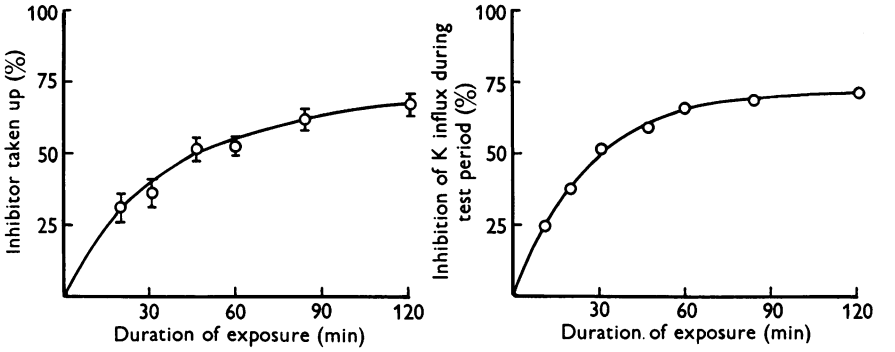


Fig. 6. The uptake of inhibitor and onset of inhibition. Expt. no. QW. 1. Haematocrit during period of exposure to scillaren A *ca.* 50%. Initial concn. of scillaren A, 2×10^{-8} g/ml.

Of course, what this experiment really measures is not uptake of scillaren but disappearance of inhibitory activity from the suspension medium. It is conceivable that the decrease in activity was brought about not by uptake but by inactivation of the inhibitor or adsorption of the inhibitor on to the walls of the flasks. The former hypothesis does not explain why the apparent uptake seemed to level off with a large amount of inhibitor still present. The latter can be excluded since the activity of a solution of scillaren was found to be unaffected by incubation at 37° C for several hours in a glass vessel.

The results shown in Fig. 6 can be a little misleading since they suggest a stoichiometrical relationship between the degree of inhibition and the amount of inhibitor taken up. This appearance arises from the fact that, with the very low concentration of scillaren used, the amount taken up was the factor limiting inhibition throughout the experiment. With a scillaren concentration of 4×10^{-7} g/ml. inhibition of potassium influx measured over the first hour of exposure is 95% of maximal—see Fig. 4—so that maximal inhibition presumably develops within the first few minutes. The uptake of scillaren is shown in Fig. 7.

It is not possible to say, from these uptake experiments, where the inhibitor that disappears from the outside solution goes. One possibility is that the glycoside diffuses slowly into the cell and acts only when it is inside. This is

compatible with the observation—see Figs. 6 and 7—that increasing the external glycoside concentration increases the rate of uptake of glycoside but leaves the half-time of entry about the same. It cannot be the whole story, however, because in every uptake experiment that has been done, the final uptake with a 50% haematocrit has been greater than 50%. With cell water occupying, say, 64% of the cell volume, equilibration between extra- and intracellular fluid should lead to an uptake of only 38%. It is possible that investigation of the uptake of cardiac glycosides by ghosts would clarify the position, but for the moment questions about the fate of the inhibitor must be left undecided.

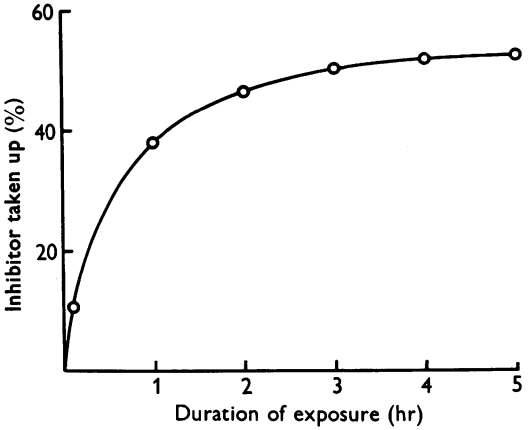


Fig. 7. Uptake of scillaren A from a solution containing 4×10^{-7} g/ml. of the drug. Expt. no. IMG. 8. Haematocrit *ca.* 50%.

The number of carrier sites. The results of the experiments on reversibility are compatible with both the 'one molecule, one site' and the 'site to site' theories. The results with very low concentrations of inhibitor make the 'site to site' theory untenable. It is therefore justifiable to use the results shown in Fig. 5 to obtain an estimate of the number of carrier sites. This will, of course, be a maximum estimate since there is no reason to suppose that every molecule of inhibitor taken up is engaged in inhibiting a carrier site. In the experiment illustrated by Fig. 5 there were present only about 600 molecules of scillaren per red cell. (A cell density of 5×10^6 red cells per mm^3 is assumed.) This amount of inhibitor was sufficient to inhibit 40% of the potassium influx; an excess of inhibitor inhibited 81%. From these figures it looks as though that component of potassium influx which is sensitive to scillaren—about four-fifths of the whole—does not involve more than about 1200 carrier sites. (The small component of potassium influx which is not inhibited by scillaren is rather a puzzle since it is too big to be accounted for entirely by the 'linear part' of the influx. Whether it represents yet a third component,

or the cardiac glycosides do not completely inhibit each site, or a few sites are resistant to inhibitor, it is not possible to say.) The figure of 1200 allows for all the inhibitor molecules present. If the estimate of the fraction of inhibitor taken up is correct, the answer should be 54% of 1200 or under 700. The results shown in Fig. 6, which are from an experiment using a slightly greater concentration of scillaren, lead to an estimate of about 2700 for the maximum possible number of sites. All these figures are remarkably low; it will be shown later that they are not impossible.

Cardiac glycosides as competitive inhibitors

It is fundamental to the argument given in the last section that the cardiac glycosides act on the carrier mechanism and not on the energy supply. The evidence for this view that has been presented above may be summarized by saying that the glycosides inhibit fluxes which are 'downhill' and which are not affected by the absence of glucose. It is convenient now to describe an experiment which provides independent evidence for the direct action on the carrier mechanism, and which suggests what this action might be.

In Fig. 2, showing the effect of digoxin on potassium influx at different external potassium concentrations, the linear portions of the two curves converge significantly. This might be explained by supposing that digoxin had some slight stimulating effect on the linear component of potassium influx, that is, that it made the membrane more leaky. A more interesting theory was suggested by Widdas (private communication). He pointed out that the difference between the two curves gives a measure of digoxin inhibition at each potassium concentration, and that this inhibition decreased at high potassium concentration. This behaviour would be expected if potassium and digoxin competed for the same sites so that excess potassium displaced digoxin and reversed the inhibition. To decide between these two theories the following experiment was performed.

Potassium influx was measured over a range of potassium concentrations, in the absence of inhibitor (control series), in the presence of a very low concentration of inhibitor (2×10^{-9} g/ml. scillaren A), and in the presence of a very high concentration of inhibitor (10^{-5} g/ml. scillaren A). Since 10^{-5} g/ml. scillaren produces maximal inhibition, the influx measurements at this inhibitor concentration gave an estimate of the component of potassium influx insensitive to scillaren. The difference between the influx in the absence of scillaren and in the presence of excess scillaren gave a measure of the potassium influx sensitive to scillaren. The difference between influx at low scillaren concentration and in the absence of scillaren gave a measure of the inhibition due to the low concentration of scillaren.

Since the uptake of inhibitor and onset of inhibition are slow at very low inhibitor concentrations, the cells were pre-incubated for 3 hr under the

conditions in which influx was to be measured. At the end of this time ^{42}K was added and its uptake over the next hour was determined. This procedure has the disadvantage that the cells in the different flasks are not identical at the beginning of the test period; for example the cells subjected to high scillaren concentrations will tend to gain sodium and therefore to have a higher internal sodium than the uninhibited cells. To minimize the effects of differences in internal sodium concentration, the experiment was performed on cells which had been cold-stored about 5 days and which therefore started with a high sodium content—about 50 mM.

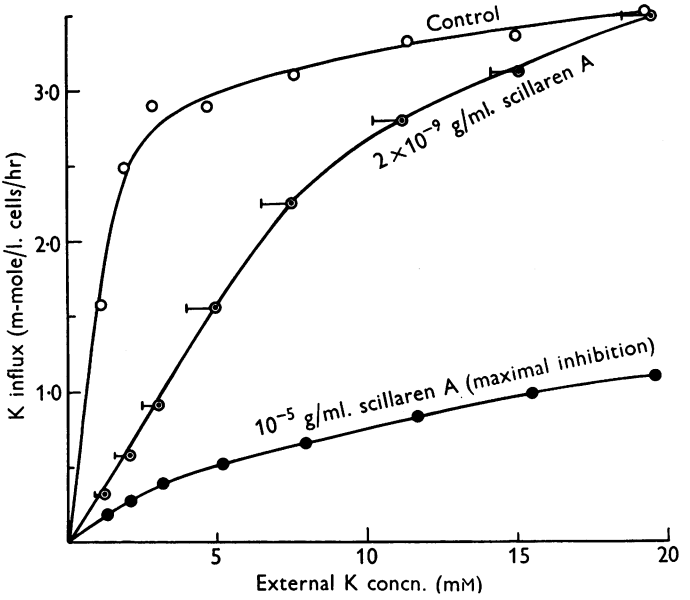


Fig. 8. The influence of potassium on the inhibitory effect of a very low concentration of scillaren A. Expt. no. WAHR. 1. Haematocrit *ca.* 3%. Cells were incubated with the test solutions for 3 hr, ^{42}K was added, and the amount of ^{42}K which entered during the next hour was measured. The lowest curve represents maximal inhibition at each K concn. The short horizontal lines indicate the greatest error that could arise from the small increase in K concn. produced by the addition of ^{42}K.

If scillaren increases the size of the linear component of potassium influx, one would expect the slope of the linear portion of the curve to be at least as great with excess scillaren as with the low scillaren concentration. On the competitive inhibition theory, reversal of inhibition with increasing potassium concentration would be expected at the low scillaren concentration but not at the very high concentration. The results obtained are shown in Fig. 8. In the presence of excess scillaren the curve at high potassium concentration runs parallel with the control curve, showing that the linear component is

unaffected. With very little scillaren present, inhibition can be completely reversed by increasing the external potassium concentration.

These results are, qualitatively, strongly in favour of competitive inhibition. Quantitative analysis, however, is not satisfactory. If potassium ions and inhibitor molecules compete for the same sites with affinity constants equal to K_m and K_I respectively, then, if the influx of potassium is proportional to the number of sites binding potassium ions, it follows that

$$m_i = \frac{k[\mathrm{K}_o]}{[\mathrm{K}_o] + K_m + \frac{K_m}{K_I}[I]},$$

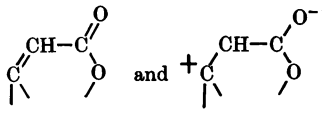
where m_i is the influx, excluding that component insensitive to scillaren, k is a constant, and $[\mathrm{K}_o]$ and $[I]$ are the respective concentrations of potassium and inhibitor. A graph of m_i against $[\mathrm{K}_o]$ based on this equation cannot be made to fit the experimental results satisfactorily over the whole range of potassium concentrations used in the experiment; depending on the value of the constants, the theoretical curve is too high at low potassium concentrations or too low at high potassium concentrations or both. The discrepancy between the theoretical curve and the experimental results is too great to be explained by random error but could be due to a systematic error in the experiment. One obvious source of error is the change in potassium concentration when ^{42}K is added, but the greatest effect this could have is small—see Fig. 8. Another error arises from differences in the sodium content of the various batches of cells at the time ^{42}K was added, but the effect of these differences should be to distort the results in the opposite direction. For example, the most inhibited cells, having the highest internal sodium, should show an influx of potassium greater than they otherwise would, because of the linkage between sodium efflux and potassium influx. An error which could be quite large, and which is in the right direction to explain the discrepancy, is that which would be produced if the cells were not allowed long enough to equilibrate with the potassium and inhibitor before tracer was added. The period allowed in the experiment was 3 hr, as the results shown in Fig. 5 suggested that this time was adequate for equilibration. But in the experiment whose results are given in Fig. 5 the external potassium concentration was 5 mM. At higher potassium concentrations more of the sites would be occupied by potassium ions, there would be fewer free sites, and equilibration with the inhibitor would be slower. It follows that a time which is adequate for equilibration at low potassium concentrations might be inadequate at higher potassium concentrations. This idea could be tested by measuring the rate of onset of inhibition with a low concentration of inhibitor at different external potassium concentrations. Unfortunately it was not possible to carry out this experiment before the work was interrupted.

DISCUSSION

The work of Schatzmann (1953) shows that the action of the cardiac glycosides on ion fluxes lies beyond the stages of glycolysis or respiration; the experiments described in the present paper suggest that the glycosides act on the ion transport mechanism itself rather than on any stage of energy metabolism. Further support for this view comes from recent experiments by Whittam (personal communication of unpublished data) who finds that digoxin does not alter the levels of organic phosphates in fresh or cold-stored red cells, and that it does not affect the re-synthesis of ATP by cold-stored cells when they are incubated with glucose.

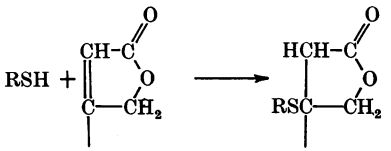
The experiments with scillaren A suggest that the cardiac glycoside molecules compete with potassium ions for the carrier sites. In the reaction between

a glycoside molecule and the membrane, it seems likely that the steroid portion of the molecule confers the necessary solubility and geometrical properties, while the lactone ring provides the 'active centre'. Contrary to earlier views (see, for example, Jacobs, Hoffman & Gustus, 1926), the work of Paist, Blout, Uhle & Elderfield (1941), Ruzicka, Reichstein & Fürst (1941) and Ruzicka, Plattner & Fürst (1941) has now established that the double bond in the five-membered ring found in cardiac glycosides from sources other than squill is in the $\alpha:\beta$ position, just as it is in the squill glycosides. This means that all the active glycosides possess unsaturated rings capable of showing resonance, the 'undisturbed' forms being



Such rings should be partly electophilic in character and should, therefore, resemble sodium and potassium ions in that they would be expected to react with nucleophilic groups in the membrane. Against this idea is the weak activity of hexahydro-scillaren A and of β-propiolactone and γ-butyrolactone (Kahn & Acheson, 1955). The activity of hexahydro-scillaren A was only 1/100 of that of scillaren A and may conceivably have been due to the presence of traces of the unsaturated compound. β-Propiolactone and γ-butyrolactone were effective only in concentrations of about 10^{-2}M and this is so much higher than the concentration of any cardiac glycoside necessary to cause inhibition that one wonders whether the two groups of substances act in the same way.

Cavallito & Haskell (1945) and Hauschka, Toennies & Swain (1945) have described reactions between a number of unsaturated lactones and reagents containing sulfhydryl groups. The general equation for these reactions is



It is possible that the cardiac glycosides react in this way with sulfhydryl groups in the cell membrane, but ultra-violet absorption measurements (unpublished) failed to show any sign of interaction *in vitro* between aqueous solutions of digoxin and of glutathione or cysteine. Furthermore, the effects on red cells of the known sulfhydryl reagents, iodoacetate (Maizels, 1951), *N*-ethyl maleimide and *p*-chlormercuribenzoate (unpublished data), are quite different from the effects of the cardiac glycosides.

Whatever the reaction between cardiac glycoside and active sites may be,

to fit the experimental facts it is necessary to suppose that the affinity of the active sites for, say, scillaren A is about six orders greater than the affinity for potassium ions. The affinity is determined only by the ratio of the velocity constants for the formation and dissociation of the complex between site and inhibitor (or site and substrate), and gives no information about the individual velocity constants. The kinetic studies, however, show that the uptake and dissociation of scillaren must be very slow, presumably because of the high molecular weight and steric effects. The results with allo-emicymarin show the importance of configuration.

It is difficult to interpret the resemblance between the cardiac glycosides and the mineralocorticoids. This resemblance is not restricted to chemical structure. Zwemmer & Lowenstein (1940) and Zwemmer, Lowenstein & Pines (1940) found that injections of strophanthin in oil (15 μg/kg) prolonged the life of adrenalectomized cats, and protected normal cats, rats and mice from the effects of insulin and of potassium administration. In normal animals injection of strophanthin caused a rise in blood sugar and a fall in plasma potassium, both effects developing within a few minutes. The experiments described in the present paper failed to provide any evidence of a connexion between the mineralocorticoids and the cardiac glycosides, but it is a well known and puzzling fact that in brief experiments *in vitro* the effects of the mineralocorticoids on ion movements have always been found to be relatively slight, and this is true even of so potent a substance as aldosterone (Flückiger & Verzar, 1954). The adrenal steroids are known to undergo rapid interconversion in the animal body, and it has been suggested that the discrepancy between their actions *in vivo* and *in vitro* is due to the formation *in vivo* of more potent substances. If this were so then it is conceivable that compounds like the cardiac aglycones were involved. Steroids with an unsaturated lactone ring in the C_{17} position have been described only once in animal tissues but this may be because they have not been looked for more often. The one situation in which they have been reported is in the skin of toads, where they are found in combination with peptides in bufotalin and bufotoxin. These poisons are generally thought to render the animal distasteful to predators, and they doubtless do this, but it is not impossible that their presence was originally related to the ion pumping activity of amphibian skin. It would be interesting to know the precursors and metabolic fate of the toad poisons.

A problem rather divorced from the main theme of this paper, but to which the experimental findings are relevant, concerns the mode of action of the cardiac glycosides on the heart. Largely owing to the very dramatic clinical effects of digitalis and squill, this problem has received a great deal of attention, and attempts to solve it date almost from the time of Withering. Most of the earlier workers sought an effect on energy metabolism—see the review by

Wollenberger (1949)—but while many of them showed that energy yielding reactions were increased, it was never proved that this was the primary effect. Ellis, in 1953, showed that provided sufficient energy was available the response of a hypodynamic frog heart to strophanthin occurred whether that energy came from oxidative metabolism or glycolysis. He concluded that the action of strophanthin occurred at the stage of energy utilization. Horvath, Kiraly & Szerb (1949) and Snellman & Gelotte (1950) have described an effect of cardiac glycosides on the viscosity of cardiac actin solutions, and have suggested that an effect on the polymerization of actin is the basis of the cardiotonic action. Wollenberger (1954), however, found that the molecular requirements for an action on the viscosity of actin solutions were less strict than those for cardiotonic action, and far higher concentrations were necessary to produce the effect on viscosity. The molecular features found to be necessary for an effect on the ion fluxes in red cells, in the experiments described in the present paper, are just those that Wollenberger found to be necessary for cardiotonic action. Furthermore, the concentration of glycoside necessary for the two effects is of the same order. The question therefore arises: might the cardiotonic action be primarily a membrane effect, the augmented contraction being secondary to an altered pattern of activity in the overlying membrane? An altered pattern of activity in the membrane is certainly suggested by the electrocardiogram, but whether such changes could alter the force of contraction depends on whether events in the membrane merely trigger the contraction or maintain and control it throughout its course. In heart muscle, where the time courses of the electrical and mechanical events are similar, the second possibility seems not unlikely, but even in skeletal muscle the recent work of Hill & Macpherson (1954) with NO_3^-, Br^-, and I^- suggests that the contraction can be modified throughout its course by events in the membrane.

Before this discussion is concluded the plausibility of the estimate of the number of carrier sites, reached earlier, must be considered. It is perhaps worth mentioning here that the estimate does not depend on the hypothesis that the cardiac glycosides act as competitive inhibitors; it assumes only that the glycosides act on the carrier mechanism in such a way that a single glycoside molecule can inhibit only one carrier site. The evidence for this view has been given. It was argued that the number of sites responsible for the major fraction of the potassium influx could not be greater than about 1000 per red cell. This figure seems remarkably small but a number of considerations suggest that it is not unreasonable.

If the entry of each potassium ion depends on the diffusion or rotation or distortion of some carrier molecule, then the time taken for each ion to enter cannot be less than about 10^{-8} sec—i.e. of the order of the molecular relaxation time of the carrier molecule. A thousand sites each transporting 10^8 ions/sec would cause an influx of roughly 10^6 m-mole/l.cells/hr. Of course, each site

is unlikely to be continuously engaged and the transit time may well be much greater than 10^{-8} sec but even so there is a very considerable margin in hand.

Another way of looking at the problem is to consider what area of membrane would need to be permeable if the whole of the potassium influx were to be accounted for by free diffusion. If the membrane is 50 Å thick and the concentration of radio-potassium is taken to be zero at the inside surface and equal to that in the bulk of the suspension medium (say 5 mM) at the outside surface,* then the area of pore necessary to account for an influx of 2 m-mole/l.cells/hr is less than 10^{-2} sq.Å. (This figure is derived from Fick's equation on the assumption that the diffusion constant for potassium is $1{\cdot}5 \times 10^{-5}$ cm^2 sec^{-1}, and of course ignores the finite size of the potassium ion.) Considerations of diffusion, then, provide no reason why even one carrier site per cell should not suffice.

Finally, the flux of potassium may be compared with that of chloride. Even at very low potassium concentrations, when the apparent permeability of the cell membrane to potassium ions is highest, the magnitude of this apparent permeability is only about $1/10^7$ of the permeability to chloride. Furthermore, the observations of Edelberg (1952) on the effect of small quantities of tannic acid on the rate of haemolysis of red cells in ammonium chloride solutions suggest that only a very small fraction of the area of the membrane is actually involved in chloride penetration. If the potassium-specific areas were as efficient—calculated on a flux per area basis—as the chloride-specific areas, only some small fraction of $1/10^7$ of the total area would need to be involved in potassium penetration to account for the observed flux. It may well be that, area for area, the potassium-specific sites are much less efficient, but since $1/10^7$ of the total area is only 1200 sq.Å, the difference in efficiency could be very considerable without being incompatible with the idea of 1000 carrier sites.

There seems to be no reason, then, to reject the estimate of the maximum number of carrier sites derived from the scillaren data. One has, of course, no idea of the size of a carrier site, but on any reasonable estimate it is difficult to avoid the conclusion that only a minute fraction of the cell surface can be employed in ion transport.

It is too early to say whether the cardiac glycosides are likely to prove as useful in the investigation of cation transport as have other specific inhibitors in other fields of physiology. In red cells the cardiac glycosides have been used to obtain an estimate of the number of carrier sites; whether they can provide any information about the nature of these sites remains to be seen. An attempt should obviously be made to find which fraction of the cell

* Application of the expression given by Carslaw & Jaeger (1947) for the temperature gradient in a conducting medium in the neighbourhood of a point sink of known strength shows that this will be roughly true.

substance is responsible for binding the inhibitor that is taken up, but to carry this analysis very far rather sensitive analytical techniques will be required. The problem of identifying the 'carriers' has never seemed easy; the fact that there are likely to be so few on each cell does not make it any easier.

SUMMARY

1. Cardiac glycosides in very low concentrations inhibit the movements of sodium and potassium ions across the human red cell membrane.

2. Among the fluxes inhibited are some which are 'downhill' and which are unaffected by removal of glucose from the cells; this suggests that the inhibitors act on the transport mechanism itself rather than on the energy supply to the pump.

3. Detailed study of the inhibition of potassium influx by scillaren A suggests that inhibitor molecules and potassium ions compete for the same sites.

4. From the effects of very small amounts of scillaren A on potassium influx, it is estimated that the number of sites responsible for the major part of the potassium influx cannot be greater than about 1000 per cell.

5. Investigation of the actions of a number of substances related to the cardiac glycosides has made it possible to define certain molecular features which seem to be necessary for the action on the ion fluxes.

6. Aldosterone and deoxycorticosterone appear to be without effect on ion movements. They neither increased nor reversed the effects of the cardiac glycosides.

7. The relevance of these findings to the action of cardiac glycosides on heart muscle is discussed.

I wish to thank Professor T. Reichstein of Basle and Professor A. Stoll of Basle for samples of glycosides, and Lord Adrian and the Trustees of the Ciba Foundation for a small quantity of aldosterone. Most of the work was done during the tenure of a Medical Research Council Scholarship. Expenses were met out of a grant from the Nuffield Foundation.

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