Effects of Intracellular Adenosine-5’-diphosphate and Orthophosphate on the Sensitivity of Sodium Efflux from Squid Axon to External Sodium and Potassium

PAUL DE WEER

From the Department of Biophysics, University of Maryland School of Medicine, Baltimore, Maryland 21201

ABSTRACT A study was made of sodium efflux from squid giant axon, and its sensitivity to external K and Na. When sodium efflux from untreated axons was strongly stimulated by K+, Na+ was inhibitory; when dependence on K+ was low, Na+ had a stimulatory effect. Incipient CN poisoning or apyrase injection, which produces high intracellular levels of ADP and Pi, rendered sodium efflux less dependent on external K and more dependent on external Na. Injection of ADP, AMP, arginine, or creatine + creatine phosphokinase, all of which raise ADP levels without raising Pi levels, had the same effect as incipient CN poisoning. Pi injection had no effect on the K sensitivity of sodium efflux. Axons depleted of arginine and phosphoarginine by injection of arginase still lost their K sensitivity when the ATP:ADP ratio was lowered and regained it partially when the ratio was raised. Rough calculations show that sodium efflux is maximally K+-dependent when the ATP:ADP ratio is about 10:1, becomes insensitive to K+ when the ratio is about 1:2, and is inhibited by K+ when the ratio is about 1:10. Deoxy-ATP mimicked ADP when injected into intact axons. Excess Mg, as well as Pi, inhibited both strophanthidin-sensitive and strophanthidin-insensitive sodium efflux. An outline is presented for a model which might explain the effects of ADP, Pi, and deoxy-ATP.

INTRODUCTION

The present work was prompted by the findings of Caldwell and his colleagues (Caldwell, 1960; Caldwell et al., 1960 a, b) concerning the dependence of ionic specificity of the sodium pump of squid giant axon on metabolic condi-

1 Abbreviations used in the text are as follows: ADP, adenosine-5’-diphosphate; AMP, adenosine-5’-monophosphate; ArgP, phosphoarginine; ATP, adenosine-5’-triphosphate; CPK, creatine phosphokinase; CreatP, phosphocreatine; d-ATP, 2’-deoxyadenosine-5’-triphosphate; DNP, 2,4-dinitrophenol; EDTA, ethylenediamine tetraacetate; Pi, inorganic phosphate.
tions inside the axoplasm. These authors found that when sodium efflux was restored by microinjection of ATP into CN-poisoned axons, the active extrusion mechanism no longer displayed its characteristic (Hodgkin and Keynes, 1955) dependence on external potassium. Contrariwise, when sodium extrusion was restored with ArgP injections, it did require external K for maximal activity. At the same time, it appeared that the “effect of [metabolic] inhibition was appreciably more complicated than it originally seemed to be” (Keynes, 1960; Caldwell et al., 1960b): incipient CN poisoning, or prolonged exposure to alkaline DNP rendered the sodium efflux insensitive to external K levels, long before the rate of sodium efflux was reduced appreciably. Analysis of axoplasm under the same conditions (Caldwell, 1960) revealed that sodium efflux disappeared when axoplasm ATP content had dropped to very low levels, but that the “K-free effect” was lost whenever the axoplasm became depleted of phosphoarginine. (The K-free effect is a reduction of Na⁺ efflux when 423 mM Na⁺, 9 mM K⁺-seawater bathing the axon is replaced with 432 mM Na⁺-seawater without K⁺.) Also, it was found that phosphoarginine injections, unlike ATP injections, were capable of restoring active K uptake in CN-poisoned axons (Caldwell et al., 1960a).

Two alternative explanations were offered for these observations (Caldwell et al., 1960a, b; Keynes, 1960; Hodgkin, 1964; Caldwell, 1968): either ArgP itself plays a part in Na-K coupling, e.g. by driving an inward K pump (a scheme to this effect was proposed by Caldwell et al., 1960b), or a high axoplasmic ATP:ADP ratio is required for the K-free effect to occur. The latter hypothesis would seem to be more in line with the discovery (Skou, 1957) of a Na⁺ K⁺ Mg-activated, glycoside-inhibited ATPase in nerve membrane preparations, but a decision between these two hypotheses was not possible on the basis of available evidence.

Specifically, the present investigation addresses itself to the question: does the dependence of sodium efflux on external potassium require the presence of phosphoarginine inside the axon, or does it require a low ADP level? Evidence is presented to support the latter alternative. If the effect of ADP on the K sensitivity of Na efflux is mediated solely through its influence on the free energy available from ATP hydrolysis, then high levels of ADP or Pᵢ should be equally capable of modifying the K-free effect; experiments designed to differentiate the effects of intracellular ADP and Pᵢ show that they are quite distinct and that high Pᵢ levels do not, in fact, reduce the K dependence of sodium efflux. Portions of this work have been briefly presented elsewhere (De Weer, 1968, 1970).

METHODS

Microinjection of Giant Axon

*Loligo pealei* specimens used in this study were either obtained at the Marine Biological Laboratory, Woods Hole, Mass. (June through August) or caught off Ocean City,
Md. (October through April) during the period 1966-1969. Only live animals were used; they were decapitated and dissected in chilled flowing seawater. Hindmost stellar axons were removed and carefully cleaned. If the nerves were not cleaned immediately after dissection, they were stored in artificial seawater at 4-10°C for periods up to 6 hr.

The microinjection apparatus used was, with minor modification, that described by Brinley and Mullins (1965). Artificial seawater (ASW) solutions were passed through the perfusion chamber (volume: 1 ml) at a rate of about 1.5 ml/min. Different solutions could be chosen by means of a manifold. In most experiments, 3 min samples were collected. Temperature control was achieved by passing the ASW solution through a plastic tubing coil immersed in ice water before it entered the chamber. By varying the length of tubing between the heat exchanger and the chamber, reasonably stable temperatures between 11°C and room temperature could be obtained. Temperature was continuously monitored with a thermistor probe located inside the chamber. Throughout the great majority of experiments, the axon was stimulated electrically and the action potential recorded through the microinjection capillary and displayed on an oscilloscope. No attempt was made to measure resting potentials, for the simple reason that most of the solutions to be injected (ATP, phosphocreatine, enzymes, etc.) could be expected to produce large and unpredictable liquid junction potentials. All the axons used in this study were excitable at the start of the experiment, and all the individual axons presented in figures in the text were excitable at the end of the experiment; some 10% of the axons listed in the various tables became inexcitable during the last 30 min of the experiment. Axons which became inexcitable at an early stage were not included. It must be said, however, that except for gross damage, or, of course, spontaneous firing, no quantitative or qualitative correlation between excitability and sodium efflux or its sensitivity to potassium, could be detected.

Artificial Seawater Solutions

The different solutions listed in Table I were used to bathe the axons under investigation. The following convenient self-explanatory shorthand (Baker, 1965) will be used in the text: “9 K(Na)SW” for artificial seawater containing 423 mM NaCl and 9 mM KCl, besides the usual amount of Mg and Ca salts, Na-EDTA, and buffer; “0 K(Li)SW” denotes a solution containing LiCl instead of NaCl, and no potassium. EDTA was included to neutralize any heavy metal contaminants. HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonate) was used as a buffer because of its adequate pK (7.55) and small temperature coefficient (ΔpK/°C = 0.014) (Good et al., 1966). When appropriate, 2 mM NaCN was also included. All experiments were performed at pH 7.8–7.95.

Most experiments in Na-free SW to be reported were carried out in Mg-mannitol SW. A common substitute for Na is Li but Li mimics K by stimulating sodium efflux from sodium-loaded muscles (Beaugé and Sjodin, 1968) and also promotes a Ca-dependent sodium efflux (Baker, Blaustein, Hodgkin, and Steinhardt, 1969). Choline seawater is not convenient in use, owing to the instability of the ion (necessitating some purification and storage at low temperatures). An artificial seawater solution of normal ionic strength was designed, using MgCl₂ + mannitol in appropriate
TABLE I
ARTIFICIAL SEAWATER SOLUTIONS*

|         | 9 K(Na)SW | 9 K(Li)SW | 9 K(Chol)SW | 9 K(Mg + mannitol)SW | 0 K(Na)SW |
|---------|-----------|-----------|-------------|----------------------|-----------|
| Na⁺     | 423       | —         | —           | —                    | 432       |
| K⁺      | 9         | 9         | 9           | 9                    | —         |
| Li⁺     | —         | 423       | —           | —                    | —         |
| Choline⁻| —         | —         | 423         | —                    | —         |
| Mg²⁺    | 48.5      | 48.5      | 48.5        | 189.5                | 48.5      |
| Ca²⁺    | 9.3       | 9.3       | 9.3         | 9.3                  | 9.3       |
| Cl⁻     | 496.6     | 496.6     | 496.6       | 355.6                | 496.5     |
| SO₄²⁻   | 25.5      | 25.5      | 25.5        | 25.5                 | 25.5      |
| EDTA    | 0.1       | 0.1       | 0.1         | 0.1                  | 0.1       |
| Na-HEPES pH 7.8 | 2 | 2 | 2 | 2 | 2 |
| Mannitol| —         | —         | —           | 423                  | —         |

* All concentrations in millimoles per liter.

amounts to replace NaCl isotonically. Mg²⁺ can hardly be expected to mimic a monovalent cation; mannitol is preferable to, say, dextrose because the resulting solution will be less dense and viscous, while still being osmotically active.

Analytical Procedures

The seawater samples collected from the chamber were dried on planchets, and counted in a low-background gas flow counter.

Samples of ATP, ADP, and phosphocreatine were assayed according to the method of Kornberg (1950), using a commercial kit; creatine phosphokinase was assayed according to Oliver (1955). The method of Rosenberg et al. (1956) was used to determine axoplasmic arginine concentrations. Axoplasmic phosphoarginine was determined by difference, after the following reactions had taken place:

\[ \text{ArgP} + \text{ADP} \rightarrow \text{ATP} + \text{arginine} \]

\[ \text{ATP} + \text{glucose} \rightarrow \text{glucose-6-P} + \text{ADP} \]

Arginine phosphokinase (lobster muscle) needed for the first reaction was obtained as a concentrated aqueous solution (Virden et al., 1965) courtesy of Dr. J. J. O'Neill.

Solutions to Be Injected

The basic solution used in injection experiments had the following composition (in mm): K₂SO₄ 500; phenol red 0.5; EDTA 0.1; MgCl₂ 0.2; pH 7.3. This solution was used as such for the injection of isotope (²¹Na or ³²Na) or enzymes, which did not modify tonicity appreciably. Whenever other substances had to be injected (MgATP etc.) the K₂SO₄ in the above mixture was reduced by an appropriate amount. The pH value of 7.3 was chosen on the basis of experience by Tasaki et al. (1965) that per-
fusion solutions at pH 7.2–7.4 gave better survival times. This pH may be a little higher than the value (7.0–7.3) found experimentally by Caldwell (1958).

Labeled sodium was obtained either as $^{23}$NaCl or as $^{22}$Na$_2$SO$_4$. In either case the solution was ashed, and taken up in the above injection medium. The following enzymes (obtained from Sigma Chemical Co., St. Louis, Mo.) were used for injection into axons: creatine phosphokinase (rabbit muscle), 32 units/mg; arginase (beef liver), 28 units/mg; apyrase (potato), 1.1 units/mg; ATP (obtained as the Mg salt) and AMP (obtained as the free acid) were neutralized with KOH. l-Arginine was obtained as the base and neutralized with HCl. Phosphocreatine, ADP, and deoxy-ATP were obtained as the sodium salt and passed over K-loaded Dowex-50 ion exchange resin, freeze-dried, and stored in a desiccator at $-20^\circ$C until needed.

**Radial Diffusion of Injected Enzymes** In a considerable number of experiments, enzymes were injected axially and allowed to diffuse radially through the axoplasm. It is not quite necessary that the enzymes reach the axolemma at all, since substrates and products are much more mobile and will undergo reaction even if the enzymes do not move from their site of injection. However, should this be the case, it may no longer be reasonable to assume that substrate and product concentrations at the membrane will be what one could calculate from equilibrium considerations, even though the quantity of injected enzyme may be nominally far in excess of what is needed to achieve almost instantaneous equilibrium. In particular, if creatine phosphokinase is not distributed throughout the axoplasm, it may be hazardous to make quantitative inferences about the ATP:ADP ratio at the membrane from the bulk CreatP:Creat ratio.

From a general equation for the time course of diffusion in a cylinder (Carslaw and Jaeger, 1947), Hodgkin and Keynes (1956) derived a particular solution for the concentration $C_m$ at the membrane, after the instantaneous introduction, at time $t = 0$, of diffusible material in the axis of the axon. When this equation is solved for a typical (500 $\mu$) axon and for a protein with diffusion coefficient $D = 0.625 \times 10^{-6}$ cm$^2$ sec$^{-1}$, it is found that after about 1000 sec, or 17 min, the protein concentration at the membrane reaches 45% of its final value. Since a vast excess of creatine phosphokinase was injected at the beginning of the experiment (i.e. at least 1 hr earlier than its substrate, phosphocreatine), the assumption of phosphagen/ATP equilibrium at the site of the membrane seems to be justified.

**Hydrolysis of Axoplasmic Arginine and Phosphoarginine**

**pH Change** Several experiments called for complete hydrolysis of axoplasmic arginine and phosphoarginine. In order to calculate the magnitude of the effect, if any, of ArgP and Arg hydrolysis to ornithine, urea, and P$_i$, the number of moles of protons produced or absorbed during hydrolysis at constant pH, say pH 7.3, should be calculated. This calculation can be done using the following list of pK values gathered from Myerhof and Lohmann (1928), Kumler and Eiler (1943), Alberty et al. (1951), Datta and Grzybowski (1961), and Greenstein and Winitz (1961):
From the table it can be deduced that at pH 7.3 the average net charge on Arg and ornithine is +0.98 and +0.96, respectively. That is to say, hydrolysis of arginine to ornithine + urea at this pH will not produce or absorb any appreciable amount of protons. As for the hydrolysis of ArgP to Arg + P1, it can be calculated that 0.18 mole of protons will be absorbed per mole of phosphagen hydrolyzed. No direct titration of axoplasm has been performed, but from the pK's of the major constituents of axoplasm (Deffner, 1961) one ought to be able to ascertain whether it can safely give off 0.6 mmole of protons per liter (to compensate for the hydrolysis of about 3 mmoles of ArgP). Considering just one major component, taurine (about 100 mmoles/kg), with pK2 = 8.74 (Greenstein and Winitz, 1961) it is calculated that upon withdrawal of 0.06 mmole of protons, this buffer system would raise its pH from 7.30 to 7.37. Thus, neglecting the probable buffer capacity of axoplasmic protein etc., a pH shift of no more than 0.07 unit is expected.

**In Vitro Efficacy of Arginase**  Beef liver arginase was obtained as a lyophilized powder. It requires Mn++, has a mol wt of 138,000 (Greenberg et al., 1956), a pH optimum of 9.2 (Bach and Killip, 1960), and catalyzes the reaction

\[
\text{Arginine} + \text{H}_2\text{O} \rightarrow \text{ornithine} + \text{urea}
\]

The commercial powder may contain other enzymes; however, any impurities do not seem to interfere with the present experiments. A stock solution was prepared containing 600 units/ml and 5 mM MnSO4 (pH 7.1). The mixture was kept at 37°C for 4 hr prior to storage in the refrigerator. In order to test the activity of the enzyme in a medium resembling axoplasm, the effect of Mn-activated arginase (10 units/ml) on a medium containing K2SO4 (0.5 mM), arginine (5 mM), Na-HEPES pH 7.3 (5 mM) was followed at 15°C by analyzing the mixture repeatedly for arginine. Arginine hydrolysis was found to be 95% complete in 10 min and 99% complete in 25 min. This rate was apparently unaffected by the inclusion of 5 mM each of ornithine and urea in the reaction mixture.

**In Vivo Efficacy of Arginase**  The ability of injected arginase to hydrolyze axoplasmic arginine + phosphoarginine was tested on five axons. Phosphoarginine is not a substrate for arginase, but in the presence of ADP, arginine phosphokinase, and an ATP-consuming system, all phosphoarginine and arginine will eventually be hydrolyzed to ornithine + urea.

Axons were injected with arginase (10 units/ml) over as long a length as possible,
and kept in (Na)SW at 15°C for a measured length of time. Axoplasm from the injected region (identified by the phenol red present in the injection mixture) was extruded and analyzed for arginine. The results showed that within 30 min, and for at least 240 min after injection, the arginine content of the axon had dropped to, or below the detection limit of the method; i.e., to 5% or less of the normal concentrations.

Effect of Injected Creatine Phosphokinase and Arginase on Na Efflux
Since some of the experiments to be reported below called for a preliminary injection of rabbit creatine phosphokinase (CPK) and beef liver arginase into axons, it first had to be established that these enzymes were sufficiently stable within the axon, and had no intrinsic effect on sodium extrusion. In order to test for stability of injected CPK, axons were injected with a known quantity of enzyme, left in (Na)SW for some time, and analyzed for residual activity. After 4 hr 40-65 % of the injected activity was recovered; this is certainly more than adequate for the purposes of the experiment. As for arginase activity, it was seen in the previous paragraph that the purpose of the injection, namely to deplete the axon of arginine, was achieved in about 30 min, and that no change in this condition occurred in the next 3.5 hr. By inference, arginase seems to be sufficiently stable for the present purpose.

In order to check for possible toxicity of the enzymes, a mixture of CPK + arginase was injected into five 22 Na-loaded axons. If anything, the injection of CPK (200 units/ml), arginase (10 units/ml) + Mn++ (0.3 mM) produced only a slight transient depression of sodium efflux into 9 K(Na)SW. The K sensitivity of the axon was not affected, nor was its ability to recover from CN inhibition.

These preliminary experiments demonstrate that CPK, arginase, and Mn++ in the concentrations used have no appreciable effect on sodium efflux; that the injected enzymes are sufficiently stable; and that axons so treated can be essentially depleted of arginine and ArgP.

RESULTS
Rate Constant for Sodium Efflux from Squid Axon
Rate constants for sodium efflux into 9 K(Na)SW were quite variable, with a mean and standard deviation of 3.93 ± 1.82 × 10^{-3} min⁻¹ (n = 115 axons). Part of the variability is ascribable to variations in diameter (range from 390 to 650 μ) and variation in temperature. When rate constants are normalized to a hypothetical diameter of 500 μ, a value of 3.6 ± 1.93 × 10^{-4} min⁻¹ is obtained. As for temperature dependence, all normalized constants for axons which happened to have been observed at a given temperature were pooled, and the means ± se plotted vs. temperature on a semilogarithmic graph (Fig. 1). A straight line was fitted by a least squares method to the unweighted means. Its slope was 0.0397 log (rate constant) per degree, corresponding to a temperature coefficient Q10 = 2.49 (or an activation energy of 15 kcal/mole). Using this Q10 value, the rate constants were further normalized to a tem-
FIGURE 1. Temperature dependence of sodium efflux from squid giant axon. Data from 115 axons. For any given temperature, rate constants for individual axons were normalized to a diameter of 500 μ, and pooled. The points shown are means (from 3-27 axons) ±se. The curve, fitted by a least squares procedure to the unweighted means, obeys equation: Log \[ \text{rate constant (in min}^{-1} \times 10^a) \] = -0.07174 + 0.3966 temperature \(^\circ\)C corresponding to a temperature coefficient \(Q_{10} = 2.49\).

FIGURE 2. Inhibition of sodium efflux by strophanthidin. Sodium efflux 20 min after addition of strophanthidin, relative to its value before addition of the drug. Temperature 13-17°C. All experiments in 9 K(Na)SW. Open circles, individual axons; filled circles, mean ±se from three to seven axons. The large se at 10^-6 M strophanthidin reflects the fact that in two out of four axons the drug had a stimulating effect on sodium efflux.

Axoplasmic Sodium Content

Four axons were analyzed for axoplasmic [Na] within 2 hr after dissection. The mean content was 66 ± 8 mmol/kg axon. This compares well with...
the reported averages of about 80 (Frumento and Mullins, 1964) and 72 (Schwartz, 1968) reported for axons from squid caught off Woods Hole at about the same time of the year. Using the above average value, it is calculated that the average rate of sodium efflux at 15°C was 45.7 ± 1.1 pmoles cm⁻² sec⁻¹.

**Effect of Strophanthidin**

Fig. 2 summarizes data on the inhibitory effect of the cardioactive steroid, strophanthidin, on sodium efflux into 9 K(Na)SW. It was found necessary to standardize the time of exposure to the drug. After 20 min of exposure to a strophanthidin concentration of 10⁻⁷ M or higher, a relatively stable plateau was reached (absolutely stable for 10⁻⁵ M). However, when drug concentrations smaller than 10⁻⁷ M were used, the rate of sodium efflux was still decreasing after 20 min. At 10⁻⁸ M, it was even seen to rise (in half the cases) before a fall would occur. The dose-response relationship for strophanthidin depicted in Fig. 2 is very similar to that published by Baker, Blaustein, Keynes, Manil, Shaw, and Steinhardt (1969) for the cardiac glycoside ouabain.

No attempt was made to investigate the point in detail, but it was found that the rate of inhibition was roughly doubled or tripled for every 10-fold increase in drug concentration. The data do not exclude the possibility that the extent of inhibition by 10⁻⁷ M strophanthidin, or even less, would eventually have reached the level obtained with 10⁻⁴ M; i.e., 19 ± 2% of control. No further fall of sodium efflux in 10⁻⁵ M strophanthidin could be observed during protracted exposure (2.5 hr); nor did 10⁻³ M strophanthidin have any additional effect.

**Effect of External Potassium**

**K-free effect** The K-free effect is the effect observed when potassium ions are removed from the seawater bathing the axon, namely, a variable (10–80%) drop in the rate of sodium efflux. In 97 axons tested, the ratio

\[
\frac{\text{Na efflux into 0 K(Na)SW}}{\text{Na efflux into 9 K(Na)SW}}
\]

ranged from 0.18 to 1.0. The average was 0.52 ± 0.02. Unlike the K-free effect, the effect of 10⁻⁸ M strophanthidin was very consistent (average reduction 81 ± 2%, range from 74 to 86%, in fresh axons). In a number of instances both K-free and strophanthidin effects were observed on the same axon: the rate of efflux was, without exception, lower in the presence of 10⁻⁸ M strophanthidin.

**Activation of sodium efflux by external potassium** The curve relating the magnitude of the K-sensitive fraction of sodium efflux to the external potassium concentration has a sigmoid shape (Fig. 3). The points
on Fig. 3 were obtained by symmetrically enclosing "test K" (Na)SW periods between 0 K(Na)SW and 9 K(Na)SW periods. The 0 K(Na)SW:9 K(Na)SW sodium efflux ratio for the individual axons varied between 0.20 and 0.46; the shape of the normalized $K_*$ activation curve was apparently independent of this ratio. The "foot" on the curve was strikingly apparent only after inclusion of the 0.5 mM K point (0.5 mM K is $1/26$, and 1 mM K is $1/6$ as effective as 9 mM K). A similar S-shaped dependence on external K has been described by Baker, Blaustein, Keynes, Manil, Shaw, and Steinhardt (1969) for the ouabain-sensitive sodium efflux from *Loligo forbesi* axons.

There is good reason to believe that the "true" curve relating K-dependent sodium efflux to $K_*$ just outside the axolemma is more strongly S-shaped than Fig. 3 would suggest. Indeed, potassium leakage from the axon into the Frankenhaeuser and Hodgkin (1956) space will raise the $K_*$ about 0.5 mM above the nominal K concentration in the seawater (see discussion below). The curve in Fig. 3 should therefore be shifted 0.5 mM to the right along the abscissa, to give the true curve relating K-stimulated sodium efflux to $K_*$.

It is apparent that even at 50 mM $K_*$, sodium efflux continues to rise with increasing $K_*$.

Baker, Blaustein, Keynes, Manil, Shaw, and Steinhardt (1969), studying the K activation of ouabain-sensitive sodium efflux in *L. forbesi*, further found that in Na-free SW's (except Li-SW) the apparent $K_m$ for $K_*$ activation of Na efflux was drastically reduced, with Na efflux tending towards the same maximum at high $K_*$ levels. Although no experiments were specifically designed to study this phenomenon, the following findings obtained from axons in good condition are in qualitative agreement with those of Baker, Blaustein, Keynes,
Manil, Shaw, and Steinhardt (1969): (a) sodium efflux into Na-free SW nominally free of K (i.e., leaving about 0.5 mM K just outside the axolemma) was three to four times larger than into K-free(Na)SW; (b) Na efflux into 10 K(CHol)SW was comparable to that in 50 K(Na)SW; and (c) Na efflux into 1 K(CHol)SW was higher than that into 10 K(Na)SW.

**Effect of CN on Sodium Efflux and its Sensitivity to K**

A number of axons were exposed for variable lengths of time to seawater containing 2 mM NaCN. Average efflux from 19 “fully poisoned” axons was 18.3 ± 3.3% of control, but for some axons it was as low as 10%.

The first noticeable phenomenon upon exposure of an axon to CN-containing seawater is the gradual disappearance, sometimes **followed by a reversal**, of the K-free effect (Caldwell et al., 1960 b). Fig. 4 is typical: it shows that within

![Figure 4](image)

**Figure 4.** Effect of CN on sodium efflux and its sensitivity to external K. Open circles, 9 mM K₀; filled circles, 0 mM K₀. Axon 71867A. Diameter 425 µ. Temperature 12°C.

![Figure 5](image)

**Figure 5.** Effect of injected ATP on sodium efflux from CN-poisoned axons. Open circles, 9 mM K₀; filled circles, 0 mM K₀. Arrow indicates time when MgATP was injected to produce a final axoplasmic concentration of 16 mM. Axon 72666. Diameter 420 µ. Temperature 16°C.
60 min of exposure to CN, and long before the sodium efflux into 9 K(Na)SW is appreciably reduced, the K-free effect has disappeared. This phenomenon is reversible, since the K-free effect reappears after CN is removed. The requirement for external potassium was usually completely lost after 30–45 min of exposure to cyanide (21 axons).

Not only does the K-free effect disappear, in many cases it even reverses, as illustrated in Fig. 13 (first 3 hr). Such a "reverse K-free effect" (defined as a rise in sodium efflux upon removal of external K), seen in eight instances, was never very pronounced (maximum observed: a 37% rise), and occurred only after exposure to CN for a considerable length of time (1.25–2 hr).

**Effect of Injected ATP**

One of four experiments involving injection of MgATP into CN-poisoned axons is illustrated in Fig. 5. These experiments confirmed the finding of Caldwell et al. (1960 a) that ATP injections are capable of restoring sodium efflux into 9 K(Na)SW (to 65–125% of control), but that the sodium efflux now is no longer dependent on external potassium.

**Effect of Injected ADP**

Since incipient CN poisoning must result in increased ADP levels, it was logical to inquire whether injection of ADP would affect the K-free effect. Five technically successful injections of ADP into axons were performed (listed in Table II). When equimolar quantities of ADP + Mg were injected, there was little effect on efflux into 9 K(Na)SW, but the K-free effect was markedly reduced (Fig. 6), at least shortly after the injection. (The K-free effect had a tendency to recover in every case in which the axon was monitored for several hours.) The extra sodium efflux into 0 K(Na)SW resulting from the ADP injection was inhibited by 10^{-6} m strophanthidin.

**Table II**

| Axon No. | ADP (nm) | Mg (nm) | Efflux in 0 K(Na)SW Before injection | After injection |
|----------|----------|---------|-------------------------------------|----------------|
| 40568    | 18       | 9       | 0.46                                | 0.95*          |
| 42368B   | 16       | 8       | 0.36                                | 0.90–0.77*     |
| 50768A   | 18       | 18      | 0.50                                | 0.93–0.77      |
| 50868A   | 8        | 9       | 0.30                                | 0.65–0.57      |
| 80868A   | 19       | 9.5     | 0.32                                | 0.90–0.81      |
| Mean     |          |         | 0.39                                | 0.87–0.73      |

* Na efflux inhibited by excess ADP.
Even with the largest amount of ADP used (19 mM final concentration, axon 80868A), the K-free effect never disappeared completely. If the myokinase and arginine phosphokinase equilibria are taken into account (see discussion), the calculated ATP:ADP ratio shortly after injection of this large amount of ADP is about 1. It would thus seem that in order to render the Na efflux insensitive to external K, the ATP:ADP ratio should be reduced to below 1.

In two instances, one of which is shown in Fig. 7, an error was made in matching the stoichiometry of ADP and Mg, and an excess of ADP was injected. This resulted not only in a disappearance of the K-free effect, but also in a drastic reduction of the sodium efflux into 9 K(Na)SW. The inhibitory effect of excess ADP (capable of binding Mg++) can be interpreted as evidence that the sodium extrusion mechanism requires free Mg. Another possibility is that free ADP or free ATP may inhibit the operation of the pump by occupying the "fuel" (MgATP) site.

**Effect of Injected AMP**

Since adenylate kinase is present in axoplasm, AMP injection should result in increased ADP levels and decreased ATP levels

$$AMP + ATP \rightarrow 2 \text{ ADP}$$

AMP does not complex Mg++ very strongly (Martell and Schwartzenbach, 1956) but, to be on the safe side, and since Mg was not anticipated to have any effect of its own, a stoichiometric amount of MgSO₄ was included when AMP was injected into axon 50768B. As a result of this injection, not only was
Figure 7. Effect of injected ADP + Mg (2:1) on sodium efflux from squid axon. Open circles, 9 mM K; filled circles, 0 mM K. Arrow indicates time when ADP + Mg was injected to give approximately the final concentrations indicated. Axon 40566. Diameter 425 μ. Temperature 11°C.

Figure 8. Effect of injected AMP on K sensitivity of sodium efflux from squid axon. Open circles, 9 mM K; filled circles, 0 mM K. Arrow indicates time of injection of AMP to make a final concentration of 20 mM. Axon 81068B. Diameter 490 μ. Temperature 18°C.

The K sensitivity diminished (as expected), but the absolute level of Na efflux into 9 K(Na)SW was reduced as well (by 50%).

This inhibition was tentatively ascribed to Mg, and another axon was injected with AMP alone. As seen in Fig. 8, no untoward effect was noticed on the rate of efflux into 9 K(Na)SW, whereas the K-free effect was initially reduced to about half its original value. Again, as was the case with ADP injection, there is a tendency for the axon to partially recover its requirement for external K after a period of several hours.

Effect of Mg++ Injection

In order to confirm the hypothesis that Mg++ might be responsible for the inhibitory effect described above, injections of MgSO₄ alone were made. Fig. 9 shows what happens when MgSO₄ is injected to produce an axoplasmic concentration of about 20 mM: a drastic reduction in sodium efflux occurs, but the K-free effect is practically unchanged.
The dose-response relationship for this inhibitory effect of Mg$^{++}$ is shown in Fig. 10. Mg concentrations in excess of 2 mM clearly depress sodium efflux. An interesting finding was that both strophanthidin-sensitive and insensitive Na effluxes were inhibited by Mg$^{++}$, the former by $41 \pm 3\%$ ($n = 4$), the latter by $26 \pm 1\%$ ($n = 3$), upon injection of 20 mM Mg$^{++}$.

**Figure 9.** Effect of injected Mg on sodium efflux from squid axon. Open circles, 9 mM K$_o$; filled circles, 0 mM K$_o$. At time indicated by arrow MgSO$_4$ was injected to a final concentration of 20 mM. Axon 80468. Diameter 450 μ. Temperature 13°C.

**Figure 10.** Inhibition of Na efflux by injected Mg. Every value (except at 40 mM) represents the mean ±se from two to four experiments. Efflux is expressed relative to that before injection.

**Effect of Arginine Injection**

Axoplasmic arginine phosphokinase activity is quite high (Caldwell et al., 1964; Mullins and Brinley, 1967) and the equilibrium constant for the reaction

$$\text{Arginine} + \text{ATP} \rightarrow \text{phosphoarginine} + \text{ADP}$$
is such (Lehmann, 1936) that one can expect appreciable amounts of ADP to be formed upon injection of arginine.

Five axons were injected with L-arginine to produce final concentrations of 10–30 mM. Fig. 11 illustrates one such experiment. It is seen that arginine injection has little effect on efflux into 9 K(Na)SW, but strongly stimulates efflux into K-free seawater. This extra efflux is inhibited by strophanthidin. The same qualitative picture was found in all axons tested: the K-free efflux rose from an average of 0.43 of control before, to an average of 0.77 of control after injection of arginine. Efflux into 9 K(Na)SW was slightly reduced to 0.92 ± 0.05 of control.

Figure 11. Effect of injected arginine on sodium efflux into normal and K-free seawater. Open circles, 9 mM K⁺; filled circles, 0 mM K⁺. Arrow indicates time of injection of L-arginine to make a final axoplasmic concentration of 20 mM. Strophanthidin 10⁻⁶ M was added at the end of the experiment. Axon 40468A. Diameter 450 μ. Temperature 14°C. Calculated ATP:ADP ratio shortly after injection, ~1.

Figure 12. Effect of injected apyrase on sodium efflux from squid axon. Open circles, 9 mM K⁺; filled circles, 0 mM K⁺. Apyrase (final concentration 20 units/ml) was injected at time indicated by arrow. Slight rise of rate constant (from 3.99 × 10⁻⁴ min⁻¹ to 4.50 × 10⁻⁴ min⁻¹) upon exposure to 10⁻⁷ M strophanthidin was statistically significant (p < 0.02). Axon 42468A. Diameter 500 μ. Temperature 12°C.
**Effect of Injected Apyrase**

Potato apyrase catalyzes the reactions:

\[
\text{ATP} \rightarrow \text{ADP} + P_i \\
\text{ADP} \rightarrow \text{AMP} + P_i
\]

In the batch of enzyme used for this study, the adenosinetriphosphatase activity was about 100 times greater than the adenosinediphosphatase activity. Consequently, injection of apyrase would be expected to reduce the axoplasmic ATP:ADP ratio, at least transiently. Four such experiments were performed. When a very large quantity (in fact, an apparently saturated solution) of enzyme was injected into an axon bathed in 0 K(Na)SW (Fig. 12), there was a rapid increase, followed by a sharp decrease in sodium efflux, to 12.9% of the control level. When \(10^{-7}\) M strophanthidin was applied, the efflux rose slightly but significantly \((p < 0.02)\) to 14.5% of the original level. Smaller quantities of apyrase led to longer lasting transient increases in efflux into 0 K(Na)SW.

**Axoplasmic Arginine and Phosphoarginine Content**

Collected in Table III are values found for free and total axoplasmic arginine. Phosphoarginine content was calculated by difference. The average \([\text{ArgP}] : [\text{Arg}]\) ratio, calculated by taking (average total Arg – average free Arg) : (average free Arg) from the four axons on which both total and free Arg were determined, was 1.7.

| Axon No. | Total Arg | Free Arg | ArgP* | ArgP/Arg |
|---------|-----------|----------|-------|----------|
|         | mmol/kg   | mmol/kg  | mmol/kg | mmol/kg  |
| 81467A  | 3.0       |          |       |          |
| 81467B  | 4.2       |          |       |          |
| 81567A  | 2.4       |          |       |          |
| 81567B  | 2.6       |          |       |          |
| 100467A | 3.9       | 1.8      | (2.1) | 1.17     |
| 100467B | 5.7       | 2.0      | (3.7) | 1.85     |
| 100667A | 4.9       | 1.1      | (3.8) | 3.45     |
| 100667B | 5.1       | 2.4      | (2.7) | 1.13     |

* Parentheses indicate that the values were obtained by difference.
Altering the ATP:ADP Ratio with an Extraneous Phosphagen System

If the sensitivity to external potassium of Na⁺ efflux in the presence of extracellular Na⁺ depends on the axoplasmic ATP:ADP ratio rather than on the presence or absence of phosphoarginine, then manipulating this ratio by injecting extraneous, nonphysiological (for squid) substances, should have predictable effects. Thus, in the presence of creatine phosphokinase, injection of creatine should result in raised ADP levels:

\[
\text{ATP} + \text{creatine} \rightarrow \text{phosphocreatine} + \text{ADP}
\]

and in a concomitant loss of requirement for external potassium. Conversely, a high ATP:ADP ratio (and, consequently, potassium requirement) should be restored by injecting phosphocreatine. However, if firm conclusions are to be drawn, these effects should also occur in axons depleted of arginine, lest the following reactions take place:

\[
\text{CreatP} + \text{ADP} \rightarrow \text{ATP} + \text{creatine}
\]
\[
\text{ATP} + \text{arginine} \rightarrow \text{ArgP} + \text{ADP}
\]

Fig. 13 illustrates an experiment in which the axon was preloaded with CPK + ³²Na. In this particular axon, the K-free effect disappeared and reversed during exposure to CN. (This phenomenon was first observed by Caldwell et al., 1960 b.) Injection of phosphocreatine now led to (partial) restoration of sodium efflux with a sizeable dependency on external potassium. Restoration of sodium efflux into 9 K(NA)SW was only to 52% of the original
level; this may be due to the fact that the axon's condition was deteriorating after 3 hr of exposure to CN. In 12 axons, the mean restored efflux obtained with $\sim 20$ mM phosphocreatine was 80% of control (Table IV).

Similar experiments on axons preloaded with CPK + $^{22}$Na + arginase gave similar results. Here again, at a time when the axon should have been essentially depleted of arginine, injection of phosphocreatine restored a K-dependent sodium efflux. A slightly different experiment on an arginine-depleted axon is depicted in Fig. 14. Here CN was applied while the axon was bathed in K-free seawater: the requirement for external K disappeared and sodium efflux rose; when phosphocreatine was injected, K-free efflux fell almost to its previous level, but efflux into 9 K(Na)SW was unchanged. The converse situation occurred when creatine was injected into an axon preloaded with CPK + arginase + $^{22}$Na. As shown in Fig. 15, injection of creatine boosted sodium efflux into 0 K(Na)SW, at least for some time.

These results make clear that whenever the ATP:ADP ratio is altered, even by means of extraneous systems, and also in the absence of detectable axoplasmic arginine, the dependence of sodium efflux on external potassium is altered in the same way as when ATP:ADP alterations are brought about by exposure to CN, injection of ADP, etc.

| Axon No. | Phosphocreatine final concentration (mM) | Restoration of Na efflux | (0 K) efflux/(9 K) efflux | Before | After |
|----------|----------------------------------------|-------------------------|--------------------------|--------|-------|
| 80666    | 15                                     | 1.05                    | 0.50                     | 0.60   |
| 70467    | 21                                     | 0.77                    | 0.48                     | 0.71   |
| 71267    | 20                                     | 0.83                    | 0.43                     | 0.48   |
| 71567    | 25                                     | 0.87                    | 0.32                     | 0.43   |
| 71867B   | 12                                     | 0.57                    | 0.30                     | 0.54   |
| 72067    | 25                                     | 0.72                    | 0.60                     | 0.53   |
| 72167    | 21                                     | 0.52                    | 0.28                     | 0.56   |
| 72967    | 25                                     | 0.83                    | 0.56                     | 0.67   |
| 80467    | 25                                     | 0.83                    | 0.42                     | 0.43   |
| 13068    | 30                                     | 0.81                    | 0.55                     | 0.71   |
| 13168    | 15                                     | 0.96                    | 0.44                     | 0.57   |
| 32768    | 30                                     | 0.83                    | 0.35                     | 0.44   |
| Mean     |                                        | 0.80                    | 0.44                     | 0.56   |
| SE       |                                        | ±0.04                   | ±0.03                    | ±0.03  |

* Na efflux into 9 K(Na)SW after injection of CPK + phosphocreatine, compared to that before CN poisoning.
Effect of Injected Deoxy-ATP

d-ATP is capable of energizing both active sodium extrusion and potassium uptake by squid giant axon (Brinley and Mullins, 1968; Mullins and Brinley, 1969). Fig. 16 illustrates one of three experiments in which this substance was injected into a normal axon: efflux into 10 K(Na)SW was unchanged, but that into K-free ASW was greatly increased. This finding is somewhat unexpected in view of the ATP-like efficacy of d-ATP in energizing active transport.

FIGURE 14. Effect of CN and phosphocreatine + creatine phosphokinase on sodium efflux from arginine-depleted axon into K-free(Na)seawater. Open circles, 9 mm K; filled circles, 0 mm K. Axon was preloaded with arginase (10 units/ml), creatine phosphokinase (200 units/ml), and 22Na. Arrow indicates time of injection of creatine phosphate to make a final concentration of 15 mm. Axon 13168. Diameter 475 μ. Temperature 14°C.

FIGURE 15. Effect of injected creatine + creatine phosphokinase on sodium efflux into 9 K and K-free(Na)seawater. Open circles, 9 mm K; filled circles, 0 mm K. Axon was preloaded with creatine phosphokinase (200 units/ml) and 22Na. Arrow indicates time of injection of creatine to a final concentration of about 20 mm. Axon 40366B. Diameter 465 μ. Temperature 12°C.
Effect of Injected Inorganic Phosphate

In view of the possibility that the mechanism by which ADP renders the Na efflux insensitive to external K might be related to its effect on the free energy of ATP hydrolysis (Caldwell, 1968), it became of interest to test the effect of P_i in this respect, since high levels of P_i will also limit free energy. When P_i was injected into axons, much the same picture was obtained as with Mg injections: a generalized inhibition of sodium efflux into both normal and K-free SW (Table V). Fig. 17 shows the dose: response curve of this inhibition. The significant finding was that the K-free effect was unaffected by the injection. In 11 axons, the ratio (Na efflux into K-free SW):(efflux into 10 K SW) averaged 0.45 ± 0.04 before, and 0.44 ± 0.04 after P_i injection. In seven of these axons, most markedly in the three axons whose [Na], had been lowered to 10–20 mmoles/kg by tetanization in 10 K(Li)SW, there was a slight but distinct increase in K sensitivity. This is perhaps not surprising since P_i may reduce ADP levels through stimulation of mitochondrial respiration.

The nature of the Na efflux inhibition by P_i is unclear, but it probably does not result from Mg complexing, since inclusion of Mg with the injected P_i (molar ratio 1:10), if anything, enhanced the inhibitory effect. It is noteworthy that even the strophanthidin-insensitive sodium efflux was inhibited by P_i.

Some caution is in order in interpreting the possible role of P_i in the K-free phenomenon. What the injection experiments show is that extra P_i in an otherwise normal axon does not render sodium extrusion less dependent on external potassium. Available evidence does not exclude the possibility, how-
ever, that some Pi may be required for increased ADP levels to have their described effect.

**Effect of Removing External Sodium**

Published data concerning the effect of removal of Na+ from the seawater on sodium extrusion are conflicting. Hodgkin and Keynes (1955) found a 20-

| Axon No. | Final concentration | Inhibition | Efflux into 0 K(Na)SW | Efflux into 10 K(Na)SW |
|----------|---------------------|------------|-----------------------|-----------------------|
|          | 10 K(Na)SW          | 0 K(Na)SW  | Before injection       | After injection        |
|          | mu Pi               | %          | %                     |                       |
| 51569A   | 2                   | 4          | %                     |                       |
| 7069A    | 2                   | 12         | %                     |                       |
| 51669B   | 5                   | 20         | %                     |                       |
| 51469B   | 5                   | 8          | %                     |                       |
| 51469C   | 5                   | 30         | %                     |                       |
| 5069A    | 10                  | 27         | 0.61                  | 0.55                  |
| 5069    | 10                  | 13         | %                     |                       |
| 51369A   | 10                  | 19         | %                     |                       |
| 51369B   | 10                  | 13         | %                     |                       |
| 51369C   | 10                  | 37         | 0.42                  | 0.37                  |
| 50669B   | 20                  | 23         | 0.55                  | 0.53                  |
| 50769A   | 20                  | 33         | 0.55                  | 0.58                  |
| 50769B   | 20                  | 41         | 0.69                  | 0.73                  |
| 70869B   | 20                  | 30         | %                     |                       |
| 70869C   | 20                  | 48         | %                     |                       |
| 70769A   | 20                  | 32         | %                     |                       |
| 70969D   | 20                  | 44         | 0.47                  | 0.44                  |
| 71669A‡  | 20                  | 45         | 0.37                  | 0.27                  |
| 71669B‡  | 20                  | 38         | 0.45                  | 0.38                  |
| 71869A‡  | 20                  | 30         | 0.41                  | 0.32                  |
| 70869A   | 40                  | 50         | %                     |                       |
| 80868B   | 20 (+2 Mg)          | 13         | 0.27                  | 0.30                  |
| 70869E   | 20 (+2 Mg)          | 36         | %                     |                       |
| 70969A   | 20 (+2 Mg)          | 50         | %                     |                       |
| 70969B   | 20 (+2 Mg)          | 38         | 0.20                  | 0.38                  |

**Mean**

| Efflux into 0 K(Na)SW | Efflux into 10 K(Na)SW |
|-----------------------|-----------------------|
| 0.45                  | 0.44                  |
| ±0.04                 | ±0.04                 |

* In the presence of strophanthin 10⁻⁶ M.
‡ [Na] reduced to 10-20 mM.

50% increase of sodium efflux from Sepia axons upon replacement of Na by choline or dextrose. The same was found for Loligo axons, upon replacement of Na by Li, choline, or dextrose (Caldwell et al., 1960; Mullins et al., 1962; Baker et al., 1969). However, on L. pealei, a negligible effect was observed by
Sjodin and Beaugé (1968), and even a lowering of sodium efflux from Na-depleted axons into (Li) SW was reported by Frumento and Mullins (1964).

**REPLACEMENT OF EXTERNAL Na WITH CHOLINE** Table VI summarizes a few observations made on axons bathed alternately in (Na)SW and (Chol)SW.

### Figure 17
Effect of injected inorganic phosphate on sodium efflux from squid axon. Efflux is expressed relative to that before injection. Every value (except at 40 mM) represents the mean ±SE from three to seven experiments.

### Table VI
COMPARISON OF Na EFFLUX INTO 0 K(Na)SW AND INTO (CHOL)SW*

| Axon No. | 0 K(Na)SW | 9 K(Chol)SW | 0 K(Chol)SW |
|----------|-----------|-------------|-------------|
| 71968A   | 0.80      | 0.90        | 0.40        |
| 71968B   | 0.29      | 1.30        | 0.65        |
| 72068A   | 0.30      | 1.40        | 0.75        |
| 72668A   | 0.24      | 1.60        | —           |

* All fluxes relative to that into 9 K(Na)SW.

The behavior of sodium efflux upon changing from (Na)SW to Na-free SW seemed erratic at first, in that efflux would sometimes rise and sometimes fall. However, it became clear that these erratic results (as well as those in (Mg+mannitol)SW described below) could be rationalized as follows: (a) whenever sodium efflux from a particular axon into K-free (Na)SW is very small relative to that in 9 K(Na)SW, the efflux into 9 K(Na-free)SW will be high; (b) axons displaying a minimal K-free effect will have a reduced efflux into 9 K(Na-free) media; (c) Na efflux from an axon with a "medium" K-free effect (i.e. about a 50% decrease) will change very little when going from 9 K(Na)SW to 9
K(Na-free)SW. In other words, there is a clear correlation between the effect of removing external K and the effect of removing external Na, for any given axon.

Replacement of external Na with Mg + Mannitol. Exactly the same picture is seen here as with the choline experiments: the higher the sodium efflux in K-free(Na)SW, the lower the efflux in 9 K(Na-free)SW will be.

Table VII summarizes data which confirm this empirical correlation between K-free effect and Na-free effect. Fig. 18 illustrates the behavior of sodium efflux from an axon displaying a "weak" K-free effect: transfer to Na-free SW results in a lowering of the sodium efflux. Furthermore, strophanthidin (10⁻⁶ M) inhibits sodium efflux to a greater extent than the combined effects of absence of sodium and absence of potassium. It is particularly interesting

Table VII
Comparison of Na efflux into 0 K(Na)SW and into (Mg + Mannitol)SW*

| Effect of reduced ATP:ADP ratio |
|---------------------------------|
| Untreated axon | Reduced ATP:ADP ratio |
|----------------|-----------------------|
| 0 K (Na) SW | 9 K (Mg + M) SW | 0 K (Mg + M) SW |
| 0 K (Na) SW | 9 K (Mg + M) SW | 0 K (Mg + M) SW |
| Agent† |
|----------------|-----------------|
| 72468 | 0.55 | 0.95 |
| 72668A | 0.24 | 1.33 |
| 72668B | 0.74 | 0.63 |
| 0.85 | 0.53 |
| 0.99 | 0.45 |
| 80669A | 0.19 | 1.42 |
| 80669B | 0.61 | 1.00 |
| 80669C | 0.38 | 1.32 |
| 80268B | 0.74 | 0.48 |
| 0.90 | 0.45 |
| 80568 | 0.64 | 0.42 |
| 73168B | 0.66 | 0.44 |
| 0.84 | 0.59 |
| 72768 | 0.76 | 0.90 |
| 0.55 |
| 73068A | 0.59 | 0.58 |
| 0.75 | 0.44 | Apyrase |
| 0.62 | 0.52 |
| 0.84 | 0.41 | Apyrase |
| 80268A | 0.66 | 0.44 |
| 0.82 | 0.36 | Arginine |
| 80368A | 0.38 | 0.76 |
| 0.82 | 0.90 | 0.70 | Arginine |
| 80368B | 0.36 | 0.61 |
| 0.74 | 0.83 | 0.50 | Arginine |
| 80769A | 0.45 | 1.25 |
| 0.95 | CN |
| 1.28‡ |

* All fluxes relative to that into 9 K(Na)SW. Data on the same row in columns 2-4 or columns 5-7 can be regarded as "simultaneous" for all practical purposes.
† Agent responsible for the lowered ATP:ADP ratio.
‡ After recovery from 20 min exposure to CN.
that the extent of strophanthidin inhibition is the same as in (Na)SW: this renders unlikely the possibility of there being much strophanthidin-insensitive, Ca-induced sodium efflux (of the type described by Baker, Blaustein, Hodgkin, and Steinhardt, 1969) into (Mg + mannitol)SW.

**Sodium Efflux from Axons with Elevated ADP Levels** It was shown earlier that whenever axoplasmic [ADP] is increased, an extra efflux of sodium into K-free seawater arises. It is logical to inquire whether here again, a loss of K sensitivity is automatically accompanied by an increased dependence on external Na. For example, if the empirical correlation alluded to in the previous paragraphs continues to hold, then injection of arginine into an axon whose sodium efflux is strongly dependent on external K should, of course,

![Figure 18. Effect of K-free seawater, K-free + Na-free seawater, and strophanthidin on sodium efflux from squid axon. Open circles, 9 mM K+; filled circles, 0 mM K+. Horizontal bars indicate periods during which (Mg + mannitol)SW or 10^{-4} M strophanthidin-containing (Na)SW, instead of (Na)SW, was bathing the axon. Axon 80568. Diameter 460 μ. Temperature 18°C.](image)

make the K-free effect weak, and should also render the sodium efflux more dependent on external sodium. The data in columns 5–7 of Table VII show that this does indeed happen. These findings support the notion that a causal link exists between the magnitude of the K-free effect and the sign and magnitude of the Na-free effect of individual axons.

Since the effect of a lowered ATP:ADP ratio on the K-free effect is reversible, its effect on the Na-free effect may be expected to be equally reversible. Reversibility was tested in one axon (No. 80769A): this axon's sodium efflux levels into 0 K(Na)SW and 9 K(Mg + mannitol)SW were respectively 0.45 and 1.25, of that into 9 K(Na)SW. After a 20 min exposure to CN, the Na-free efflux had decreased to 0.95, but when CN was removed, the Na-free efflux level returned to 1.28 of that in 9 K(Na)SW; the phenomenon thus seems to be reversible.

**Empirical Relationship between "K-free Effect" and "Na-free Effect" or "(Na + K)-free Effect"**

In order to formulate the empirical relationship between K-free and Na-free effects, a scatter plot was prepared from all the data listed in Tables VI and
VII (Fig. 19). (All fluxes are expressed relative to that into 9 K(Na)SW.) Every point on the plot represents two measurements: a K-free test on the one hand, and a Na-free or (Na + K)-free test on the other. A straight line fitted to the points relating K-free(Na)SW efflux and Na-free efflux rate obeyed equation:

\[(\text{Na-free efflux}) = 1.74 - 1.25 (\text{K-free efflux}) \]  \hspace{1cm} (1)

Similarly, the line relating K-free efflux rate and (Na + K)-free efflux rate obeyed equation:

\[(\text{(Na + K)-free efflux}) = 0.83 - 0.47 (\text{K-free efflux}) \]  \hspace{1cm} (2)

From the curves, it can now be predicted that an axon whose sodium efflux suffers, for example, a 45% decrease in K-free seawater, will show no change upon transfer from (Na)SW to (Na-free)SW, either in the presence or in the absence of external potassium. If an axon's sodium efflux drops to 0.20 in 0 K(Na)SW, its efflux into 9 K(Na-free)SW would be expected to rise 50%;
changeover from (Na)SW to (Na-free)SW in the absence of K would result in a three- to fourfold increase in sodium efflux.

In five axons with artificially increased ADP levels (Table VII) the empirical relationship between K-free effect and Na-free effect was the same as that in untreated axons. In fact, the corresponding data were included in the above calculations because they were statistically indistinguishable from the data obtained on untreated axons. In other words, it appears that increasing [ADP] reversibly moves the axon’s characteristics along the slopes of Fig. 19, without modifying the underlying empirical equations.

No experiments were performed specifically to study reverse K-free effects, but extrapolation of Fig. 19 A to the right ought to afford a theoretical upper limit to the magnitude of the reverse K-free effect. From equation (1) it follows that the line of Fig. 19 A intersects the abscissa at 1.39. Since Na+ efflux into 9 K(Na-free)SW cannot be negative (and neglecting passive efflux), this means that the predicted upper limit to the reverse K-free effect is a 39% increase. In fact, the highest reverse K-free effect found in this study was +37%, while Caldwell et al. (1960 a) never found more than +40%.

DISCUSSION

The K-Free Effect of Untreated Axons

The variability of the K-free effect of “normal” axons is well-known. For Sepia axons Hodgkin and Keynes (1955) found a mean relative sodium efflux of 0.30 (range from 0.19 to 0.57) in 0 K(Na)SW compared to 10.4 K(Na)SW. For Loligo axons, a mean of 0.35 with a range between 0.2 and 0.5 (Caldwell et al., 1960 a) or even between 0.1 and 0.5 (Baker and Manil, 1968), and a mean of 0.42, range from 0.22 to 0.63 (Sjodin and Beaugé, 1968) have been reported. For 97 untreated axons tested in this report, the mean relative efflux into K-free seawater was found to be 0.52 ± 0.02 (range from 0.18 to 1.00).

THE FRANKENHAUSER-HODGKIN SPACE It may be asked whether the variability of the K-free effect might not result from a variable leak of K+ into the Frankenhaeuser-Hodgkin (F-H) space just outside the axolemma. Frankenhaeuser and Hodgkin (1956) calculated this region to be about 300 A thick, and limited by a nonspecific permeability barrier of permeability $P = 6 \times 10^{-2} \text{ cm} \cdot \text{sec}^{-1}$. If excessive potassium accumulation in the F-H space were the cause of weak K-free effects, then an axon displaying no K-free effect at all should have a rather constant K+ concentration—say 10 mm—in its F-H space, independent of the K+ concentration in the seawater. It is easily calculated that the potassium efflux rate needed to assure a concentration of this magnitude in the F-H space when the axon is bathed in K-free SW, should be about 600 pmoles·cm$^{-2}$·sec$^{-1}$. This is inordinately high indeed—
15 times higher than the value published by Caldwell and Keynes (1960) for
$^{42}$K-injected Loligo axons bathed in 10 K(Na)SW.

Another argument against the F-H space hypothesis is the following: if some axons did not respond to K-free seawater because their F-H space was
being provided with $K^+$ leaking out of the axon, then the positive phase (or
"underswing") of action potentials from these same axons should undergo
little modification upon transfer from 9 K(Na)SW to 0 K(Na)SW. This was
not the case. For example, action potentials from axon 52467A, which had
no K-free effect, displayed underswings of about 15 mv in 9 K(Na)SW, and
31 mv in 0 K(Na)SW; this is almost exactly what would be predicted from
Fig. 5 of Frankenhaeuser and Hodgkin (1956).

**K-Free Effect and Damage**

In view of the lack of objective criteria
with which the variable K-free effects could be correlated, it is tempting to
look for less easily controllable parameters which might influence the per-
formance of this delicate preparation. In this respect, it may not be altogether
unreasonable to suspect that at least part of the "spontaneous" variability in
K-free effect might be related to the traumata suffered by the membrane dur-
ing dissection, cleaning, and manipulation. If this suggestion is correct, the
amount of damage required to alter the K-free effect is very subtle. It is not
extensive enough to affect electrical excitability or passive or active sodium
efflux. As already shown above, reduction of K sensitivity is invariably accom-
panied by increased Na sensitivity (Fig. 19). The findings then suggest that
whatever it is that produces the spontaneous variability of the K-free effect,
causes a variable fraction of the sodium efflux to shift from a $K_\infty$-stimulated mode to a
$Na_\infty$-stimulated mode.

**K-Free Effect and Axoplasmic ADP**

Evidence shows that the K sensitivity of sodium efflux from squid giant axon
is reversibly reduced, abolished, and even reversed whenever the axoplasmic
ratios of ATP:ADP or ArgP:Arg are reduced. This reduction can be brought
about by any one or a combination of the following mechanisms:

| Reaction | Mechanism                        |
|----------|----------------------------------|
| ATP $\rightarrow$ ADP + P$_1$   | CN poisoning or apyrase injection|
| ADP      | ADP injection                     |
| AMP + ATP $\rightarrow$ 2 ADP   | AMP injection                     |
| Arg + ATP $\rightarrow$ ArgP + ADP | Arg injection                |
| Creat + ATP $\rightarrow$ CreatP + ADP | Creatine injection         |

The first two mechanisms produce high levels of both ADP and P$_1$. No effect
of the latter on the K sensitivity was seen, however, in experiments designed
to differentiate the two effects.

Published November 1, 1970
It has been shown here that axons can be essentially depleted of arginine and phosphoarginine with no ill effects and no change in the behavior of the K-free effect with respect to intracellular [ADP]. Raising or lowering [ADP] by means of an extraneous phosphagen system, has the predicted effects on the K sensitivity of sodium efflux: strong dependence on external K when [ADP] is low; weak or no dependence on external K when the [ADP] is high. This finding satisfactorily proves that axoplasmic phosphoarginine is not required for the Na pump to be dependent on external potassium, but that a low ADP level is required.

Because of the presence of adenylate kinase in axoplasm the question of whether the ATP:ADP ratio rather than the ATP:AMP ratio is important cannot be resolved with the data at hand. However, considering that the product of the "pump ATPase" is ADP and not AMP (Skou, 1957), it seems unlikely that AMP, rather than ADP, should have an effect on the pump's behavior.

A further necessary consideration is the following. It is possible that not ADP, but another substance (a metabolite, for example) whose concentration level varies concomitantly with that of ADP, is responsible for the phenomena now ascribed to ADP itself.

**Quantitative Considerations** There seems to be a relationship between K dependence of the sodium efflux and axoplasmic [ADP] or the ATP:ADP ratio. Microinjection techniques do not allow rigorous and quantitative control of intracellular constituents. For example, it is nearly impossible to increase [ADP] without at the same time modifying [ATP]: CN poisoning or arginine injection will increase [ADP] and reduce [ATP]; ADP injections will increase both [ADP] and [ATP], etc. Because of this it cannot be decided, at the present time, whether reduction of K sensitivity of the sodium pump results solely from high absolute [ADP] levels, rather than from a combination of increased [ADP] and reduced [ATP]; i.e., from a lowered ATP:ADP concentration ratio. In view of this uncertainty it is preferable to use the more general variable, "ATP:ADP ratio," with the understanding that future experiments (for example, with internally dialyzed axons) should allow an assessment of the respective roles of [ATP] and [ADP] in these phenomena. Furthermore, in view of the inherent (spontaneous) variability of the initial K-free effect in untreated axons, it is difficult to formulate a very quantitative correlation between the ATP:ADP ratio and the (efflux into 0 K):(efflux into 9 K) ratio. Rather, as a first approximation, one can try to evaluate the approximate ATP:ADP ratio at which the K-free effect becomes very small or even reverses.

**ATP:ADP Ratio of Untreated Axons** The experimentally found ATP:ADP ratio in normal axons is about 11 (Mullins and Brinley, 1967) but
this figure may not be very accurate in view of the difficulty of measuring low levels of ADP. The experimentally determined ArgP:Arg ratio may be more reliable; this ratio was found here to be about 1.7 (confirming Caldwell and Schirmer, 1965). From the scant data concerning the arginine phosphokinase equilibrium (Lehmann, 1936; Griffiths et al., 1957), one estimates that at pH 7.3 and with excess Mg, the (Arg)(ATP):(ArgP)(ADP) equilibrium value lies around 4. This leads to a calculated axoplasmic ATP:ADP ratio of about 7. Since increasing this ratio does not improve the K-free effect, it would seem that the ATP:ADP ratio of normal axons (roughly 10) assures the maximal K sensitivity for any given axon.

**ATP:ADP ratio for minimal K-free effect**  When reasonable values are taken for total axoplasmic adenosine (3.7 mM), total ~P (2 per ATP and 1 per ADP and ArgP molecule) (10 mM), and total arginine (5 mM) and using the value of Atkinson et al. (1961) for the adenylate kinase equilibrium 

\[
[\text{ADP}]^2 : [\text{ATP}] [\text{AMP}] = 2
\]

one can calculate the ATP:ADP ratio immediately after an injection of, say, ADP or arginine. It is found that an ATP:ADP ratio of 1 or somewhat less is required to reduce the K-free effect from a 60% drop to a 10–15% drop.

**ATP:ADP ratio for reverse K-free effect**  This phenomenon was seen only in axons that had been CN-poisoned for at least 75 min. Sodium efflux levels were usually reduced to about 15 pmole·cm⁻²·sec⁻¹, and from Fig. 7 of Brinley and Mullins (1968) it can be estimated that the ATP level was probably around 50 μM. With this figure, and a total (ATP + ADP + AMP) concentration of 3.7 mM, one finds 

\[
[\text{ADP}]^2 : [\text{ATP}] [\text{AMP}] = [0.55]^2 : [0.05] [3.1] = 2.0
\]

That is to say, when the K-free effect is reversed, the axoplasmic ATP:ADP ratio is about 1/11. Since reverse K-free effects were only seen at very low ATP levels, the possibility should be kept in mind that this phenomenon may be related to the Na⁺-activated, K⁺-inhibited, ouabain-sensitive ATPase discovered by Czerwinski et al. (1967).

**The Dependence of Sodium Extrusion on External Potassium Concentration**  When K-stimulated sodium efflux from squid axon is plotted against nominal external potassium concentration, an S-shaped curve results (Fig. 3 and Baker, Blaustein, Keynes, Manil, Shaw, and Steinhardt, 1969). It is important to know whether this shape truly reflects a property of the underlying extrusion mechanism rather than an artifact resulting from the peculiar anatomy of the squid axon. There is undoubtedly some potassium in the Frankenhaeuser-Hodgkin space when the axon is bathed in nominally K-free seawater. When the permeability coefficient \( P = 6 \times 10^{-4} \text{ cm·sec}^{-1} \) for the external barrier proposed by Frankenhaeuser and Hodgkin (1956) and a value of 30 pmole·cm⁻²·sec⁻¹ for the potassium efflux from squid axon
(Caldwell and Keynes, 1960) are used, one arrives at an excess potassium concentration of 0.5 mM in the F-H space surrounding the axolemma. It is obvious that this excess K\textsuperscript+ concentration will effectively truncate the leftmost part of the curve in Fig. 3. If anything then, the true curve relating K-stimulated Na efflux to effective (not nominal) K\textsubscript{e} must be more, not less, strongly S-shaped than Fig. 3 suggests.

**Effect of external sodium on the K activation curve**

Removal of external sodium has a remarkable effect on the potassium-dependence of sodium extrusion. In red blood cells, removal of Na\textsubscript{e} drastically reduces the apparent K\textsubscript{m} for K activation of pumping rate (Post et al., 1960; Garrahan and Glynn, 1967). In the absence of Na\textsubscript{e}, K activation of active transport apparently obeys hyperbolic kinetics with a low K\textsubscript{m}, but in the presence of external sodium, the curve is shifted to the right and acquires a sigmoid shape; maximal stimulation at high K\textsubscript{e} concentrations is identical in the presence or absence of external sodium. Analogous phenomena have been noticed in other preparations, including crab nerve (Baker and Connelly, 1966) and nonmyelinated mammalian fibers (Rang and Ritchie, 1968).

A similar effect occurs in squid giant axon (Baker, Blaustein, Keynes, Manil, Shaw, and Steinhardt, 1969). Whereas the curve relating K-dependent sodium efflux to external potassium concentration is clearly sigmoid in (Na)SW, the curve in (Na-free)SW is shifted to the left, apparently hyperbolic with a K\textsubscript{m} definitely below 1 mM. As a matter of fact, many efflux rates into nominally K-free (Na-free)SW are substantially higher than in 0 K(Na)SW (Tables VI and VII); this can easily be explained if one considers the truncating effect of residual K in the Frankenhaeuser-Hodgkin space, as discussed earlier. For a rectangular hyperbolic curve with K\textsubscript{m} = 0.5 mM, a residual F-H potassium concentration of about 0.5 mM would displace the intercept on the ordinate upward to about halfway V\textsubscript{max}.

**Relation between K-free and Na-free effects**

As shown in Fig. 19, a striking correlation was found between the K-free effect and the Na-free effect displayed by a given axon at a given time. A similar correlation has recently been described by Sjodin and Beaugé (1969). These authors, who used Li and Tris as substitutes for Na, interpreted their findings on the basis of a carrier model in which the affinities for external K and Na, as well as the rate constant for Na-activated sodium efflux, were assumed to vary from axon to axon. (Their correlation, although superficially similar, was not quite identical to the one described here—this difference might be due to the fact that the cations replacing sodium were different in the two studies.) In the present paper, the alternative view is held that sodium efflux represents a summation of two distinct operations with constant ionic affinities, but whose
relative importance varies from axon to axon, and for a given axon, also depends on the intracellular ATP:ADP ratio.

The following points are relevant to the discussion: (a) sodium efflux by passive diffusion amounts to only 2% of the normal efflux (Mullins and Brinley, 1967) and will be neglected here; (strophanthidin sensitivity is inadequate as a criterion for active or carrier-mediated flux); (b) in the presence of external sodium, K-dependent sodium efflux is stimulated by K₀ along a sigmoid curve which approaches a plateau of about 1.7 times the value at 9 mM K₀; the exact nature of the mechanism responsible for this cooperativity, as well as the nature of the interaction between external Na and K, is irrelevant to the present discussion: all that is required is an activation curve with apparent Kₐ about 10 times higher than in the absence of external sodium; (c) in the absence of external sodium, K-dependent sodium efflux is activated by K₀ along a hyperbolic curve with the same maximum as the sigmoid curve (Baker, Blaustein, Keynes, Manil, Shaw, and Steinhardt, 1969), but with a Kₐ of about 0.5 mM (in fact, Kₐ = 0.45 mM was used for a better fit); (d) sodium-dependent sodium efflux is partly inhibited by external potassium: in the presence of 9 mM K₀, it is only 2/3 as large as in the absence of K₀ (this is, of course, the reverse K-free effect seen in axons with very low ATP:ADP ratios); (e) continuous leakage of potassium from the axon creates an excess K+ concentration of 0.5 mM in the Frankenhaeuser-Hodgkin space: nominally K-free SW then leaves about 0.5 mM K in the immediate vicinity of the axon.

The most economical model which will account for the features of Fig. 19 simply assumes that, besides a negligible (2%) passive diffusion component, sodium efflux from squid axon is composed of only two distinct and additive processes: (a) K₀-stimulated sodium efflux (subject to inhibition by Na₀), and (b) Na₀-stimulated Na efflux (inhibitable by K₀).

From the above conditions, the following equations are easily derived:

\[(\text{Na-free efflux}) = 1.7[1 - (\frac{3}{4}) (\text{K-free efflux})]\]

or

\[(\text{Na-free efflux}) = 1.7 - 1.13 (\text{K-free efflux}) \quad (3)\]

and

\[(\text{Na + K)-free efflux} = 0.9 [1 - (\frac{3}{4}) (\text{K-free efflux})]\]

or

\[(\text{Na + K)-free efflux} = 0.9 - 0.6 (\text{K-free efflux}) \quad (4)\]

The theoretical equations (3) and (4) were entered as dashed lines in Fig.
PAUL DE WEER  Sodium Pump of Squid Axon

19; considering the simplicity of the assumptions, the fit is rather satisfactory. It is interesting to analyze two extreme situations. The first extreme is an axon with exclusively K-stimulated sodium efflux: removal of K would practically abolish the efflux; removal of Na would increase efflux to 1.7; and removal of both Na and K would result in a relative efflux of about 0.9 (due to the residual 0.5 mM K in the F-H space). The other extreme alternative is an axon whose sodium efflux is solely of the Na-stimulated variety: removal of Na (with or without K) would abolish the efflux whereas removal of K would increase the efflux by about 50%; i.e., a reverse K-free effect.

It is clear that in this scheme, axons whose Na efflux does not decrease when K is removed from the SW still possess an appreciable fraction of K-stimulated sodium efflux, and that studies of “pure” Na-stimulated sodium efflux should preferably be carried out on axons displaying a reverse K-free effect. A few experiments were tried in which large amounts of Arg2AMP were injected, but without much success (sodium efflux into both K-free and 9 K(Na)SW was inhibited). Internal dialysis (Brinley and Mullins, 1967), during which a wide variety of axoplasmic ATP:ADP ratios can be imposed, seems very promising in this respect.

One way in which the above model might be improved is to consider the nature of the K inhibition of Na-stimulated efflux. If this inhibition is by way of transforming a Na-stimulated fraction into a K-stimulated one (as has been proposed for red blood cells, Garrahan and Glynn, 1967 c), a more realistic picture might be obtained.

**Nature of K-stimulated and Na-stimulated Effluxes**

The K-stimulated sodium efflux can conveniently be ascribed to the operation of a coupled Na:K pump. However, the nature of the Na-stimulated sodium efflux is less immediately apparent, and the present experiments were not designed to throw any light on this aspect of the question. The sodium pump of red blood cells is said to be capable of switching from a Na:K exchanging mode of operation to a Na:Na exchanging one when the intracellular ATP:P ratio is low (Garrahan and Glynn, 1967 a-c). In ATP-reactivated, CN-poisoned axons, Caldwell et al. (1960 a) found an increased Na influx “which would be qualitatively consistent with the idea [of] an exchange diffusion of Na across the membrane.” Working with axons partially poisoned with DNP (which have low ATP and high ADP + P_i levels), whose sodium efflux was little affected by removal of external K, Baker, Blaustein, Keynes, Manil, Shaw, and Steinhardt (1969) found a large ouabain-sensitive sodium influx. It seems quite probable therefore, that the reversible, ADP-induced, Na-requiring mode of operation represents a Na:Na exchange phenomenon taking place across the pump machinery.

On the other hand, little can be said yet about the variable, spontaneous, Na-requiring mode of operation found in untreated axons. From the data
presented here, this mode of operation and the one resulting from high ADP levels cannot be distinguished (except, of course, that the latter is reversible). No strophanthidin-sensitive sodium influx has been found in untreated axons (Mullins and Brinley, 1969; Baker, Blaustein, Keynes, Manil, Shaw, and Steinhardt, 1969), which suggests that the two Na+ stimulated mechanisms are intrinsically different. Should there be a strophanthidin-sensitive sodium influx in untreated axons, however, it will be most easily found (a) in axons whose spontaneous K-free effect is known to be poor, and (b) in the absence of external potassium.

**Mode of Action of ADP, Pi, and Deoxy-ATP**

The ability of ADP, but not Pi, to render sodium efflux from squid axon less sensitive to external K, even though either will reduce the free energy available from ATP hydrolysis, puts an interesting constraint on any proposed model for the sodium pump. No detailed mechanisms can be proposed yet, but the findings do suggest an outline for a possible model.

Several intermediates have now been described in the operation of the (Na + K)-activated ATPase or pump ATPase (Post et al., 1965; Albers, 1967; Albers et al., 1968; Post et al., 1969). One of the early steps is a reaction whereby the transport enzyme \( E \) is phosphorylated:

\[
E + \text{ATP} \leftrightarrow E \cdot \text{ATP} \leftrightarrow E_1 \sim P \cdot \text{ADP} \leftrightarrow E_1 \sim P + \text{ADP}
\]

\( E_1 \), in the terminology of the quoted authors, refers to the transport enzyme in a particular conformation.) Binding of ATP to the enzyme does not require Na+, but the transphosphorylation reaction proper does. High intracellular ADP levels may be expected to reverse the above reaction sequence, with accumulation of \( E_1 \sim P \cdot \text{ADP} \) and \( E \cdot \text{ATP} \). We now assume that transphosphorylation and sodium translocation are concomitant, i.e. interconversion of the Na-binding forms

\[
E \cdot \text{ATP} \leftrightarrow E_1 \sim P \cdot \text{ADP}
\]

is accompanied by a translocation of the Na-binding site from the inside of the membrane to the outside (Fig. 20). If \( E_1 \sim P \) is destined for eventual reentry via a K-carrying step, it is clear that the ADP level will determine the relative proportions of the pump enzyme engaging in the Na:Na exchange and in Na:K exchange. In the presence of low ADP levels, sodium efflux will acquire a measure of irreversibility. High ADP levels, on the other hand, will promote Na:Na exchange and reduce K uptake.

As for the site of action of \( P_i \), Fig. 20 suggests that excess \( P_i \) could act by limiting the availability of \( E \) (i.e. product inhibition), thus reducing the potentiality for both Na:Na exchange and Na:K transport to the same extent.
Finally, the following explanation for the ADP-like effect of injected deoxy-ATP can be offered. It has been shown that d-ATP, like ATP (but not as efficiently), is capable of energizing both sodium efflux and potassium influx (Brinley and Mullins, 1968; Mullins and Brinley, 1969). If one assumes that the equilibrium of reaction

\[ E_1 \sim P \cdot dADP \leftrightarrow E_1 \sim P + dADP \]

lies more to the left than that of the equivalent reaction with ADP, it is easy to see that phosphorylation of the enzyme with d-ATP may lead to high levels of Na:Na exchange. At the same time, the scheme shows why d-ATP will support active K uptake only when d-ADP is kept at a very low level (Mullins and Brinley, 1969).

This study was supported by a grant from National Institute of Neurological Diseases and Stroke (NS 05846) and one from the National Science Foundation (GB8279).

I thank the Director, Marine Biological Laboratory, Woods Hole, Mass., for the use of facilities, Dr. L. J. Mullins for much help and advice, and Drs. F. J. Brinley, Jr., and R. A. Sjodin for helpful comments.

The able technical assistance of Mr. D. Saul and Mr. R. Saul is acknowledged.

Received for publication 19 January 1970.
REFERENCES

ALERES, R. W. 1967. Biochemical aspects of active transport. Annu. Rev. Biochem. 36:727.
ALERES, R. W., G. J. KOVAL, and G. J. SIEGEL. 1968. Studies on the interaction of ouabain and other cardioactive steroids with sodium-potassium-activated adenosine triphosphatase. Mol. Pharmacol. 4:524.
ALERETY, R. A., R. M. SMITH, and R. M. BORK. 1951. The apparent ionization constants of the adenosinephosphates and related compounds. J. Biol. Chem. 193:425.
ATKINSON, M. R., R. M. BURTON, and R. K. MORTON. 1961. Equilibrium constant of phosphoryl transfer from adenosine triphosphate to galactose in the presence of galactokinase. Biochem. J. 78:813.
BACH, S. J., and J. D. KILLIP. 1960. The crystallization and properties of hepatic arginase of ox, sheep and horse. Biochem. J. 77:7P.
BAKER, P. F. 1965. Phosphorus metabolism of intact crab nerve and its relation to the active transport of ions. J. Physiol. (London). 180:383.
BAKER, P. F., M. P. BLAUSTEIN, A. L. HODGKIN, and R. A. STEINHARDT. 1969. The influence of calcium on sodium efflux in squid axons. J. Physiol. (London). 200:431.
BAKER, P. F., M. P. BLAUSTEIN, R. D. KEYNES, J. MANIL, T. I. SHAW, and R. A. STEINHARDT. 1969. The ouabain-sensitive fluxes of sodium and potassium in squid giant axons. J. Physiol. (London). 200:459.
BAKER, P. F., and C. M. CONNELLY. 1966. Some properties of the external activation site of the sodium pump in crab nerve. J. Physiol. (London). 185:270.
BAKER, P. F., and J. MANIL. 1968. The rates of action of K+ and ouabain on the sodium pump in squid axons. Biochim. Biophys. Acta. 150:328.
BEAUGAY, L. A., and R. A. JØDIN. 1968. The dual effect of lithium ions on sodium efflux in skeletal muscle. J. Gen. Physiol. 52:408.
BRINLEY, F. J., JR., and L. J. MULLINS. 1965. Ion fluxes and transference numbers in squid axons. J. Neurophysiol. 28:526.
BRINLEY, F. J., JR. and L. J. MULLINS. 1967. Sodium extrusion by internally dialyzed squid axons. J. Gen. Physiol. 50:2303.
Caldwell, P. C. 1958. Studies on the internal pH of large muscle and nerve fibers. J. Physiol. (London). 142:22.
Caldwell, P. C. 1960. The phosphorus metabolism of squid axons and its relationship to the active transport of sodium. J. Physiol. (London). 142:22.
Caldwell, P. C. 1968. Factors governing movement and distribution of inorganic ions in nerve and muscle. Physiol. Rev. 48:1.
Caldwell, P. C., A. L. Hodgkin, R. D. Keynes, and T. I. Shaw. 1960 a. The effect of injecting "energy-rich" phosphate compounds on the active transport of ions in the giant axons of Loligo. J. Physiol. (London). 152:561.
Caldwell, P. C., A. L. Hodgkin, R. D. Keynes, and T. I. Shaw. 1960 b. Partial inhibition of the active transport of cations in the giant axons of Loligo. J. Physiol. (London). 152:561.
Caldwell, P. C., and R. D. Keynes. 1960. The permeability of the squid giant axon to radioactive potassium and chloride ions. J. Physiol. (London). 171:119.
Caldwell, P. C., and H. Schërmer. 1965. The free energy available to the sodium pump of squid giant axons and changes in the sodium efflux on removal of the extracellular potassium. J. Physiol. (London). 181:25P.
CARSLAW, H. S., and J. C. JAEGER. 1947. Conduction of Heat in Solids. Oxford University Press, London.
CEZEREWSKI, A., H. T. GITELMAN, and L. G. WELT. 1967. A new member of the ATPase family. Amer. J. Physiol. 213:786.
DATTA, S. P., and A. K. GRZYBOWSKI. 1961. Thermodynamic quantities for the dissociation
equilibria of biologically important compounds. 8. The first and second acid dissociation of arginine. Biochem. J. 78:289.

Deffner, G. G. J. 1961. The dialyzable free organic constituents of squid blood; a comparison with nerve axoplasm. Biochim. Biophys. Acta. 47:578.

De Weer, P. 1968. Restoration of a potassium-requiring sodium pump in squid giant axons poisoned with CN and depleted of arginine. Nature (London). 219:730.

De Weer, P. 1970. Effects of intracellular ADP and Pi on the sodium pump of squid giant axon. Nature (London). 226:1251.

Frankenhaeuser, B., and A. L. Hodgkin. 1956. The after-effects of impulses in the giant nerve fibers of Loligo. J. Physiol. (London). 131:341.

Frumento, A. S., and L. J. Mullins. 1964. Potassium-free effect in squid axons. Nature (London). 214:1312.

Garrahan, P. J., and I. M. Glynn. 1967 a. The behaviour of the sodium pump in red cells in the absence of external potassium. J. Physiol. (London). 192:159.

Garrahan, P. J., and I. M. Glynn. 1967 b. The sensitivity of the sodium pump to external sodium. J. Physiol. (London). 192:175.

Garrahan, P. J., and I. M. Glynn. 1967 c. Factors affecting the relative magnitudes of the sodium: potassium and the sodium: sodium exchanges catalysed by the sodium pump. J. Physiol. (London). 192:189.

Good, N. E., G. D. Winget, W. Winter, T. N. Connolly, S. Izwawa, and R. M. M. Singh. 1966. Hydrogen ion buffers for biological research. Biochemistry. 5:467.

Greenberg, D. M., A. E. Bagot, and O. A. Roholt, Jr. 1956. Liver arginase. III. Properties of highly purified arginase. Arch. Biochem. Biophys. 62:946.

Greenstein, J. P., and M. Wintz. 1961. Chemistry of the Amino Acids. John Wiley & Sons, Inc., New York. i-

Griffiths, D. E., J. E. Morrison, and A. H. Ennor. 1957. A study of the kinetics of the reactions catalysed by arginine phosphokinase. Biochem. J. 65:153.

Hodgkin, A. L. 1964. The Conduction of the Nervous Impulse. Liverpool University Press, Liverpool, England.

Hodgkin, A. L., and R. D. Keynes. 1955. Active transport of cations in giant axons from Sepia and Loligo. J. Physiol. (London). 128:228.

Hodgkin, A. L., and R. D. Keynes. 1956. Experiments on the injection of substances into squid giant axons by means of a microsyringe. J. Physiol. (London). 131:592.

Keynes, R. D. 1960. The effect of complete and partial inhibition of metabolism on active transport in nerve and muscle. Ciba Found. Study Group Pap. No. 5. Regulation of the inorganic ion content of cells. p. 77.

Kornberg, A. 1950. Reversible enzymatic synthesis of diphosphopyridine nucleotide and inorganic pyrophosphate. J. Biol. Chem. 182:779.

Kübler, W. D., and J. J. Eiler. 1943. The acid strength of mono and diesters of phosphoric acid. The n-alkyl esters from methyl to butyl, the esters of biological importance, and the natural guanidine phosphoric acids. J. Amer. Chem. Soc. 65:2353.

Lehmann, H. 1936. Ueber die Umesterung des Adenylatsystems mit Phosphagenen. Biochem. Z. 285:336.

Martell, A., and S. Schwartzenzbach. 1956. Adenosinphosphat und Triphosphat als Komplexbildner für Calcium und Magnesium. Helv. Chim. Acta. 39:553.

Meyerhof, O., and K. Lohmann. 1928. Ueber die natürlichen Guanidinophosphorsäuren (Phosphagen) in der quergestreiften Muskulatur. II. Mitteilung: Die physikalisch-chemischen Eigenschaften der Guanidinophosphorsäuren. Biochem. Z. 196:49.

Mullins, L. J., J. W. Adelman, Jr., and R. A. Sjoedin. 1962. Sodium and potassium ion effluxes from squid axons under voltage clamp conditions. Biophys. J. 2:257.

Mullins, L. J., and F. J. Brinley, Jr. 1967. Some factors influencing sodium extrusion by internally dialyzed squid axons. J. Gen. Physiol. 50:2333.

Mullins, L. J., and F. J. Brinley, Jr. 1969. Potassium fluxes in dialyzed squid axons. J. Gen. Physiol. 53:704.
OLIVER, I. T. 1955. A spectrophotometric method for the determination of creatine phosphokinase and myokinase. *Biochem. J.* 61:116.

POST, R. L., S. KUME, T. TOBIN, B. ORCUTT, and A. K. SEN. 1969. Flexibility of an active center in sodium-plus-potassium adenosine triphosphatase. *J. Gen. Physiol.* 54(1, Pt. 2):306.

POST, R. L., C. R. MERRITT, C. R. KINNSOLVING, and C. D. ALBRIGHT. 1960. Membrane adenosine triphosphatase as a participant in the active transport of sodium and potassium in the human erythrocyte. *J. Biol. Chem.* 235:1796.

POST, R. L., A. K. SEN, and A. S. ROSENTHAL. 1965. A phosphorylated intermediate in adenosine triphosphate-dependent sodium and potassium transport across kidney membranes. *J. Biol. Chem.* 240:1437.

RANG, H. P., and J. M. RITCHIE. 1968. On the electrogenic sodium pump in mammalian non-myelinated nerve fibres and its activation by various external cations. *J. Physiol. (London).* 196:183.

ROSENBERG, H., A. H. ENNOR, and J. F. MORRISON. 1956. The estimation of arginine. *Biochem. J.* 63:153.

SCHWARTZ, E. A. 1968. Effect of diethylether on sodium efflux from squid axons. *Curr. Mod. Biol.* 2:1.

SJODIN, R. A., and L. A. BEAUGÉ. 1968. Coupling and selectivity of sodium and potassium transport in squid giant axons. *J. Gen. Physiol.* 51(5, Pt. 2):152.

SJODIN, R. A., and L. A. BEAUGÉ. 1969. The influence of potassium- and sodium-free solutions on sodium efflux from squid giant axons. *J. Gen. Physiol.* 54:664.

SKOU, J. C. 1957. The influence of some cations on an adenosine triphosphatase from peripheral nerves. *Biochim. Biophys. Acta.* 23:394.

TASAKI, I., I. SINGER, and T. TAKENAKA. 1965. Effects of internal and external ionic environment on excitability of squid giant axon. *J. Gen. Physiol.* 48:1095.

VIRDEN, R., D. C. WATTS, and E. BALDWIN. 1965. Adenosine 5'-triphosphate-arginine phosphotransferase from lobster muscle: purification and properties. *Biochem. J.* 94:536.