J. Physiol. (1972), **224**, *pp.* 463–475
With 6 text-figures
Printed in Great Britain

INHIBITION OF THE SODIUM PUMP IN SQUID GIANT AXONS BY CARDIAC GLYCOSIDES: DEPENDENCE ON EXTRACELLULAR IONS AND METABOLISM

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(*Received 10 February 1972*)

SUMMARY

1. The rate of inhibition of the Na pump by ouabain was examined both by direct measurement of the rate of decline of the Na efflux and by the binding of [^{3}H]ouabain.

2. The onset of inhibition of the Na efflux was concentration-dependent; but did not follow simple first order kinetics. The time course of inhibition was roughly exponential although in about 30% of the axons inhibition was preceded by a transient stimulation of the Na efflux.

3. Inhibition of the Na efflux by both ouabain and strophanthidin was apparently irreversible.

4. The onset of inhibition was slowed markedly at low temperatures.

5. Replacement of external Na by choline, dextrose or potassium slowed the rate of inhibition. Li behaved like Na and inhibition was faster in K-ASW than in choline-ASW.

6. The rate of inhibition of Na-Na exchange was similar to that of Na-K exchange, but ouabain failed to bind securely to fully poisoned axons.

7. Two components of [^{3}H]ouabain-binding could be distinguished. A linear component which probably reflects uptake into the cells and a saturable component which seems to reflect binding to Na-pumping sites.

8. The saturable component of binding followed a similar time course to the inhibition of the Na efflux and the rate of binding was reduced in choline-ASW and in fully poisoned axons.

9. Measurements of [^{3}H]ouabain-binding indicate that the number of Na pumping sites in the axon membrane is probably between 10^3 and $10^4/\mu^2$.

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INTRODUCTION

The Na-K exchange pump in squid axons is inhibited very specifically by a number of cardiac glycosides including ouabain and strophanthidin. Micromolar concentrations of these glycosides produce complete inhibition when included in the external medium, but much higher concentrations of ouabain are without effect when injected into the interior of the axon (Caldwell & Keynes, 1959; Brinley & Mullins, 1968; Baker, Blaustein, Keynes, Manil, Shaw & Steinhardt, 1969). The mechanisms by which glycosides inhibit the sodium pump is poorly understood. In many mammalian tissues glycoside-induced inhibition is antagonized by external potassium ions (Glynn, 1964; Baker & Willis, 1970, 1972), but this is less pronounced in the squid axon where inhibition is much more sensitive to external Na ions than to K ions (Baker & Manil, 1968; Baker, 1968).

The present experiments were designed to extend the results of Baker & Manil (1968) on the rates of glycoside-induced inhibition of the sodium pump and to compare these results with the binding of [^{3}H]ouabain. The main conclusions are that in squid axons the binding of ouabain is dependent both on the ionic composition of the external medium and also on the metabolic state of the cell.

METHODS

Material

The hindmost pair of giant axons from the squid *Loligo forbesi* were used throughout. Unless otherwise stated, axons were dissected from mantles which had been refrigerated for 1–4 hr. The internal Na content of these axons was about 75 m-mole/kg axoplasm.

Solutions

Axons were immersed in bicarbonate-CO_2 buffered artificial sea water (ASW) of standard composition, NaCl, 460 mM; KCl, 10 mM; $CaCl_2$, 11 mM; $MgCl_2$, 55 mM; $NaHCO_3$, 2·5 mM (10 K(Na)ASW). Na-free sea waters were made by replacing NaCl isosmotically by LiCl, choline Cl, KCl or dextrose. Ouabain (strophanthin-G) was obtained from British Drug Houses and was used in aqueous solution, 2 mM-CN was used as a convenient metabolic inhibitor because its action on squid axons is readily reversible.

Measurements of Na efflux

These were essentially the same as described by Baker *et al.* (1969). Axons were loaded with ^{22}Na by microinjection and the rate of action of glycoside was determined using the special apparatus described by Baker & Manil (1968) for effecting high speed changes in external solutions. An axon, carefully cleaned of small nerve fibres, is mounted in the outlet arm of a 3-way tap. Flow rates of up to 3 ml./sec were used enabling the external solution to be 90% changed in about 3 sec.

Measurement of the binding of [^{3}H]ouabain

In order to increase the quantity of ouabain bound, whole nerve trunks were immersed in sea water containing [^{3}H]ouabain (New England Nuclear) and the binding to both giant axon and surrounding small nerve fibres and Schwann cells

was measured after removing superficial radioactivity in a series of washes in ice-cold ASW containing 1 mM cold ouabain. Before counting, the axoplasm was normally extruded from the giant axon and the uptakes into axoplasm and surrounding sheath were measured separately as described by Baker & Willis (1969, 1972). Ouabain bound to the sheath was expressed as uptake/mg wet wt.

RESULTS

Concentration-dependence of the rate of inhibition

The squid axon is very sensitive to cardiac glycosides. Baker *et al.* (1969) showed that the Na efflux is slowly inhibited by 10^{-7} M ouabain.

The main features of inhibition by the glycoside ouabain are illustrated in Figs. 1, 2 and 3. In many axons the onset of inhibition followed a roughly exponential time course (Fig. 2) but in some, inhibition was preceded by a transient stimulation of the Na efflux (Fig. 1*A*). The time to half inhibition decreased as the concentration of ouabain was increased (Table 1); but

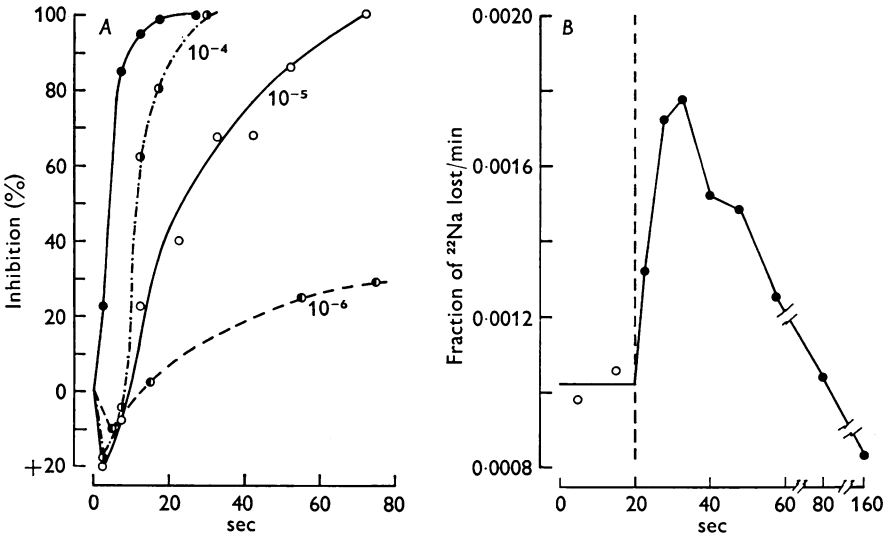

Fig. 1. Experiments comparing the rate of action of ouabain on the Na pump with that of external K ions.

A, comparison of the rate of inhibition of the Na pump by different concentrations of ouabain (○, ◑, ◐) with that of a switch from sea water containing 10 mM-K to K-free sea water (●). Ordinate: % inhibition of the ouabain sensitive Na efflux. Abscissa: time (sec) after addition of ouabain. Temp. 18–20° C. The concentrations of ouabain (M) are given on the graph. 10^{-3} M ouabain is not shown, but followed a similar time course to 10^{-4} M ouabain. The curves are drawn by eye. Note the stimulation of the Na efflux preceding inhibition by ouabain. This was seen in about one third of the preparations.

B, changes in Na efflux following rapid transfer of a nerve from K-free (Na)ASW (○) to 10 K(Na)ASW containing 10^{-5} M ouabain (●). Temp. 20° C.

even with 10^{-3} M ouabain which is a concentration 10^4 times greater than is needed to inhibit the Na pump in this tissue, the onset of inhibition was slower than the reduction in Na efflux which follows a switch from K-containing to K-free ASW (Fig. 1*A*). This slow action of ouabain is illustrated in Fig. 1*B* where an axon immersed in K-free ASW was suddenly exposed to 10 K(Na) ASW containing 10^{-5} M ouabain. The Na efflux underwent a transient stimulation presumably because the Na pump was activated by K ions before the onset of glycoside inhibition.

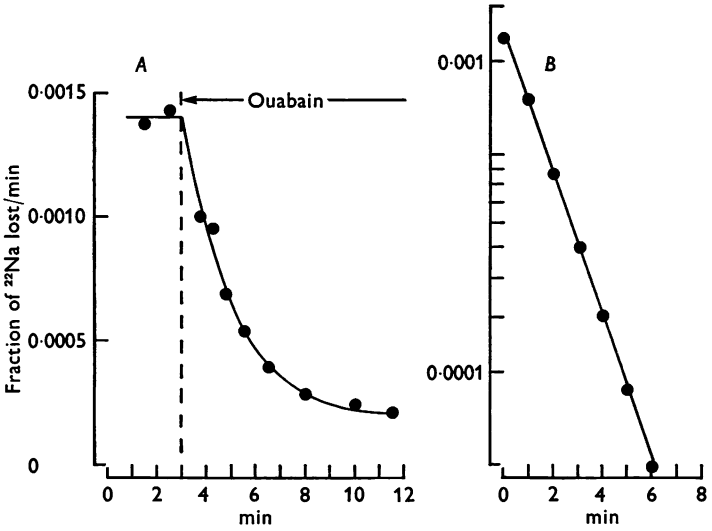

Fig. 2. Rate of action of ouabain in an artificial sea water containing 100 mM-K and 400 mM-Na. The ouabain concentration was 10^{-5} M.

A, experimental data. *B*, semi-logarithmic plot of the ouabain-sensitive fraction of the Na efflux. Abscissa: time in minutes after addition of ouabain. Axon diameter 800 μ. Temp. 17° C.

TABLE 1. Times to half-inhibition of the Na efflux by ouabain. Each value was obtained on a different axon. The external solution was 10 K(Na) ASW throughout. For comparison, the time to half-inhibition of the Na efflux following transfer from 10 K(Na) ASW to 0 K(Na) ASW was $3{\cdot}3 \pm 0{\cdot}33$ sec

Ouabain concn. (M)	Times to half-inhibition (sec)	
	22° C	12° C
10^{-7}	~ 720	No detectable inhibition in 15 min
10^{-6}	75, 144, 130	840
10^{-5}	15, 50, 30, 30, 28, 32	200
10^{-4}	10·5	—
10^{-3}	13	—

An interesting feature of the inhibition is that the observed changes in half-time as a function of glycoside concentration, especially at high glycoside concentrations, are not consistent with a simple first order process. Glycoside binding to mammalian cells has been shown to follow first-order kinetics (Baker & Willis, 1970) and it is possible that the failure to detect similar kinetics in the squid is merely because too few measurements have been made, but an alternative explanation of the discrepancy is that the rate of binding is limited by diffusion of ouabain to its binding sites. More data is required to clarify this point.

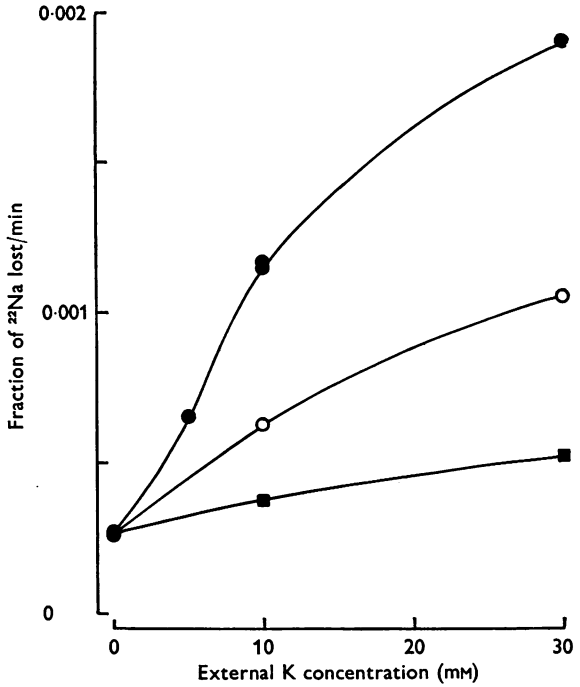

Fig. 3. Partial inhibition of the Na efflux by ouabain. ●, before application of ouabain; ○, after exposure to 10^{-5} M ouabain for 15 sec, followed by thorough washing in ouabain-free sea water and ■, after exposure to 10^{-5} M ouabain for 10 min.

Note that the Na efflux into 10 K(Na)ASW was quite stable in the partially inhibited condition. Axon diameter 900 μ. Temp. 19° C.

Inhibition is very difficult to reverse. Continuous washing for over 2 hr in ouabain-free 10 K(Na) ASW failed to recover any appreciable ouabain-sensitive Na efflux. Similar results were obtained with 0-K (choline) ASW and 100 K(Na) ASW. Fig. 3 shows that if the exposure to ouabain is brief, it is possible to arrest the inhibition at some intermediate level. The K-

sensitivity of this partially inhibited preparation was identical to that before application of glycoside indicating that in the squid axon ouabain acts as a non-competitive inhibitor of the Na pump, reducing the number of available pumping sites in an apparently irreversible manner.

Effects of temperature

Table 1 and Fig. 4*A* show that reducing the temperature from 22 to 12° C produces a six- to tenfold slowing in the rate of action of ouabain. Although based on rather few determinations the data suggest that over the temperature range 22–12° C, the Q_{10} is considerably greater than 3. The binding of [^{3}H]ouabain to mammalian cells also exhibits a Q_{10} greater than 3 (Baker & Willis, 1969, 1972).

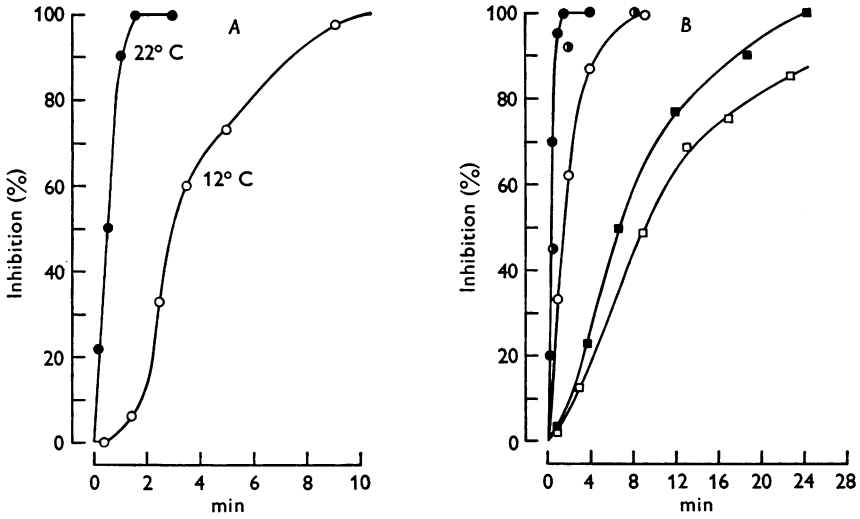

Fig. 4. Effect of temperature and ions on the rate of inhibition of the Na pump by ouabain. The ouabain concentration was 10^{-5} M throughout. Ordinate: inhibition of the ouabain-sensitive Na efflux. Abscissa: time (min) after addition of ouabain.

A, effect of a 10° C difference in temperature.

B, effect of complete replacement of external Na by various Na substitutes: ●, Na; ◑, Li; ○, K; ■, dextrose; □, choline. Temp. 18–20° C.

Dependence on external ions

The binding of ouabain to mammalian cells is faster in K-free media than in media containing K ions. The squid data are superficially similar. Table 2 shows that raising the external K concentration from 10 to 100 mM almost doubled the time to half inhibition of the Na efflux and complete replacement of external Na by K further reduced the rate. It was not

possible to determine directly whether the rate of inhibition is increased in K-free ASW because the Na pump is already inhibited under these conditions. In two experiments axons were exposed to glycoside in 0 K(Na) ASW and then returned to glycoside-free 10 K(Na) ASW to measure the rate of pumping. Exposure to K-free ASW containing 10^{-5} M ouabain for 5 min resulted in complete inhibition of the pump which suggests that inhibition is not appreciably slower than in 10 K(Na) ASW; but the time to half inhibition could not be determined with sufficient accuracy to decide whether the rate of action is increased.

TABLE 2. Influence of external ions on the time to half inhibition of the Na efflux. The ouabain concentration was 10^{-5} M throughout. Temperature 18–22° C

External solution	Time to half-inhibition (sec)
10 K (460 Na) ASW	31 ± 4·5
10 K(Na) ASW + 2 mM-CN (unpoisoned axon)	30
0 K(Na) ASW, axon partially poisoned with alkaline DNP	15, 30
10 K (460 Na) O Ca 55 Mg	27
100 K (400 Na) ASW	78, 84
(460 K) ASW	100,* 192
10 K(Li) ASW	60
10 K(Ch) ASW	390,† 510, 378
0 K(Ch) ASW	260, 540
0 K (Ch) 0 Ca, 0 Mg	420
0 K (dextrose) ASW	420
0 K (92 Na-Ch) ASW	240*
100 K(Ch) ASW	180

* †, refer to pairs of axons from the same squid.

The pair to * was half inhibited in 10 K(Na) ASW in 15 sec. A single experiment in 0 K(Ch) ASW at 12° C gave a half-time of 1020 sec. The ouabain-sensitive Na efflux in 100 K(Na) ASW is intermediate between that in 0 K and 10 K(Ch) ASW. The data in Fig. 5 indicate that in axons fully poisoned in 10 K(Na) ASW about half the Na pumping sites are irreversibly inhibited by 10^{-5} M ouabain in 300 sec.

A criticism of these experiments is that raising the external K concentration necessitated a corresponding fall in external Na and this reduction in Na may have influenced the rate of glycoside action. Table 2 and Fig. 4*B* show this to be true. The rate of glycoside action was reduced to one tenth in choline or dextrose, but not Li, sea waters. In choline-ASW the rate of inhibition was not appreciably affected by the presence of 10 mM-K, but in a single experiment was increased by 92 mM-K. The rate of action was not affected by changes in the concentration of external Ca or Mg ions.

These experiments suggest that in squid axons the extracellular concentration of Na is the primary factor in determining the rate of action of

ouabain. The order of effectiveness of different external ions in promoting inhibition by ouabain is Na = Li $\gg$ K $\gg$ Ch = dextrose. Two experiments with strophanthidin suggest that inhibition by this glycoside also requires external Na ions. At 22° C the time to half inhibition by 10^{-7} M strophanthidin was 330 sec in 10 K(Na) ASW and 960 sec in 0 K(Ch) ASW. As with ouabain, inhibition by strophanthidin was effectively irreversible.

The high speed apparatus made it possible to change the external solution during the course of inhibition by ouabain. When the Na efflux was 50 % inhibited in Na, it was approximately 50 % inhibited in choline and vice versa, indicating that despite the differences in its rate of action, ouabain is acting at the same site in both solutions.

Dependence on metabolism

It is possible to maintain the squid axon in three stable metabolic states: (*a*) unpoisoned, in which the axon contains ATP and arginine phosphate (Arg P) and has a high ATP/ADP ratio; (*b*) partially poisoned, in which the concentration of Arg P and the ATP/ADP ratio are reduced, and (*c*) fully poisoned, in which the ATP concentration is reduced to about 100 μM (Caldwell, 1960). Each state is associated with different behaviour of the ouabain-sensitive Na pump (Baker *et al.* 1969). In the unpoisoned state, pumping is largely Na-K exchange; in the partially poisoned state Na–Na exchange predominates, whereas in the fully poisoned state no pumping is detectable.

It seemed of interest to determine whether there was any difference in the ease of combination of ouabain with the pumping sites in these three metabolic states. The rate of inhibition of Na-Na exchange can be measured quite simply and the rates were similar to those in unpoisoned axons (Table 2), but in order to determine the rate of action on fully poisoned axons it was necessary to examine whether the pump could be reactivated after application of the glycoside. The results of two experiments are illustrated in Fig. 5. 10^{-5} M ouabain was applied for 5 min to a fully poisoned axon immersed in Na–ASW. The ouabain was then washed away. Under identical conditions the Na pump in an unpoisoned axon would have been completely and irreversibly inhibited. When the poisoned axon was allowed to recover in sea water lacking both ouabain and cyanide, the Na efflux increased to about half its original size and subsequent application of ouabain established that this efflux was ouabain-sensitive.

CN *per se* does not protect the axon against ouabain because application to an unpoisoned axon of 10 K(Na) ASW containing 2 mM-CN and 10^{-5} M ouabain resulted in rapid inhibition (Table 2). It follows that either the rate of binding of ouabain is dramatically reduced in fully poisoned axons or that binding occurs but is easily reversed. One piece of evidence against

the second possibility is that if the sodium pump is first inhibited by ouabain and then the axon fully poisoned in ouabain-free ASW containing 2 mM-CN, subsequent removal of the cyanide fails to recover any ouabain-sensitive Na efflux. The first possibility is supported by data presented in the next section which shows that the rate of glycoside binding is reduced in fully poisoned axons (Table 3).

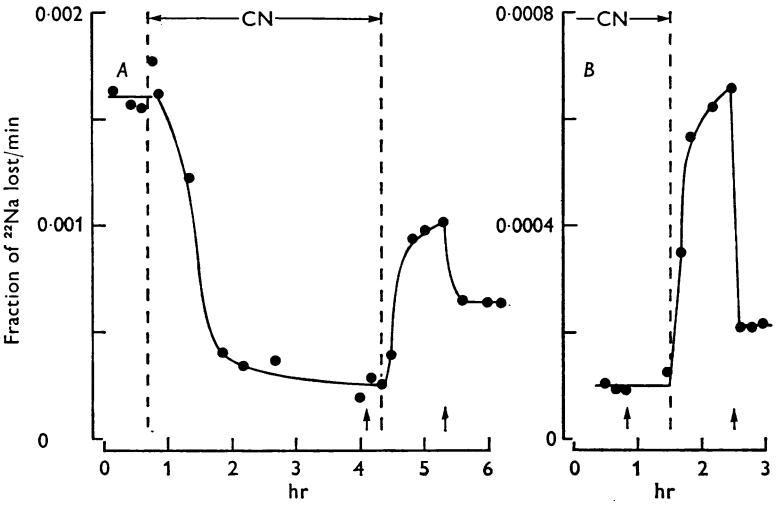

Fig. 5. Failure of ouabain to inhibit the Na pump irreversibly when applied to fully poisoned axons. *A*, complete experiment. *B*, part of a long experiment showing the period following addition of ouabain and removal of cyanide. External ASW, 10 K (Na) ASW throughout. Arrows indicate times of exposure to 10^{-5} M ouabain. Ouabain was applied for 5 min in every instance. After the first application of ouabain, the unbound ouabain was removed by repeated washes in ASW containing cyanide. The CN was then removed and the efflux allowed to recover. At the second arrow the axons were re-exposed for 5 min to CN-ASW containing 10^{-5} M ouabain. Temp. 17° C.

In both experiments the Na efflux recovered to about half of its initial value. This suggests that in fully poisoned axons the time to half inhibition of the Na pumping sites by 10^{-5} M ouabain is about 300 sec. In the unpoisoned axon it averages 30 sec. As it is rare for the Na efflux to recover completely after application of CN, these experiments suggest that in fully poisoned axons the rate of action of ouabain is reduced to less than one tenth of the rate in unpoisoned axons.

Measurements of glycoside binding

As the squid axon is inhibited by very low concentrations of ouabain, it is a particularly suitable preparation for measuring the binding of [^{3}H]-ouabain to Na pumping sites without the complication that is especially

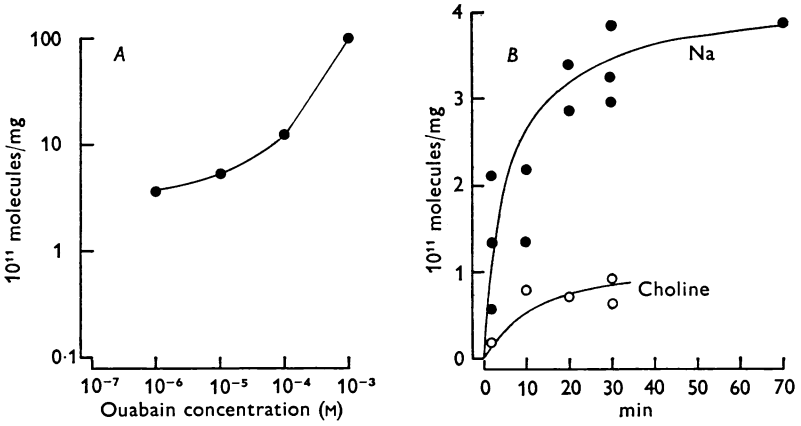

Fig. 6. Binding of [^{3}H]ouabain to squid axons.

A, dependence of binding on the external concentration of ouabain. Ordinate: number of molecules of ouabain bound/mg wet weight. Note that the number has been divided by 10^{11}. Abscissa: external ouabain concentration (M). Note that both scales are logarithmic. Temp. 20° C.

B, effect of replacing external Na by choline on the rate of ^{3}[H]ouabain-binding. Ouabain concentration 10^{-6} M. Ordinate: as for Fig. 6*A* except that the scale is linear. Abscissa: time after addition of ouabain in minutes. After 120 min exposure to [^{3}H]ouabain in Ch-ASW, binding was 2×10^{11} molecules/mg.

In two experiments, the internal Na was elevated by tetanizing the nerve in ASW. In both cases the amount of glycoside bound after 30 min was somewhat lower in the Na loaded nerves.

TABLE 3. Binding of [^{3}H]ouabain to squid axons. Data is also included for the uptake of ouabain into the axoplasm of the giant axon: most points are averages of two or three experiments. Axons were exposed to ouabain for 30 min. All axons were excitable after exposure to [^{3}H]ouabain. Temperature 18–20° C

Solution	Ouabain concn. (M)	10^{11} molecules of ouabain bound/mg wet wt.	Uptake into axoplasm (10^{10} molecules/ mg wet wt.)
10 K(Na) ASW	10^{-6}	$3{\cdot}6 \pm 0{\cdot}2$	0·33
10 K(Na) ASW	10^{-5}	$5{\cdot}5 \pm 0{\cdot}5$	4·2
10 K(Na) ASW	10^{-4}	$12{\cdot}2 \pm 3{\cdot}0$	29·4
10 K(Na) ASW	10^{-3}	100·0	405·0*
10 K(Ch) ASW	10^{-6}	$0{\cdot}8 \pm 0{\cdot}07$	1·4
10 K(Na) ASW + 2 mM-CN Axons fully poisoned	10^{-6}	$1{\cdot}3 \pm 0{\cdot}33$	0·30†

* For an axon of average diameter 600 μ, this is equivalent to an influx of $5{\cdot}5 \times 10^{-14}$ moles/cm^2.sec. The data in the last column is consistent with a permeability constant for ouabain of about $5{\cdot}5 \times 10^{-8}$ cm/sec.

† In a single experiment with 10^{-3} M ouabain, uptake into the axoplasm of a CN-poisoned axon was about one third of that into a control axon.

noticeable at high glycoside concentrations of appreciable binding to non-pumping sites (Baker & Willis, 1970, 1972). Non-specific binding is characterized as a linear increase in binding with increasing glycoside concentration. Fig. 6*A* shows that in squid axons this type of binding becomes appreciable at concentrations above 10^{-5} M. Between 10^{-6} and 10^{-5} M, the amount of glycoside bound is rather constant and the properties of this binding suggest that it is associated with inhibition of the Na pump. Table 3 shows that the uptake of ouabain into axoplasm extruded from the giant axon increased linearly with the external glycoside concentration which supports the view that the linear component of glycoside binding represents passive uptake into the cell interior. Much evidence linking the saturable component of binding to the Na pump has been obtained in other preparations (Baker & Willis, 1969, 1970, 1972; Landowne & Ritchie, 1970) and the following observations indicate that a similar interpretation is also applicable to the squid axon:

(*a*) *The rate of binding.* Fig. 6*B* shows that binding to axons immersed in 10 K(Na) ASW containing 10^{-6} M ouabain is half complete in about 4 min. In comparable measurements on the rate of inhibition of the Na efflux, this concentration of ouabain takes about 2 min to produce half-maximal inhibition. A discrepancy in this direction is not unreasonable, as the axons used for measurement of [^{3}H]ouabain binding were uncleaned.

(*b*) *Dependence of binding on Na.* Fig. 6*B* also shows that the rate and extent of binding is very much reduced in choline-ASW. This observation is also fully consistent with the data on the rate of inhibition of the Na efflux. Uptake into the axoplasm was somewhat greater in the absence of external Na.

(*c*) *The effect of metabolic poisons.* Table 3 includes two experiments on fully poisoned axons. After 30 min exposure to 10^{-6} M ouabain in 10 K(Na) ASW the binding was only one third of that in the absence of cyanide.

Although the data is somewhat sparse, it seems very likely that the saturable component of [^{3}H]ouabain binding to squid axons is associated with inhibition of the Na pump. As most of the binding data was obtained on uncleaned axons it is difficult to calculate the number of glycoside molecules bound per μ^2 of axon membrane, but a single determination on a cleaned axon gave a value of 5000 molecules/μ^2 of membrane. This must be considered an upper limit because non-specific binding and binding to the Schwann cells have been ignored, but even allowing for this it seems unlikely that the value is less than 1000 molecules/μ^2. Provided 1 molecule of glycoside binds to one pumping site, the density of pumping sites is 10–100 times higher than the number of Na channels determined in other nerves from the binding of tetrodotoxin (Moore, Narahashi & Shaw, 1967; Keynes, Ritchie & Rojas, 1971; Hafemann & Houston, 1971).

The figure for the number of ouabain-binding sites can be used to calculate a rough value for the rate of turnover of ATP at each pumping site. At 20° C the maximum ouabain-sensitive Na efflux is about 120 p-mole/cm^2.sec (Baker *et al.* 1969), which gives a molecular turnover of 2900 min^{-1} on the assumption that 3 Na ions are pumped for each energy-rich phosphate bond split. Allowing for a Q_{10} of 3, this figure is equivalent to a turnover of about 15,000 min^{-1} at 35° C which is within the range found for other tissues (Baker & Willis, 1969, 1972).

DISCUSSION

The techniques described in this paper provide two complementary methods for measuring the rate of combination of cardiac glycosides with the sodium pump.

A curious feature of glycoside inhibition in some axons was the transient stimulation of the Na efflux. This was seen in both Na and choline sea waters and we have no information whether it reflects an increase in the Na-K exchange flux or in some other component of the Na efflux. It might, for instance, be related to the small increase in Na efflux from dialysed squid axons which is induced by strophanthidin (Brinley & Mullins, 1968).

The most striking aspect of this study is the dependence of glycoside (ouabain and strophanthidin) action on extracellular Na ions. Of the various Na substitutes tested, only Li behaved like Na. In squid axons both external Na and Li ions competitively inhibit the activation of the Na pump by external K (Baker *et al.* 1969), and one explanation for their action in promoting glycoside binding is that they slow the rate of pumping and in so doing increase the time that some essential structure or conformation is available at the outer face of the membrane. While this may be partly true, simply slowing the rate of pumping is unlikely to be a complete explanation of the action of Na because under conditions where the rate of pumping in Na-ASW is adjusted to be the same as that in choline-ASW, the rate of inhibition is still much faster in the presence of Na ions. The simplest explanation of this data is that combination of the Na pump with external Na or Li ions maintains a conformation that has a high affinity for ouabain or strophanthidin. Thus if we write the following reactions as occurring at the outer face of the Na pump (designated A),

$$A\ \mathrm{Na_m} \overset{m\mathrm{Na}}{\rightleftharpoons} A \overset{n\mathrm{K}}{\rightleftharpoons} A\ K_\mathrm{n} \longrightarrow \text{pumping}$$

the form $A\ \mathrm{Na_m}$ binds glycoside much more readily than A. In view of the fact that $A\ \mathrm{K_n}$ promotes pumping and probably is only available at the outer face of the membrane for a very short period, the observation that glycoside binding is faster in K-ASW than in choline-ASW suggests that the affinity of $A\ \mathrm{K_n}$ for glycoside must be considerably greater than that of A and may be comparable to that of $A\ \mathrm{Na_m}$.

The reduction in the rate of glycoside binding in fully poisoned axons suggests that the metabolic state of the cell can in some way influence the availability of A. Fully poisoned axons differ in a number of respects from unpoisoned or partially poisoned axons. They have much less ATP, but more ADP, AMP and P_i (Caldwell, 1960; Baker & Shaw, 1965) and the intracellular concentration of ionized Ca is increased about thirtyfold (Baker, Hodgkin & Ridgway, 1971). Any or a combination of these factors might be responsible for the reduced rate of glycoside binding.

We wish to thank the Director and staff of the Laboratory of the Marine Biological Association, Plymouth, for providing material and facilities for this work.

REFERENCES

BAKER, P. F. (1968). Recent experiments on the properties of the sodium efflux from squid axons. *J. gen. Physiol.* **51** (suppl.), 172–179.

BAKER, P. F., BLAUSTEIN, M. P., KEYNES, R. D., MANIL, J., SHAW, T. I. & STEINHARDT, R. A. (1969). The ouabain-sensitive fluxes of sodium and potassium in squid giant axons. *J. Physiol.* **200**, 459–496.

BAKER, P. F., HODGKIN, A. L. & RIDGWAY, E. B. (1971). Depolarization and calcium entry in squid giant axons. *J. Physiol.* **218**, 709–755.

BAKER, P. F. & MANIL, J. (1968). The rates of action of K^+ and ouabain on the sodium pump in squid axons. *Biochim. biophys. Acta* **150**, 328–331.

BAKER, P. F. & SHAW, T. I. (1965). A comparison of the phosphorus metabolism of intact squid nerve with that of the isolated axoplasm and sheath. *J. Physiol.* **180**, 424–438.

BAKER, P. F. & WILLIS, J. S. (1969). On the number of sodium pumping sites in cell membranes. *Biochim. biophys. Acta* **183**, 646–649.

BAKER, P. F. & WILLIS, J. S. (1970). Potassium ions and the binding of cardiac glycosides to mammalian cells. *Nature, Lond.* **226**, 521–523.

BAKER, P. F. & WILLIS, J. S. (1972). Binding of the cardiac glycoside ouabain to intact cells. *J. Physiol.* **224**, 441–462.

BRINLEY, F. J. & MULLINS, L. J. (1968). Sodium fluxes in internally dialysed squid axons. *J. gen. Physiol.* **52**, 181–211.

CALDWELL, P. C. (1960). The phosphorus metabolism of squid axons and its relationship to the active transport of sodium. *J. Physiol.* **152**, 545–560.

CALDWELL, P. C. & KEYNES, R. D. (1959). The effect of ouabain on the efflux of sodium from a squid giant axon. *J. Physiol.* **148**, 8*P*.

GLYNN, I. M. (1964). The action of cardiac glycosides on ion movements. *Pharmac. Rev.* **16**, 381–407.

HAFEMANN, D. R. & HOUSTON, A. H. (1971). Specificity of binding of radioactive tetrodotoxin. *Fedn Proc.* **30**, 255.

KEYNES, R. D., RITCHIE, J. M. & ROJAS, E. (1971). The binding of tetrodotoxin to nerve membranes. *J. Physiol.* **213**, 235–254.

LANDOWNE, D. & RITCHIE, J. M. (1970). The binding of tritiated ouabain to mammalian non-myelinated nerve fibres. *J. Physiol.* **207**, 529–557.

MOORE, J. W., NARAHASHI, T. & SHAW, T. I. (1967). An upper limit to the number of sodium channels in nerve membrane. *J. Physiol.* **188**, 99–105.