THE ACTION OF CARDIAC GLYCOSIDES ON ION MOVEMENTS

I. M. GLYNN

Physiological Laboratory, Cambridge, England

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I. INTRODUCTION

When most animal tissues are cooled the cells lose potassium and gain sodium, so that the intracellular concentration of each of these ions moves towards the concentration in the solution outside. If tissues that have been stored in the cold are subsequently warmed in the presence of suitable substrates, the cells expel the sodium they have gained and take up potassium until the original composition is restored. The movements of ions that restore the original composition are active, in the sense that they are movements against the electrochemical potential gradient, and they are achieved at the expense of energy from metabolism. In 1953, Schatzmann (147) showed that the active movements of sodium and potassium in red cells were prevented by low concentrations of strophanthin k, one of the cardiac glycosides found in *Strophanthus gratus*. There had earlier been observations that cardiac glycosides could lead to changes in the ionic composition of cardiac muscle, but it was never clear that these changes were not secondary to effects on the mechanisms responsible for contractility or excitability. In red cells no such explanation was possible, and since Schatzmann also showed that strophanthin did not affect oxygen consumption or lactic acid production, it seemed unlikely that the energy-yielding reactions in the cell were being interfered with. Attention was focused on the active transport of sodium and potassium across the cell membrane. Since 1953 a great deal of work has been done on the effects of cardiac glycosides on the movements of sodium and

potassium, and of certain other ions, in a great variety of tissues. This work is the subject of the present review.

The review is divided into four sections. The first describes work on the effects of cardiac glycosides on sodium and potassium movements in various tissues and on the adenosinetriphosphatase activity now thought to be associated with active transport. The second discusses the relation between the actions of cardiac glycosides and of steroid hormones, particularly those affecting mineral metabolism. The third describes work on the inhibition by cardiac glycosides of the movements of ions other than sodium and potassium. The final section considers the connection between the effects of cardiac glycosides on ion movements and on the contractility of cardiac muscle. In writing this review I have made use of recent reviews by Bush (21), Hajdu and Leonard (77), Repke (141), Weatherall (188), and Wollenberger (209), and also of a collection of papers on cardiac glycosides edited by Wilbrandt (199). I am grateful to Dr. Schatzmann of the University of Berne and to Dr. Niedergerke of University College, London, who have given me helpful advice.

II. THE ACTIONS OF CARDIAC GLYCOSIDES ON THE MOVEMENTS OF SODIUM AND POTASSIUM AND ON $(Na^+ + K^+)$-ACTIVATED ADENOSINETRIPHOSPHATASES

A. Sodium and potassium movements in red cells

In his work on red cells, Schatzmann (147) noticed that strophanthin did not accelerate the ionic changes that took place during cold storage. He therefore thought it unlikely that an increase in the passive permeability of the cell membrane accounted for the failure of the cells to expel sodium and regain potassium when they were subsequently incubated. Inhibition of the active movements of sodium and potassium was the obvious alternative explanation. Any uncertainty was removed when Joyce and Weatherall (96) showed that digoxin and other glycosides inhibited the uptake of ^{42}K by red cells.

More detailed studies of the effects of cardiac glycosides on sodium and potassium movements in red cells have been made by Kahn and Acheson (99), Solomon *et al.* (168) and Glynn (67). It is known from the work of Shaw (160, 161) and of Glynn (66) that potassium enters red cells by two pathways; in one the rate of entry varies linearly with the external potassium concentration; the other pathway shows a saturation effect formally resembling the saturation effect in Michaelis-Menten enzyme kinetics. The "Michaelis" pathway seems to be the "active" route, for it alone is affected by depriving the cells of glucose. It turns out that only the Michaelis pathway is inhibited by cardiac glycosides. There are two curious features about this inhibition which have not been satisfactorily explained. The first is that although most of the influx by the Michaelis pathway is sensitive to quite low concentrations of inhibitor—the precise sensitivity will be discussed later—a small part of the flux remains uninhibited even at high concentrations. The other, and perhaps more intriguing feature, is that with low concentrations of glycoside the percentage inhibition of the Michaelis component of influx depends on the external potassium concentration. If the

external potassium concentration is raised, inhibition does not occur. This kind of behaviour would be expected if cardiac glycosides and potassium ions competed for the same sites, and the results obtained are qualitatively strongly suggestive of competitive inhibition. Quantitative analysis of the data, however, shows deviations from the behaviour to be expected for simple competitive inhibition (67).

Sodium efflux from red cells seems to consist of at least two components. About a third of the efflux varies with the external potassium concentration in precisely the same way as does the Michaelis component of potassium influx (66). Since this part of the sodium efflux occurs only when metabolisable substrates are available, it is presumably active; and the dependence on external potassium is usually explained by supposing that active extrusion of sodium depends on the exchange of sodium for potassium (not necessarily in 1:1 ratio) across the membrane. This component of sodium efflux is abolished by cardiac glycosides (67). The sodium efflux which occurs in the absence of external potassium seems to be passive, because it is not affected by depriving the cells of glucose (66). Furthermore, in the absence of glucose and of external potassium, cells do not show a net loss of sodium even if the sodium concentration outside is only slightly greater than inside. It is therefore at first sight surprising that this part of the sodium efflux was found to be inhibited to a considerable extent by digoxin (67). However, proof that this component of sodium efflux is passive does not imply that it occurs by simple diffusion and there is in fact evidence that it does not. For simple diffusion through a membrane the ratio of the fluxes in the two directions is given by the Ussing (180) formula

$$\frac{m_o}{m_i} = \frac{f_i c_i}{f_o c_o} e^{zEF/RT}$$

where m_o and m_i denote the outward and inward fluxes, f_i and c_i and f_o and c_o denote the activity coefficients and concentrations of the ion under consideration on the two sides of the membrane (inside and outside), z is the valency of the ion, E is the electrical potential and R, T and F have their usual meanings. For red cells, and on the assumption that chloride is passively distributed,

$$E = \frac{RT}{F} \ln \frac{[\mathrm{Cl}]_i}{[\mathrm{Cl}]_o}$$

and for a univalent ion whose activity coefficient is assumed to be the same on the two sides of the membrane, Ussing's equation becomes (160)

$$\frac{m_0}{m_i} = \frac{c_i}{c_o} \frac{[\mathrm{Cl}]_i}{[\mathrm{Cl}]_o}.$$

If the values of the sodium and chloride concentrations inside and outside the cells are substituted in this equation, it turns out that the ratio of sodium efflux to sodium influx must be about 1 to 15. The size of the potassium-independent sodium efflux is between 1 and 2 mmol/l cells/hour, so that if simple diffusion

were responsible there would have to be a passive influx of sodium of between 14 and 28 mmol/l cells/hour. The observed influx is about 3 mmol/l cells/hour. A further reason for rejecting simple diffusion is that there is some evidence that the efflux of sodium in the absence of external potassium is affected by the concentration of sodium outside the cells (66). If the efflux of sodium occurs by some kind of "facilitated diffusion" mechanism, then it is less surprising that it should be inhibited by cardiac glycosides. An attractive possibility is that in the absence of potassium the carriers which are usually responsible for the active transport of sodium out of the cells still shuttle sodium across the membrane. When potassium is absent from the solution bathing the cells, the carrier system seems not to be driven by metabolism, but the mechanism could still be sensitive to cardiac glycosides.

Potassium influx and sodium efflux, being "uphill," must be active at least in part. The remaining fluxes to be considered, potassium efflux and sodium influx, would be expected to be entirely passive, and there is no reason to doubt that they are. Sodium influx under ordinary conditions is not affected by cardiac glycosides. If, however, red cells are suspended in a solution containing no potassium, the influx of sodium is increased (66, 160) and this extra influx is abolished by digoxin (10^{-5} g/ml) (67). The explanation of the increased sodium influx is not clear, but an attractive hypothesis is that the sodium is carried in by carriers that would normally be transporting potassium. On this hypothesis the sensitivity to cardiac glycosides is to be expected. The extra sodium influx is not affected by depriving the cells of glucose, so it is again necessary to postulate that external potassium is necessary if the carrier system is to be driven by metabolism.

Potassium efflux in human red cells [*cf.* Shaw (160) on horse cells] cannot be attributed entirely to simple diffusion through the membrane because application of the Ussing formula shows that the efflux would have to be accompanied by an influx about twice as big as the observed linear influx of potassium. If about half of the efflux of potassium occurs by diffusion and the rest by some other route—a "backwash" through the pump perhaps—it is not too surprising that digoxin (10^{-5} g/ml) inhibits the efflux by about 25%.

The conclusions from the work on red cells that has been described are 1) the main effects of the cardiac glycosides are on the active uptake of potassium and the active extrusion of sodium; 2) the effects cannot be explained satisfactorily by supposing that cardiac glycosides simply disconnect the energy supply; 3) where cardiac glycosides inhibit passive fluxes, these fluxes are not the result of simple diffusion; it is possible that they involve the same carrier system as is involved in active transport.

B. Sodium and potassium movements in cells other than red cells

Work on other tissues has been less detailed than that on red cells partly because, except perhaps in nerve and muscle, less is known of the detailed pattern of ion movements and partly because analysis of the effects of a drug on ion fluxes is complicated by inhomogeneity of the cells and the existence of an extra-

cellular space. Nevertheless, it is clear from tracer studies, or from observations of net movements of sodium and potassium, that cardiac glycosides inhibit active sodium or potassium movements in the following tissues: skeletal muscle (35, 56, 95, 123), cardiac muscle (19, 33, 65, 74a, 76, 78, 87, 105, 109, 138, 154, 179, 179a, 183, 189, 211), intestinal smooth muscle (71, 72), uterine smooth muscle (49), nerve axon (24), mouse ascites tumour cells (122), lens (102), frog skin (20, 104, 106, 125, 192, 197), kidney (22, 130, 152, 171, 184, 196, 203), gall bladder (52), salivary gland (207), and the alga *Nitella* (121).

The experiments of Caldwell and Keynes (24) on the giant axon of the squid are of particular interest because with this preparation it was possible to compare the effects of injecting the glycoside inside the fibre, with the effects of adding it to the surrounding solution. It turned out that inhibition of sodium extrusion occurred only when the glycoside was present outside the cell. It is possible that the injected glycoside was bound in the axoplasm so that little reached the internal surface of the membrane, or that the high internal potassium concentration prevented the inhibitory action, but much the most likely explanation is that the cardiac glycoside has to combine with sites on the outer surface of the cell if it is to inhibit. A similar directional effect has been described by Koefoed-Johnsen (106) in frog skin and by Diamond (52) in gall bladder. In both of these tissues sodium chloride is moved actively across an epithelial membrane and in both it is a necessary condition for transport to occur that potassium should be present on the side towards which sodium is being pumped. And in frog skin and gall bladder, just as in the squid axon, it is only at this surface that the cardiac glycosides have any effect.

The effects of cardiac glycosides on the passive movements of sodium and potassium in cells other than red cells are uncertain, though inhibition of potassium efflux has been reported in both cardiac and skeletal muscle (74a, 87, 153, 179a). In most cells such effects have not been looked for.

C. The effects of cardiac glycosides on ($Na^+ + K^+$)-activated adenosinetriphosphatases

Within the last few years it has been established that the active transport of sodium and potassium across cell membranes is associated with the splitting of adenosinetriphosphate by the membranes (23, 54, 63, 84, 85, 137, 164). From work on red cell ghosts it seems that though the ATP split is intracellular, splitting occurs only if potassium is present at the outside surface of the membrane and magnesium and sodium are present at the inside surface (68, 193, 194). Calcium ions are inactive at the outside surface of the membrane but inhibit the glycoside-sensitive splitting of ATP when present in low concentrations at the inside surface (54, 70).

There is indirect evidence in red cells (54, 137), nerve (165), brain (166, 169), and kidney (166, 191) that the sodium and potassium ions activating the ATPase combine with sites which are more or less selective for these ions. The "transport ATPase" is highly sensitive to cardiac glycosides—indeed this sensitivity was one of the reasons for regarding the splitting of ATP as part of the transport

mechanism—and in red cells, kidney, cardiac muscle, brain, and nerve, at least, inhibition by glycosides shows the same curious dependence on potassium concentration as does the inhibition of potassium influx mentioned above (8, 17, 54, 103, 150). Again, the picture does not quite fit simple competitive inhibition (54, 103, 150); the effect of potassium seems to depend in a complicated way on the sodium concentration.

Adenosinetriphosphatases activated by sodium-plus-potassium and inhibited by cardiac glycosides have now been identified in particulate fractions prepared from a very large number of tissues and from a wide range of animals (2, 3, 4, 6, 7, 14, 15, 16, 17, 18, 29, 30, 46, 51, 54, 57, 68, 69, 70, 84, 85, 86, 93, 94, 103, 111, 112, 134, 135, 137, 142, 146, 150, 155, 156, 157, 164, 165, 166, 169, 177, 181, 191, 193, 194, 195, 210). In every case the enzyme activity seems to be associated with that fraction which is thought to contain fragments of the original cell membrane, and there seems to be a good correlation between the activity of the ATPase in a particular tissue and the amount of pumping going on in that tissue [see for example (15)]. The richest sources of "transport ATPase" are mammalian kidney and brain, and the electric organs of electric eels and torpedoes. Ironically, red cells, the cells in which the evidence for an association between ion transport and ATPase activity is most convincing, provide the poorest source of the enzyme.

D. The sensitivity of sodium and potassium transport and of "transport ATPase" to cardiac glycosides

So far nothing has been said about the concentrations of cardiac glycosides necessary to inhibit sodium or potassium fluxes in intact cells or the sodium-plus-potassium-activated ATPases of membrane preparations. The concentrations which have been found to inhibit range from less than 10^{-8} M to more than 10^{-4} M. There seems to be rather wide variation in the sensitivity of different tissues, and there is also some variation in the potencies of different cardiac glycosides. Furthermore, with low concentrations of glycoside the degree of inhibition may vary greatly with the time during which the inhibitor is allowed to act. For example, when red cells were exposed to 4.8×10^{-9} M scillaren A for different periods of time, inhibition increased from about 14% of the susceptible flux in cells exposed for 30 minutes to nearly 50% in cells exposed for 3 hours (67). In a similar experiment with a slightly higher concentration of scillaren A (2.9×10^{-8} M) the gradually increasing inhibition was paralleled by increasing uptake of inhibitor from the surrounding solution. With high concentrations of inhibitor, inhibition has been shown to reach a maximum within a few minutes in red cells (67), atrial muscle (138, 188), and skeletal muscle (206).

Most of the experiments in the literature on inhibition of ion fluxes or ATPase activity by cardiac glycosides have not been designed to detect a slowly increasing effect with low concentrations of inhibitor; it follows that they may underestimate the potency of the cardiac glycosides. The concentration necessary for 50% inhibition is generally of the order of 10^{-7} to 10^{-6} M (see for example 15, 54, 137), though a few preparations seem to be much less sensitive. The ATPase

in the microsomal fraction from crab nerves described by Skou (164) required more than 10^{-4} M ouabain for 50% inhibition.

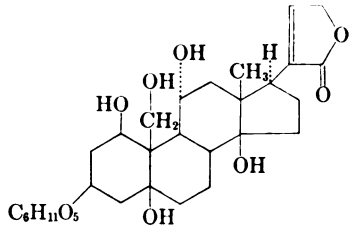
E. The mode of action of cardiac glycosides on ion movements

It is now clear that the active movement of sodium and potassium across cell membranes involves the splitting of ATP and that the system responsible is inhibited by cardiac glycosides. But what the cardiac glycosides do is not clear, and it is not likely to become clear until the transport system itself is better understood. To refer to this system as an ATPase is convenient, but it is very likely that the liberation of the terminal phosphate of the ATP as inorganic phosphate is preceded by its transfer to some group in the membrane, and there may, of course, be more than one intermediate compound. Attempts are at present being made in a number of laboratories to analyse the transport mechanism further and to identify any phosphorylated intermediate involved. Certain preliminary results have been published (2, 29, 30, 82, 86, 146) but it would be premature to attempt to review them. The sensitivity of the sodium-plus-potassium-activated ATPase and of sodium transport to oligomycin (69, 93, 94, 181) raises interesting questions about the relation of ion transport to the terminal stages of oxidative phosphorylation, but such questions are beyond the scope of this review.

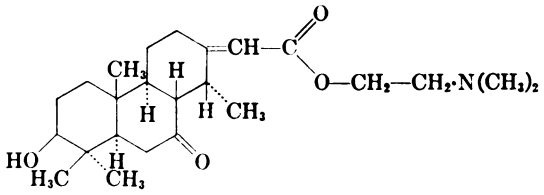
Another approach to the problem of the mode of action of cardiac glycosides is to try to discover which features of the glycoside molecule are essential for inhibition to occur. Inhibition of active ion movements or of sodium-plus-potassium-activated ATPase has been reported with the glycosides: ouabain, strophanthin k, digoxin, digitoxin, lanatoside C, lanatoside B, desacetyl lanatoside C, cymarin, emicymarin, and scillaren A, and with the genins: digoxigenin, digitoxigenin, strophanthidin, and 3-acetyl strophanthidin (54, 67, 99, 140, 168). All these substances possess an unsaturated lactone ring attached in the β configuration to the C_{17} of a cyclopentanophenanthrene nucleus. Partial or complete loss of activity is caused by saturation of the lactone ring, α configuration at C_{17}, dehydrogenation of the hydroxyl at C_3 or epimerization of this hydroxyl from the β to the α position (9, 54, 67, 99, 140, 168, 175). Certain simple lactones lacking steroid rings have been shown to inhibit ion transport, but only at extremely high concentrations (10^{-2} M) (99). Rather surprisingly, in view of the results obtained with the steroid lactones, butyrolactone, a saturated γ-lactone, and propiolactone, a saturated β-lactone, were found to be as potent as the unsaturated angelica lactone.

Two years ago Kahn (98, 98a) showed that the erythrophleum alkaloids, which resemble the cardiac glycosides pharmacologically but differ from them in chemical structure (see figure), inhibit the entry of potassium into red cells. More recently these alkaloids have been shown to inhibit ($Na^+ + K^+$)-activated ATPases prepared from brain, kidney, ciliary body, and choroid plexus (17). In experiments on a preparation of rabbit brain ATPase, inhibition by a low concentration of erythrophleine (5×10^{-7} M) could be largely reversed by increasing the potassium concentration in the assay medium (17). Any theory which is

to explain the action of the cardiac glycosides in terms of their chemical structure will therefore have to take into account the very similar action of the chemically different erythrophleum alkaloids. So far, no satisfactory theory of this kind has been suggested.



Ouabain ($C_{29}H_{44}O_{12}$)



Cassaine ($C_{24}H_{39}O_4N$)

III. THE RELATION BETWEEN CARDIAC GLYCOSIDES AND ADRENAL STEROIDS

A question which has attracted a good deal of attention is: what is the relation between the actions, on ion movements, of cardiac glycosides and of the salt-retaining hormones of the adrenal cortex? Many workers in this field have tended to think of the salt-retaining steroids as having a direct effect on sodium and potassium balance of many kinds of cell, and they have been attracted by the possibility that the cardiac glycosides act by replacing the natural hormones at the receptor sites. The most specific suggestions have been put forward by Wilbrandt and his collaborators (28, 92, 173, 174, 198, 202), who postulate that the salt-retaining steroids act on ion transport by chelating metal ions, and so acting as lipid-soluble ion carriers. The cardiac glycoside with its unsaturated lactone ring is supposed to resemble the chelate complex sufficiently to act as a competitive inhibitor.

The evidence on which theories linking cardiac glycosides and salt-retaining steroids is based is as follows:

1) Both groups of compounds act on sodium and potassium movements; both are steroids; it is therefore *prima facie* likely that they act at the same site.

2) In a large number of experiments *in vivo*, cardiac glycosides have effects opposite to those of the salt-retaining steroids.

3) Cardiac glycosides cause contraction of strips of aorta and of taenia coli, and this contraction is inhibited by certain steroid hormones (148, 149). With aortic strips the inhibition seems to be competitive.

4) Experiments *in vitro* have been reported in which steroid hormones appear

to have actions opposite to those of the cardiac glycosides on sodium transport in toad bladder (38, 39) and frog skin (133, 176 but *cf.* 20), on potassium movements in guinea pig heart (110, 172), and on sodium and potassium movements in red cells (174).

5) Competition between cardiac glycosides and a chelate complex formed between a potassium ion and an adrenal steroid would explain why raising the potassium concentration appears to protect both the pump and the transport ATPase of red cells against inhibition by low concentrations of glycosides.

Each of these lines of evidence will be considered in turn.

1) Bush (21) has pointed out that the resemblance between the adrenal steroids and the cardiac glycosides is less than the usual representation of their formulae on paper would suggest. In the adrenal steroids the A/B and C/D ring junctions are both *trans*, giving the molecule a more or less planar form, whereas in the cardiac glycosides these junctions are both *cis*, giving a "bunched-up" molecule with the A and B rings bent back towards the α side of the plane of the C and D rings. Referring to the more specific theories of Wilbrandt, Bush argues that there is no evidence that the ketol side-chain of the salt-retaining steroids does in fact form chelates with alkali metal ions, and that some of the 21 deoxysteroids still possess sodium-retaining properties.

2) The opposite effects of the cardiac glycosides and the salt-retaining steroids *in vivo*, however suggestive they may be, are always ambiguous because it is never clear that the two groups of compounds are acting at the same site. How likely a common site is depends on what view one takes about the action of the adrenal steroids. On the older view that their action was probably fairly general on cell membranes, it was attractive to suppose that the opposing but similarly general action of the cardiac glycosides might occur at the same sites. But this view of the adrenal steroids is now difficult to maintain. If attention is restricted to those steroids, like aldosterone and cortexone (DOC), in which glucocorticoid activity is absent or very weak relative to the salt-retaining activity, the only tissues in which an unequivocal effect has been demonstrated are kidney (see 21), salivary gland (73), colon (10), sweat glands (see 156a), frog skin (176 but *cf.* 20), toad bladder (38, 39) and fish gills (159). All these tissues are concerned with the secretion or absorption of salt, and in all of them salt is transported across an epithelial membrane. If it is fair to regard the amphibian skin and bladder as models for the other membranes, it seems likely that in every case we are dealing with a sheet of cells of which one face expels sodium in exchange for potassium, while at the other face sodium enters passively down the concentration gradient (but *cf.* 52). In such a system the cardiac glycosides inhibit the transport of sodium chloride by preventing the active extrusion of sodium from the cell. In toad bladder, at least, it is known that antidiuretic hormone increases salt transport by increasing the permeability to sodium at the other end of the cell (60), and very recently Crabbé has described experiments suggesting that aldosterone acts in a similar way (39). What he finds is that the increased sodium chloride transport produced by aldosterone is accompanied by a *rise* in the intracellular concentration of sodium; if the effect were primarily on the pump mech-

anism a fall would be expected. Another important paper that has appeared recently is by Edelman *et al.* (55), who also studied the action of aldosterone on sodium chloride transport in toad bladder. They found 1) there was a considerable latent period between the addition of aldosterone and the increase in transport; 2) with tritiated aldosterone, most of the aldosterone taken up appeared to be associated with cell nuclei; 3) the effect on sodium chloride transport could be prevented by actinomycin D, which inhibits DNA-controlled RNA synthesis, or by puromycin, which inhibits protein synthesis at the ribosomal level. The conclusion that Edelman and his colleagues drew is that aldosterone promotes synthesis of the enzyme or enzymes concerned in active sodium extrusion. An alternative and, in view of Crabbé's work, more attractive explanation is that the hormone promotes synthesis of the enzyme concerned in the passive penetration of sodium at the opposite end of the cell. Edelman *et al.* did not discuss this possibility, perhaps because they assumed that passive entry takes place by simple diffusion and perhaps because puromycin and actinomycin did not interfere with the action of antidiuretic hormone. Since the "passive" end of the cell allows the passage of sodium ions but not the smaller potassium ions, simple diffusion can be ruled out. Failure of actinomycin D and of puromycin to interfere with the effect of antidiuretic hormone is to be expected if the antidiuretic hormone acts on the permeability mechanism *in situ* in the cell membrane.

If this interpretation of recent work is correct, then aldosterone and cardiac glycosides, though they both affect the same epithelial transport process, are acting at opposite ends of the epithelial cell.

3) The effects of cardiac glycosides and of certain steroid hormones on the contraction of smooth muscle in aortic strips (148) and taenia coli (149) are difficult to interpret. There is no doubt that the effects may be antagonistic, but it is remarkable that a list of the steroids that antagonise the action of strophanthin *excludes* aldosterone, corticosterone and cortisol, and *includes* cortexone (DOC), progesterone, oestradiol and testosterone. The action of the steroids therefore seems to be of a different kind from the general salt-retaining action of aldosterone and cortexone, and it would be rash to assume that the antagonism between the steroids that are effective and strophanthin necessarily denotes opposing actions at the same site.

4) The antagonistic effects of cardiac glycosides and salt-retaining steroids on frog skin potential follow from their antagonistic effects on sodium chloride transport across the skin; the cause of this antagonism has been discussed already under 2. The action of steroids in preventing the loss of potassium from guinea pig heart muscle poisoned by strophanthin k (Kunz and Wilbrandt, 110) is peculiar in that the steroid that is most effective is progesterone, followed by testosterone, cortisol, cortexone, corticosterone, and aldosterone in that order. The synthetic 9α fluorocortisol, which is more potent than cortexone in salt-retaining activity and more potent than cortisol in carbohydrate activity (Liddle *et al.*, 116), is almost without effect. Where such a wide range of steroids is active, where there is no relation between the known physiological activities of the different steroids and their relative effects on the phenomenon under consideration, and where the doses involved are very high compared with the concentra-

tions likely to be found in the body, it seems reasonable to doubt whether the effects observed have much physiological significance. This is not to deny that they require an explanation.

Reports of the action on red cells of adrenal steroids either alone or in the presence of cardiac glycosides are conflicting but mostly negative. Glynn (67) found that aldosterone (10^{-5} g/ml) had no effect on potassium influx or sodium efflux when tested alone, and did not affect the inhibition caused by cardiac glycosides when present at about 20 times the concentration of the glycoside. Experiments with cortexone (10^{-5} g/ml) were restricted to studies of potassium influx but gave similarly negative results. Streeten and Solomon (170) found a very slight diminution of potassium influx with cortisol (3.6×10^{-5} M) but Solomon *et al.* (168) found no inhibition with aldosterone (2.5×10^{-6}) or fluorocortisol (10^{-5} M). Ashwini *et al.* (5) found that hydrocortisone (2×10^{-3} g/ml) alone had no effect on the net loss of sodium that occurs when cold-stored red cells are warmed. Cortexone (2×10^{-3} g/ml) caused little or no effect alone and did not interfere with the inhibitory action of digoxin (10^{-7} g/ml). D'Amico and Cesana (47), and Sulser and Wilbrandt (174) found aldosterone to be without effect on the net sodium and potassium movements of cold-stored red cells incubated at 37°C. A claim by Sulser and Wilbrandt that aldosterone, and to a lesser extent cortexone, antagonised the inhibitory effects of cardiac glycosides, has been withdrawn in a more recent paper by Iff *et al.* (92). Using cold-stored blood under a variety of experimental conditions, these authors failed to find any evidence of antagonism between cardiac glycosides and aldosterone, 9α fluorocortisol, cortexone, or the esters of cortisol, cortisone, prednisolone, or dexamethasone. Sherwood Jones (162) claimed that cortexone, at an unstated concentration, blocked sodium output from cold-stored red cells incubated at 37°, but in a later paper (163) he showed that large effects were obtained only after long exposures to very high concentrations (10^{-3} g/ml), and were accompanied by increased mechanical fragility of the cells. He therefore thought it unlikely that the effect was a specific hormonal response. Friedman and Friedman (62) found that both hydrocortisone (0.2 to 5×10^{-6} g/ml) and aldosterone (1 to 2×10^{-6} g/ml) depressed the rate of sodium extrusion when cold-stored cells were warmed. It is difficult to explain these results in the face of all the negative evidence, but the suggestion made by the authors that aldosterone is effective provided that the cells are suspended in plasma, is contradicted by the work of d'Amico and Cesana (47) and Sulser and Wilbrandt (174), since both these groups of workers used whole blood for their experiments. It is remarkable that the decrease in sodium loss in Friedman and Friedman's experiments was not accompanied by a decrease in potassium uptake; as changes in cell composition were inferred from changes in the plasma concentration, alterations in potassium content should have been easy to detect.

Attempts to demonstrate an interaction between cardiac glycosides and aldosterone in experiments on the ATPase activity of red cell membranes have not been successful. Aldosterone had no action itself and did not antagonise the action of ouabain (151).

If cardiac glycosides did act by competing with or displacing the natural

salt-retaining steroids, one might expect that the spirolactones, which are antagonists of these steroids (97, 97a, 115), would also inhibit active transport. This expectation might seem even more reasonable because both cardiac glycosides and spirolactones have a lactone ring on C_{17}. D'Amico and Cesana (48) found that sodium and potassium movements in cold-stored red cells incubated at 37° were unaffected by the compound SC 9420 at a concentration of 3×10^{-3} M. In similar experiments by Ashwini *et al.* (5) a marked inhibition of sodium loss was obtained with SC 5233 at a concentration of 2×10^{-4} g/ml and a slight effect with one-tenth of this concentration. But the theory that spirolactones act by antagonising the effect of natural salt-retaining steroids was turned topsy-turvy by the demonstration that the effect of SC 5233 was greatly *increased* by the simultaneous presence of cortexone at a concentration of 2×10^{-3} g/ml.

The effects of SC 5233 and SC 9420 on red cell fluxes have also been investigated by Iff (91), who found that, when added as a dry powder to give a concentration of 10^{-3} g/ml, these spirolactones inhibited both sodium and potassium movements. However, the action seemed to be different from that of the cardiac glycosides because the spirolactones caused inhibition additional to that caused by a supramaximal concentration of ouabain (5×10^{-4} g/ml).

5) The competitive or quasi-competitive behaviour of potassium and cardiac glycosides noted in studies of potassium influx in red cells, and of several sodium-plus-potassium-activated ATPases, would be nicely explained by Wilbrandt's chelation hypothesis. A particularly attractive feature of the explanation is that the failure of potassium to be effective beyond a certain concentration (54) is predictable, since once all the steroid present is saturated, raising the potassium concentration will have no effect. But there is nothing in the experimental evidence to incriminate adrenal steroids, and potassium ions might quite well combine with something else in the membrane to form a complex which competes with the cardiac glycosides, if indeed this is the mechanism of the effect.

IV. THE EFFECTS OF CARDIAC GLYCOSIDES ON THE MOVEMENTS OF IONS OTHER THAN SODIUM AND POTASSIUM

Work on the effects of cardiac glycosides on ions other than sodium and potassium has not been extensive and where effects have been obtained their interpretation is generally not clear.

Surprisingly little is known about the transport of the divalent cations and its sensitivity to cardiac glycosides; effects of cardiac glycosides on calcium movements have excited some interest in relation to cardiotonic action but this work is more conveniently considered in the final section.

Inhibition of iodide transport by low concentrations of cardiac glycosides or their aglycones has been demonstrated by Wolff (207), and Wolff and Maurey (208) in thyroid, thyroid tumour, mammary gland, and submaxillary gland. The experiments did not show whether the inhibition was the result of a direct effect on the iodide transport mechanism or was secondary to interference with potassium uptake. Some connection with potassium seems certain because iodide accumulation by thyroid slices did not occur in the absence of external potassium

(207), and because the inhibition of iodide uptake by cardiac glycosides could be reversed by raising the external potassium concentration. Three possible explanations are: 1) that the mechanism for the assimilation of iodide is intimately linked to the mechanism for uptake of potassium or the coupled extrusion of sodium; 2) that iodide accumulation depends on the intracellular concentration of potassium, which runs down in the presence of cardiac glycosides; 3) that the membrane potential is important for iodide accumulation and is altered by changes in extracellular or intracellular potassium concentration.

Results similar to those just described have been obtained in studies of iodide uptake by the rabbit choroid plexus (190); glycoside inhibition of ^{131}I uptake from cerebrospinal fluid *in vivo* has also been reported recently (50).

A little work has been done on the effects of cardiac glycosides on chloride, bicarbonate, and hydrogen ion secretion but again interpretation is difficult. In *in vivo* experiments, Cooperstein and Brockman (37) demonstrated inhibition of bicarbonate secretion by the intestine, and Orloff and Burg (130) observed a reduction in hydrogen ion excretion by the kidney. In both tissues the observed inhibition may have been secondary to disturbances of sodium and potassium movements in the epithelial cells responsible for transport. A detailed investigation of the effects of strophanthidin on hydrogen ion and chloride secretion by the isolated gastric mucosa of the bullfrog has been made by Cooperstein (36) using a combination of electrical and flux measurements. Strophanthidin in doses of 10^{-6} to 10^{-5} M caused a marked reduction of the spontaneous potential and short circuit current, and stopped the secretion of hydrogen and chloride. Membrane conductance and sodium flux were unaltered but the exchange diffusion component of chloride flux seemed to be increased. Whether these changes were brought about directly or as a result of a disturbance of sodium and potassium movements could not be determined. Inhibition of chloride transport in frog skin is to be expected since the cardiac glycosides inhibit the sodium transport that generates the electrical potential responsible for the movement of chloride. In gall bladder, however, Diamond (52) has shown that sodium and chloride ions are transported across the epithelium in 1:1 ratio without the generation of an electrical potential. Ouabain inhibited the transport of both ions. In the alga *Nitella*, MacRobbie (121) found that chloride flux was unaffected by concentrations of ouabain that inhibited potassium uptake. Recently Keynes (101) found that ouabain did not inhibit the active uptake of chloride which he was able to demonstrate in the squid axon.

The uptake of amino acids by various tissues has been shown to be more or less sensitive to cardiac glycosides. Paine and Heinz (132) found rather little effect of ouabain on glycine uptake by Ehrlich mouse ascites tumour cells, but later experiments by Bittner and Heinz (13) showed that with higher concentrations of inhibitor and longer exposures marked inhibition could be produced. The concentrations that were effective also inhibited the movements of sodium and potassium, but the effect on glycine uptake seemed not to be secondary to changes in the internal concentrations of these ions. With the right concentration of inhibitor and the right exposure, it was possible to demonstrate marked inhibition of

glycine uptake in cells that had lost very little potassium; conversely, Kromphardt *et al.* (108) found that replacing some of the intracellular potassium with sodium, by cooling the cells, did not affect glycine uptake (but *cf.* 143). In the same paper, Kromphardt *et al.* showed that glycine uptake was highly sensitive to the external sodium concentration, tending towards zero when sodium was removed (see also 31). They drew attention to the similar effect of external sodium on the absorption of glucose by the intestine (11, 12, 40, 44, 45, 144), and it is interesting that this absorption is also sensitive to glycosides (43). In the experiments on ascites cells it is not clear whether external sodium acts externally or is necessary simply to prevent the cells from becoming depleted of sodium. A possible explanation of the need for sodium is that glycine uptake is somehow linked to active sodium extrusion, which of course would soon be brought to a stop in a sodium-free medium. This hypothesis would explain the sensitivity to glycosides, but it is less easy to explain the earlier observations of Riggs *et al.* (143), also confirmed by Kromphardt *et al.*, that glycine uptake into Ehrlich ascites cells is greatly reduced by lowering the external potassium concentration. A similar dependence of glycine uptake on the extracellular concentrations of sodium and potassium has recently been demonstrated in slices of rat kidney cortex (59).

Transport of amino acids across the gut wall has also been shown to be sensitive to cardiac glycosides. Csáky (41), working with isolated loops of frog gut, found that 10^{-6} M ouabain caused marked inhibition of the transport of L-tyrosine, DL-alanine, 3-methylglucoside, and the pyrimidine uracil. In a later paper (42) he showed that, at concentrations of 10^{-6} to 10^{-5} M, several different glycosides and the aglycone strophanthidin caused marked inhibition of the transport of DL-phenylalanine and also of methylglycoside. Hexahydroscillaren A, which is very much less potent in inhibiting the entry of potassium into red cells, had to be present at 1000 times the concentration of ouabain to produce a comparable effect. Some care is needed in interpreting these results because, in similar experiments with guinea pig gut, Csáky (41) found that the absorption of "substances which are not likely to be actively transported, such as ethanol or antipyrin," was also inhibited by ouabain.

Working with slices of brain cortex, Gonda and Quastel (74) showed that ouabain inhibited the *influx* of glutamate and of creatine but increased the *efflux* of several amino acids. The influx of glutamate was more marked if the potassium concentration in the medium was increased or if ammonium ions were added. With avian erythrocytes, Günther and Winkelmann (75) found that a fairly high concentration of ouabain (1.7×10^{-4} M) inhibited glycine entry, and the inhibition was reversed by cortexone at a concentration of 7.6×10^{-3} M. Cortexone and various other steroids acting alone accelerated the entry of glycine, but the concentrations used were high (6.7 μg/ml for cortexone; 33 to 66 μg/ml for the other steroids), and the relative effectiveness of different steroids did not fit any obvious pattern: cortexone, aldosterone, cortisol, testosterone, and oestradiol were effective; cortisone, oestrone, and progesterone were not.

Kostyo and Schmidt (107) studied the effects of ouabain, scillaren, and scilliroside on the uptake of the non-metabolisable α amino-isobuytric acid by rat diaphragm muscle and found that inhibition was obtained only with 10^{-3} M glycoside. Much lower concentrations were sufficient to disturb the distribution of sodium and potassium.

It is not possible to draw many conclusions from all this work but certain things are clear. In the first place there is no doubt that cardiac glycosides *at reasonably low concentrations* may interfere with the movements of ions other than sodium and potassium. Secondly, in no case has it been demonstrated that the inhibition of the movements of these ions is entirely independent of the inhibition of the movements of sodium and potassium. Thirdly, in Ehrlich ascites tumour cells the inhibition by cardiac glycosides of the uptake of amino acids is not secondary to changes in intracellular sodium or potassium concentrations. In these cells, at least, there seems to be a more intimate link between the uptake of amino acids and the movements of sodium and potassium across the membrane. Fourthly, it is only when chloride and sodium are transported together that cardiac glycosides appear to inhibit chloride movements. But the usual explanation—that in this situation cardiac glycosides act on chloride movements by switching off the potential generated by sodium transport—does not apply to the gall bladder.

V. ION MOVEMENTS AND THE CARDIOTONIC ACTION OF CARDIAC GLYCOSIDES

In previous sections of this review there are no doubt omissions, and perhaps important omissions, but an attempt has been made to cover the literature with some thoroughness. This last section deals with a subject on which the literature is so voluminous that no such attempt has been made. Instead certain crucial questions will be discussed and a few experiments that bear on them will be referred to.

A. Sodium and potassium in cardiac muscle

The most obvious question is: can the positive inotropic effect of cardiac glycosides on heart muscle be secondary to changes in the intracellular concentrations of sodium or potassium which result from inhibition of active movements of these ions across the muscle membrane? The features of the cardiac glycoside molecule that confer the ability to inhibit the movements of sodium and potassium (see section II E) are the same as those that are essential for cardiotonic activity. Furthermore the very different sensitivities to strophanthin of the hearts of rat, guinea pig, toad, and man are fairly well correlated with the different sensitivities of the ATPases in their red cells (140, 141), though the cause of this difference between species is not known. There is no doubt that in digitalis poisoning heart muscle loses potassium and gains sodium, but there has been a good deal of controversy about whether similar changes occur with therapeutic doses of cardiac glycosides. Hagen (78), Wedd (189), Boyer and Poindexter (19), Vick

and Kahn (183), Kühns (109), Lee *et al.* (114), Tuttle *et al.* (179, 179a), and Klaus *et al.* (105) all found little change, or even a slight increase, in intracellular potassium during the positive inotropic phase of the action of cardiac glycosides. Several of these investigators also claimed that the intracellular sodium content was unaffected, and Klaus *et al.* that it was decreased, but the difficulties in measuring intracellular sodium are so great that it is unwise to place too much reliance on the results. The sodium concentration outside the cells is so much higher than inside that small errors in estimates of extracellular fluid volume lead to enormous errors in calculating the internal sodium concentration. And estimates of extracellular space vary widely (179). Inulin has been used by several investigators in attempts to measure the extracellular fluid volume, but the rapid uptake of inulin into the extracellular space is followed by a slow uptake, perhaps due to adsorption, which is difficult to allow for satisfactorily.

If there is reason to doubt that cardiac glycosides in therapeutic doses cause a loss of potassium and it is difficult to tell whether they cause a gain in sodium, it is pertinent to ask whether at low concentrations they do in fact affect the active fluxes of sodium and potassium in heart muscle. Rayner and Weatherall (138) and Klaus *et al.* (105) found that 10^{-6} M ouabain was necessary to inhibit ^{42}K entry, and Carslake and Weatherall (26) found that the loss of ^{24}Na from loaded auricles was the same in controls and in auricles brought to a standstill with 10^{-5} ouabain. However, Carslake and Weatherall also found that, under identical conditions, total tissue sodium was increased while tracer influx seemed to be reduced. These results are clearly incompatible and Carslake and Weatherall gave reasons for believing that the sodium efflux was in fact reduced in their experiments. Because of the uncertainties of direct efflux measurements, they also considered that the apparent threshold of 10^{-6} M for inhibition of ^{42}K entry given by Rayner and Weatherall may need revision. Much greater sensitivity to ouabain was found by Grupp and Charles (74a) in experiments on dog hearts perfused with blood *in situ*. A significant decrease in ^{42}K influx was produced by the intravenous injection of 8.5 μg ouabain/kg body weight. Recently Schatzmann (150) has shown that a sodium-plus-potassium-activated ATPase prepared from pig heart muscle may be inhibited by concentrations of ouabain as low as 10^{-9} to 10^{-8} M. With a similar ATPase preparation from guinea pig heart muscle, Repke and Portius (142) claimed that inhibition can be obtained with concentrations of ouabain down to 10^{-7} M, and that lower concentrations cause stimulation. This effect is surprising and needs confirmation.

It is perhaps worth pointing out that because there is so much potassium in the cell, and because the membrane has a high passive permeability to potassium, it should be much easier to produce appreciable changes in the internal sodium than in the internal potassium concentration. A gain in internal sodium during the therapeutic phase might be expected to reduce the size of the overshoot of the rising phase of the action potential, and such a reduction has sometimes been reported, but according to Kassebaum (100) the increase in contractile force may precede any change in the action potential. Of course the action potential is measured only in one or two of the surface fibres, whereas the contractile force is

determined by all the fibres, but it is difficult to see why the surface fibres should be less affected.

There are two pieces of indirect evidence for the idea that the cardiotonic activity depends on the inhibition of the sodium and potassium movements through the membrane. The first is the observation of Wilbrandt *et al.* (200) that the time taken to reach the maximal inotropic effect varies inversely with the rate of beating. Since each beat is associated with the entry of a certain amount of sodium, the rise in internal sodium concentration when the sodium pump is inhibited will depend on the number of beats. However, as beats are also associated with an entry of calcium, an alternative explanation of the effect of rate of beating is that the internal calcium level is critical. More will be said about this later. The other piece of evidence is the observation that the effect of the cardiac glycosides on contractility is increased if the potassium level in the medium is reduced, and re-reduced if the potassium level in the medium is increased (28, 32, 89, 117).

B. A direct effect on the contractile proteins

Several investigators have attempted to explain the cardiotonic action of cardiac glycosides on the basis of a direct effect on the actomyosin system. The fact that inhibition of sodium and potassium transport by cardiac glycosides involves inhibition of ATPase activity makes such a theory attractive. On the other hand, glycosides do not inhibit the ATPase activity of extracted myosin (187) or of heart muscle myofibril preparations (1). It is known that cardiac glycosides can attach themselves to muscle proteins and modify the physical properties of these proteins (90, 167), and though some of these effects are rather non-specific, Waser (185) has shown that a decrease in the thixotropy of actomyosin solutions is produced only by cardioactive glycosides—inactive analogues produced an increase in thixotropy. Waser (186) also claimed that the cardioactive glycosides caused an increased binding of potassium to actomyosin, but as potassium was the chief cation in his actomyosin solutions there is no reason to suppose that the increased binding capacity was specific for potassium; it may simply be that the surface charge on the protein was increased. Robb and Mallov (145) made fibres of cardiac actomyosin by compressing surface films and measured the speed and force with which these films contracted when ATP was added. They found that fibres made in the presence of very low concentrations of cardiac glycosides (7×10^{-9} g/ml) contracted more quickly and more forcibly, and they concluded that a direct action on the actomyosin system was probably the basis of the cardiotonic action of the cardiac glycosides. A criticism of Robb and Mallov's experiments is that although the concentration of glycoside was extremely low in the bulk of the solution on which the actomyosin was spread, the concentration at the surface may have been extremely high. The fact that the glycoside affected the thickness and strength of the surface films, and the gross appearance and mechanical properties of the fibres, suggests that it may have been acting simply as a surface-active agent. The specificity of the effects described by Waser makes his observations more convincing, but there are stoichiometric difficulties. Waser suggests that about 1 μmole of cardiac glycoside combines with 1 gram of

actomyosin, but estimates of the amount of cardiac glycoside actually present in hearts showing a positive inotropic response give a figure about one hundred times less (61, 128). A full discussion of this problem is given by Wollenberger (209).

C. The action potential mechanism

Another theory that is attractive at first sight is that the cardiotonic effect is primarily on the action potential mechanism. On this theory the selectively cardiac action of the cardiac glycosides could be related to the curious action potentials peculiar to heart muscle. Unfortunately the theory is almost certainly untrue. For one thing, the action of cardiac glycosides is not limited to contractions produced by an action potential: contractions produced by immersion in high potassium media are also augmented (131). For another, when ventricular muscle is treated with strophanthin, the contractile force starts to increase before there are any changes in the action potential (100), and continues to increase while the plateau at first lengthens and then becomes shorter and lower (53, 100). The effects of toxic concentrations of ouabain on the Purkinje fibres of sheep have recently been investigated by Müller (124), who concluded that most of the electrical changes can be explained on the basis of a decreased intracellular potassium concentration. The decrease in the size of the overshoot is presumably caused by an increase in intracellular sodium.

D. Calcium

It has been known for fifty years (32) that the action of cardiac glycosides on heart muscle is highly sensitive to the concentration of calcium in the medium, and in the last few years there has been a great deal of work on the effect of cardiac glycosides on the calcium in heart muscle. This work has been stimulated by recent advances in knowledge of the rôle of calcium in what has become known as "excitation-contraction coupling" (81, 83, 120, 126, 127, 201, 204). The observation of Wilbrandt and Koller (201) that the force of contraction of frog heart muscle depends on the ratio $[Ca^{++}]/[Na^{+}]^2$ in the outside solution has been confirmed and extended by Lüttgau and Niedergerke (120). These authors suggest that the effect arises from the competition of calcium and sodium ions for a carrier R at the surface, and that when the membrane is depolarised CaR moves inwards, carrying calcium into the fibre and so initiating a contraction. In a recent paper Niedergerke (127) has shown that strips of frog ventricle take up extra amounts of calcium when they are made to contract either by reducing the external sodium concentration (thus decreasing the $[Ca^{++}]/[Na^{+}]^2$ ratio) or by depolarising with high external potassium concentrations. In each case the final increase in tension roughly paralleled the increase in calcium uptake (see also 204), but the time courses were different; cellular calcium concentration continued to increase for some time after peak tension was reached. Furthermore when the muscle was replaced in ordinary Ringer solution, relaxation was complete in 10 to 20 seconds, whereas net calcium release occurred for more than five minutes. These results suggested that only a portion of the exchangeable intracellular calcium was concerned with the initiation and maintenance of

contraction. It was therefore proposed that the contraction was initiated by the release of some form of "active calcium" (possibly ionic calcium) at the inner surface of the cell membrane, and that after somehow catalysing the contraction the "active calcium" was inactivated and stored in the cell until it was expelled. [A similar hypothesis for skeletal muscle fibres has been discussed by Hodgkin and Horowicz (83)].

On this theory the cardiac glycosides might increase the force of contraction either by increasing the liberation of "active calcium" at the inner surface of the membrane, or by delaying its inactivation. Unfortunately, although it is quite clear that cardiac glycosides, both in therapeutic and in toxic doses, have effects on muscle calcium, it is difficult to be precise about what these effects are. Apart from the two hypothetical components of the exchangeable intracellular calcium, there is a large amount of unexchangeable intracellular calcium, a large amount of free extracellular calcium and an uncertain amount of extracellular calcium bound, probably, to connective tissue. In frog muscle, it is clear from Niedergerke's results that though most of the calcium external to the fibres may be washed out within five minutes, a large part of the calcium taken up in association with a contraction can also be lost in this time (127). This makes interpretation of experiments on the uptake of ^{45}Ca extremely difficult; it is clearly not permissible to equate ^{45}Ca uptake with calcium influx.

Most of those who have investigated the effects of cardiac glycosides on calcium movements find that total muscle calcium is little affected, or even decreased, by concentrations of glycosides producing a therapeutic effect, and is increased only by toxic concentrations (105, 114, 119). An increase in exchangeable calcium, or in ^{45}Ca uptake, in the presence of therapeutic concentrations has been demonstrated by Lüllmann and Holland (119) and by Gersmeyer and Holland (64), but was found not to occur by Harvey and Daniel (79), Thomas (178) and Witt (205). Most of these authors, and also Holland and Sekul (88, 89), found an increase in ^{45}Ca uptake with higher concentrations of glycoside. Sekul and Holland (158) found that in rabbit atria treated with 4×10^{-7} M ouabain there was no increase in ^{45}Ca uptake if the atria were quiescent, but, if the atria were made to beat, the extra calcium uptake per beat was increased nearly five-fold. They concluded that the cardiac glycosides affected only the calcium exchange that accompanied excitation. A related observation (89) was that atria which were protected against ouabain contracture by pre-incubation in a Ringer solution containing twice the normal concentration of potassium, failed to show the increased ^{45}Ca uptake that otherwise occurred under the influence of ouabain.

The hypothesis that the cardiac glycosides alter the relative affinities of the hypothetical membrane receptors towards sodium and calcium, so that calcium is more readily taken up, has been tested by Reiter (139), and is not satisfactory. He found that the increase produced by a cardiac glycoside in the rate of development of force during the isometric contraction of guinea pig papillary muscle was almost independent of the $[Ca^{++}]/[Na^{+}]^2$ ratio, but was more than doubled by increasing sodium concentration in the medium from 70 to 140 mM.

A possible explanation for this effect is that in the presence of glycoside the increase in external sodium concentration leads to an increased internal sodium concentration and that this interferes with the inactivation of "active calcium." Such a hypothesis could explain the cardiotonic effect of cardiac glycosides entirely on the basis of raised internal sodium, and we have already seen that it is possible, though by no means certain, that a rise in internal sodium is brought about by cardiac glycosides in therapeutic doses. If this hypothesis is correct it should be possible to produce a positive inotropic response by raising the internal sodium concentration in either of two ways: by incubating the muscle for some time in a Ringer solution low in potassium, or by cooling it for some time in a normal Ringer solution. Unfortunately both potassium deprivation and cooling have other effects so that even if the expected result was obtained the interpretation would be to some extent ambiguous.

A more fashionable theory to explain the cardiotonic effect is that "active calcium" is sequestered in vesicles like those obtained by Hasselbach and Makinose (80) from skeletal muscle, and that cardiac glycosides inhibit the uptake of calcium by these vesicles. It is difficult to assess how likely this theory is. Inhibition of calcium absorption by cardiac glycosides has been reported by Vogel (184) in kidney tubules but the detailed mechanism of the inhibition is not known. Calcium ions at quite low concentration appear to inhibit the sodium-plus-potassium-activated ATPase of *Torpedo* (70) and of red cell ghosts (54), though they greatly stimulate the glycoside-insensitive part of the ghost ATPase activity (54). Whether this calcium-stimulated activity is associated with calcium transport is not known, but in any event if it is not sensitive to cardiac glycosides it is not relevant to the theory. In experiments on a membrane ATPase prepared from heart muscle, calcium was found to stimulate activity in the absence of sodium, but here again the calcium-stimulated activity was not sensitive to digitalis (136).

Indirect evidence in favour of an action of cardiac glycosides on the uptake of calcium into vesicles comes from some interesting experiments of Lee (113). He measured the effect of ouabain (10^{-6} g/ml) on the tension produced by adding ATP to strips of dog heart muscle that had been stored in cold glycerol for 3 to 15 days, and he also tested for "relaxing factor" by adding phosphocreatine to the muscle strips after the ATP. It appeared that only those muscles responded to glycoside which also possessed "relaxing factor." Since relaxing factor activity is now generally believed to be the result of uptake of calcium by vesicles, the result suggests that ouabain had an effect only when such uptake was occurring. The difficulty about this interpretation of Lee's results is that it is rather doubtful whether cardiac glycosides do in fact inhibit the uptake of calcium by the vesicles responsible for relaxing factor activity. In experiments on vesicles prepared from skeletal muscle, Fairhurst and Jenden (58) found that 10^{-4} M ouabain had no effect on calcium uptake, and Portius and Repke (136) using 10^{-4} M strophanthin k found at most 10% inhibition. Abe *et al.* (1) investigated the effect of digitalis on the relaxing factor activity of heart muscle granules and found no effect even with a concentration of 10^{-3} M. On the other hand, more positive results have been obtained by Luckenbach and Lüllmann (118), who

investigated the effect of ouabain on the calcium content of granules from guinea pig heart muscle. They exposed the tissue to the glycoside in three different ways: by infusing the glycoside into the intact heart; by homogenising the heart in a solution containing the glycoside; and by treating the isolated granules with glycoside. All three methods showed a diminished calcium content under the influence of glycoside, but the second and third methods required 10^{-5} M ouabain to be effective. The first method was said to require less but the concentration used was not stated. Calcium uptake into liver mitochondria appears not to be affected by cardiac glycosides (182).

E. Summary of Section V

It is difficult to summarise when so much that has been said is tentative but it is probably fair to state that:

1) The cardiotonic effect is not primarily on the action potential mechanism or on the contractile mechanism but is on "excitation-contraction coupling."

2) The sensitivity to cardiac glycosides of the sodium and potassium transport mechanism in the muscle membrane is sufficient for sodium and potassium transport to be affected by cardiac glycosides in therapeutic concentrations. Nevertheless

3) it is doubtful whether therapeutic doses of cardiac glycosides cause appreciable lowering of the internal potassium concentration. A gain in sodium may occur.

4) A positive inotropic action is probably associated with increased uptake of calcium.

5) It is possible that the cardiotonic action of the cardiac glycosides is caused by interference with the removal or inactivation of the calcium that enters the muscle at each contraction. Such interference might be a primary effect of the cardiac glycoside or it might be secondary to a rise in the intracellular sodium concentration.

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