ENERGY-TRANSDUCING REACTIONS IN BIOLOGICAL MEMBRANES

III. A MODEL OF THE SODIUM PUMP AND ITS POSSIBLE RELEVANCE TO SOME OTHER ACTIVE TRANSPORT PROCESSES

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Summary

A model of a sodium pump is presented which utilizes an alkali metal carrier in the form of a polypeptide “spring” containing about four consecutive phosphatidylinerine residues bound to lipid molecules embedded as a bilipid, rubber-like structure, attached to the matrix of a biological membrane. Electrostatic repulsive forces between the anions of the spring and osmosis, which are opposed by the elasticity of the bilipids, tend to maintain it in an expanded configuration of restricted dimensions, in which hydrated Na+ is preferred to K+ as a diffuse ionic atmosphere. Compression of the spring forms a helical structure in which unhydrated K+ is preferred to Na+ and is solvated by the oxygen atoms of carbonyl (or ether) groups within the peptide and the lipid.

The springs are attached to polymeric structures which can be crosslinked reversibly. In the Na+ configuration the polymers are not crosslinked; in the K+ configuration the polymers are crosslinked and the springs are contracted.

The crosslink is produced by a reversible reaction between ATP and a carboxylate residue R¹COO⁻ on one polymer chain to form R¹COO²⁻ which reacts with a thiol on a second polymer chain to form a thioester crosslink. The free energy change of the crosslinking reaction depends on the phosphorylation potential and the Na⁺:K⁺ ratio and ionic strength on either side of the membrane. Since K⁺ is normally required for the spring to contract, K⁺ favours crosslinking but Na⁺ facilitates hydrolysis of the crosslink by forming an ion-association complex with R¹COO⁻ which promotes hydrolysis of the thioester and phosphorylation; Na⁺ also assists hydrolysis by stabilizing the expanded configuration.

The model is consistent with a large number of experimental observations and suggests principles for some other active-transport processes which are energized by ion concentration gradients. Some salt-link crosslinking mechanisms are proposed for such pumps.

I. INTRODUCTION

The sodium pump utilizes ATP to actively transport Na⁺ across a membrane in exchange for K⁺. This paper proposes a model of the sodium pump which postulates participation of the phospholipopeptide “spring” of the action potential model (see Part II of this series, Weiss 1969b) in the ion-exchange activities of the pump. The model postulates a dilation–contraction mechanism in which ATP energizes contraction of the spring by forming a thioester crosslink, and the resulting increase in K⁺ selectivity enables adsorption of K⁺ to reject previously adsorbed Na⁺ against its concentration and electrochemical gradients. The energy of the phosphorylation reaction thereby interacts with the ion-interaction energies of the exchange sites, and

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with elastic changes in the spring bilipids, according to principles discussed in Part I (Weiss 1969a).

The ATPase of the sodium pump has several features which distinguish it from all other ATPases: its operation requires the presence of Na\(^+\) and Mg\(^{2+}\) within, and of K\(^+\) outside, the membrane, and it is inhibited by cardiac glycosides, such as ouabain, applied to the outer side of the membrane. The sodium pump is widely distributed in mammalian membranes. For recent reviews of the subject see Skou (1964, 1965), Baker (1966), Glynn (1968), and Post (1968).

Skou first succeeded in isolating, from a homogenate of the peripheral nerves of crab, a submicroscopic particulate ATPase whose activation requires Mg\(^{2+}\), Na\(^+\), and K\(^+\), and which is inhibited by ouabain. Its behaviour therefore parallels that of the sodium pump in intact membranes (see Skou 1965; Glynn 1968). Such preparations will be referred to as (Na\(^++\)K\(^+)\)-induced ATPase. Since the first isolation of this enzyme system in 1957, many similar preparations have been made from a large number of different mammalian membrane sources (Baker 1965; Skou 1965; Barclay et al. 1967; Bader, Post, and Bond 1968; Glynn 1968). The purified enzyme preparation has an apparent molecular weight of 670,000 (Medzihradsky, Kline, and Hokin 1967); this size is sufficient for it to extend through the membrane.

II. Earlier Models

Many earlier models postulate that reaction of a membrane component with ATP induces a conformational change which alters the ion selectivity (see Skou 1964, 1965). Recent models of this type have been proposed by Opit and Charnock (1965), Jardetzky (1966), Müller (1967), and Lowe (1968). The present model is an extension of their ideas.

III. Components of the Model

(a) Assembly

A phospholipopeptide spring is postulated to be bound in bilayers to membrane lipids so that when contracted into the K\(^+\)-selective configuration the exchange sites contact the outer solution with reduced access to the inner solution (Fig. 1). When expanded into the Na\(^+\) configuration, the springs contact the inner solution and access to the outer solution is reduced. This anisotropic action is essential for pumping and could arise from substituents in the spring polymers blocking appropriate channels in the contracted or expanded configurations.

The spring is postulated to be crosslinked reversibly by a thioester formed between a thiol and a carboxyl group, such as a glutamate residue for example, contained in polymers attached to either end of the spring. Phosphorylation of R\(^1\)COO\(^-\) by ATP energizes crosslinking which stabilizes the K\(^+\) configuration. The reactions are:

\[
\text{ATP}^{4-} + \text{R}^1\text{COO}^- \rightleftharpoons \text{R}^1\text{COOP}^{2-} + \text{ADP}^{3-}, \tag{1}
\]

\[
\text{R}^1\text{COOP}^{2-} + \text{R}^2\text{SH} \rightleftharpoons \text{R}^1\text{COSR}^{2-} + \text{P}_2^{2-}. \tag{2}
\]

Experimental data will be reviewed before describing the pumping action of the model to establish the extent to which postulation of the proposed components of the model is justified.
(b) Role of Lipids and Phospholipopeptide

Lipids are essential for the functioning of \((\text{Na}^+ + \text{K}^+)\)-induced ATPase. Incubation with phospholipases destroys the activating effect of Na\(^+\) and K\(^+\) and suggests that lipid is important as the site of interaction of Na\(^+\) and K\(^+\) (Skou 1965). An inactive component separated from \((\text{Na}^+ + \text{K}^+)\)-induced ATPase from beef brain, and lacking phospholipid, is activated by phospholipid (Tanaka and Strickland 1965), particularly phosphatidylserine (Fenster and Copenhaver 1967). A contractile protein has been extracted from horse erythrocyte membranes with the properties of \((\text{Na}^+ + \text{K}^+)\)-induced ATPase; it is activated also to a small extent by phosphatidylserine but not by phosphatidylinositol or phosphatidylethanolamine (Ohnishi, Nskamuru, and Kawamura 1964).

![Diagram](image)

Fig. 1.—Diagrammatic representation of the sodium pump model.

The occurrence of Heald’s lipophosphatidylserine peptide in biological membranes has been considered in Part I, as has its suitability for its postulated task as an alkali metal ion carrier.

(c) Phosphatidylserine as a Na\(^+\)-K\(^+\) Carrier

When dissolved in chloroform, phosphatidylserine selectivity extracts K\(^+\) rather than Na\(^+\) from an aqueous solution (Solomon, Lionetti, and Curran 1956). Phosphatidylserine has been extracted from erythrocytes as the Na\(^+\) salt even though the erythrocytes contained mainly K\(^+\), thus suggesting a role for the lipid as a Na\(^+\) carrier (Kirschner 1957). These seemingly conflicting results are, however, consistent with the proposed dual role of contracted and expanded configurations of a phosphatidylserine peptide as a carrier of Na\(^+\) and K\(^+\).

(d) Reaction with ATP

The reaction of \((\text{Na}^+ + \text{K}^+)\)-induced ATPase with ATP is not specific, but reactivity with other nucleotide triphosphates is low (Skou 1965). The reaction tends to be inhibited with increasing concentrations of ADP. A small ATP–ADP exchange reaction has been observed (Stahl, Sattin, and McIlwain 1966). A reversal of the reaction by the addition of P\(_1\) has been observed in an intact erythrocyte ghost membrane (Garrahan and Glynn 1967e). These reactions are consistent with the model.
(e) Evidence for an Acyl Phosphate

There is substantial evidence for the involvement of a high-energy acyl phosphate intermediate produced by the interaction of ATP, Mg$^{2+}$, and Na$^+$ with (Na$^+$+K$^+$)-induced ATPase. Two types of bound phosphate have been observed.

With low concentrations of ATP, in the presence of Mg$^{2+}$ and Na$^+$ and with contact times of a few seconds, a labile phosphorylated intermediate appears with a high phosphate turnover rate. Its formation is facilitated by Na$^+$, but it is rapidly dephosphorylated in the presence of K$^+$ ions at low concentrations. Its pH stability characteristics, its reactivity towards hydroxylamine, alcohols, molybdate, and an acyl phosphate identify it as an acyl phosphate (Bader, Post, and Bond 1968; Kahlenberg, Galsworthy, and Hokin 1968; Post 1968). It is unlikely that the acyl phosphate actually isolated is an artefact due to a major migration within the enzyme system as a result of stopping the reaction by the addition of acid to a pH value of 2.5, since bound phosphate can be isolated when the reaction is stopped at neutral pH with a detergent (Rodnight, Hems, and Lavin 1966; Nagano et al. 1967). The acyl phosphate can be formed also, in the presence of Na$^+$, from acetyl phosphate (Israel and Titus 1967). The acyl phosphate is probably an L-glutamyl-$\gamma$-phosphate residue (Kahlenberg, Galsworthy, and Hokin 1968).

With incubation times of a few minutes, and higher ATP concentrations of 1–3 mM in the presence of Mg$^{2+}$ and Na$^+$, phosphate becomes bound as phosphorylsine (Rodnight, Hems, and Lavin 1966). The (Na$^+$+K$^+$)-induced ATPase reaction is inhibited irreversibly by diisopropylfluorophosphate (Hokin and Yoda 1964), which forms a phosphorylsine derivative associated with the ATP substrate site. As aspartate or glutamate residues have been found adjacent to active serine residues in a considerable number of enzymes of animal origin (Oosterbaan and Cohen 1964), this finding is consistent also with the probability of an L-glutamyl-$\gamma$-phosphate residue in the phosphorylated enzyme.

The experimental evidence is therefore consistent with active carboxyl residues, and with the formation from ATP of a carboxyl-$\gamma$-phosphate intermediate, as postulated in the model.

(f) Evidence for a Thiol Activity

The following sulphhydryl reagents inhibit the activity of (Na$^+$+K$^+$)-induced ATPase: Hg$^{2+}$ (Bader and Sen 1966), N-ethylmaleimide (Skou 1965; Fahn et al. 1966), N-butylmaleimide (Fahn et al. 1966), p-chloromercuribenzoate (Skou 1965; Fahn et al. 1966), p-chloromercuri sulphate (Fahn et al. 1966), and 2,4-dinitrofluorobenzene (Skou 1965). The inhibitory effect of p-chloromercuribenzoate is reversed by cysteine (Skou 1965). Bader, Post, and Jean (1967) have shown that the acyl phosphate intermediate is attached to a protein containing one or more sulphhydryl groups.

Cardiac glycosides, such as ouabain, inhibit the dephosphorylation reaction of (Na$^+$+K$^+$)-induced ATPase at low concentrations (Skou 1965). Inhibition by ouabain, strophanthidin, and strophanthidin-3-acetate is reversible, but with strophanthidin-3-haloacetates (SHA) about 70% of the inhibition is irreversible.
(Hokin, Mokotoff, and Kupchan 1966). Strophanthidin protects against irreversible inhibition by SHA. Thiol compounds added to SHA prior to the reaction abolish the irreversible inhibition, but only reduce the inhibition when added to the enzyme at the same time as the addition of SHA. These facts, plus the pH dependence of the SHA reactions, suggest that the irreversible inhibition of the enzyme system by SHA is probably due to alkylation of the enzyme at a thiol at, or adjacent to, the site where cardiac glycosides act (Hokin, Mokotoff, and Kupchan 1966). This conclusion is reinforced by later studies with hellebrigenin-3-bromoacetate, a compound with a structure related to that of SHA but with much higher biological activity (Ruoho et al. 1968). Iodoacetate does not inhibit the enzyme system but does so when esterified with strophanthidin. Such a behaviour is consistent also with the presence in the model of the anionic site R^1COO^− near the thiol group, since the anionic charge would tend to repel anionic iodoacetate but not its ester.

The sulphhydryl reagents, cysteine, glutathione, and 2-mercaptoethanol enhance the release of P_i by (Na^++K^+)−induced ATPase in the presence of ATP, Mg^{2+}, Na^+, and K^+ (Tanaka and Strickland 1965). Their effect in the model would be to compete with R^2SH in reacting with R^1COOP^2−.

There is therefore substantial evidence for the presence in (Na^++K^+)−induced ATPase of an active thiol and justifies the postulate of R^2SH in the model.

\( \text{(g) Location of the Pump in the Membrane} \)

In an intact membrane, the sodium pump requires Na^+ inside and K^+ outside for operation under optimum conditions. There is kinetic evidence for the presence of two sites for the reaction of cations; at one, in the case of the enzyme system in crab nerve, the affinity for Na^+ is about 6–8 times that for K^+; the other has a high affinity for K^+ but has a very low affinity for Na^+ (Skou 1964, 1965). Maximum activity requires Na^+ at the first site and K^+ at the second site. Experiments on intact cells show that Na^+ must be inside the membrane whilst K^+ is required outside (Baker 1965). The model is consistent with such observations. The dimensions of the spring are about 0.2 pm, so that it could be accommodated within a 0.7–1.0 pm biological membrane.

Having established some factual basis for the postulated participation of lipid structure, phosphatidylserine, ATP, acyl phosphate, and a thiol in the model, the operation of the pump under physiological conditions will be considered next before discussing evidence for the crosslinking reaction.

IV. Normal Mode of Operation of the Model

\( \text{(a) Operation} \)

It is a characteristic of the sodium pump that phosphorylation is stimulated by the internal addition of Na^+. Two mechanisms are postulated in the model to account for this.

Carboxyl ion-exchange resins (Gregor et al. 1956), and di(ethylhexyl)phosphate dissolved in kerosene, show a preference for Na^+ rather than for K^+; in the latter case the titration curve of the acid is markedly flat and indicates strong ion association
(see Part I). It is postulated that $\text{R}^+\text{COO}^-$ in the lipid phase of the model membrane possesses a marked selectivity for $\text{Na}^+$:

$$\text{R}^+\text{COO}^- + \text{Na}^+ \rightleftharpoons \text{R}^+\text{COONa}^+ + \text{H}^+. \quad (3)$$

By shifting the equilibrium of reaction (3) to the right, the addition of $\text{Na}^+$ stimulates the formation of $\text{R}^+\text{COONa}^+$ and thereby promotes hydrolysis of thioester crosslinks and their subsequent phosphorylation [reaction (1)]. The latter involves nucleophilic attack of the terminal phosphorus atom of ATP by $\text{R}^+\text{COO}^-$. The formation of an ionic bond, rather than a covalent bond, between $\text{Na}^+$ and $\text{R}^+\text{COO}^-$ is therefore an important feature of the reaction, for which purpose alkali metal cations have a unique advantage. This effect of $\text{Na}^+$ is considered to be saturated at relatively low concentrations of $\text{Na}^+$.

At higher $\text{Na}^+$ concentrations, mass action effects at the spring exchange sites tend to replace $\text{K}^+$ with $\text{Na}^+$ and to thereby expand the springs into the $\text{Na}^+$ configuration. The tendency for expansion, being coupled with the crosslinking reaction, also promotes hydrolysis of the thioester crosslinks. The resulting increase in the concentration of $\text{R}^+\text{COOH}$ stimulates further phosphorylation, through reactions (1) and (3). Hence the ability of $\text{Na}^+$ to increase the stability of $\text{R}^+\text{COO}^-$ and of expanded spring sites cooperates to promote hydrolysis of the thioester and subsequent phosphorylation of $\text{R}^+\text{COOH}$.

$\text{K}^+$ will have the opposite effect to $\text{Na}^+$ and will favour dephosphorylation and crosslinking. Because of the high interaction energy postulated between $\text{K}^+$ and the contracted spring sites, the addition of $\text{K}^+$ will favour contraction of the spring and concomitant formation of the thioester crosslinks with release of $\text{P}_1$. Optimum activity of $(\text{Na}^++\text{K}^+)$-induced ATPase requires a balance between $\text{Na}^+$-stimulated thioester hydrolysis and phosphorylation, and $\text{K}^+$-stimulated dephosphorylation; hence excessive amounts of $\text{Na}^+$ are inhibitory at concentrations where they can compete with $\text{K}^+$ for the contracted spring sites in accordance with observations by Schatzmann (1965) and Whittam and Ager (1964).

$\text{Mg}^{2+}$ is an essential requirement for the enzyme preparation and forms a complex with ATP which enables it to bind to the phosphorylating enzyme in the model. $\text{Ca}^{2+}$ is considered to inhibit by competing with $\text{Mg}^{2+}$ and thereby distorting the enzyme complex into an unfavourable conformation (see Epstein and Whittam 1966).

Inhibition of $(\text{Na}^++\text{K}^+)$-induced ATPase by low concentrations of ouabain inhibits $\text{K}^+$-stimulated dephosphorylation. In the model, such inhibition is accounted for by adsorption of the inhibitor in the expanded polymer near the thiol, where its presence prevents closure for crosslinking. $\text{K}^+$ opposes ouabain inhibition (Ahmed and Judah 1965; Schatzmann 1965) by favouring crosslinking since ouabain cannot penetrate the contracted system. $\text{K}^+$ and ouabain therefore form a system where the extent of mutual interaction between the two is determined by their relative binding energies and concentrations (cf. drug actions in nerve; see Part II).

Consider now the behaviour of the model in a membrane, where anisotropic conditions apply. The inner solution is enriched with $\text{K}^+$ but contains little $\text{Na}^+$; the outer solution is enriched with $\text{Na}^+$ but contains some $\text{K}^+$. 

$$\text{R}^+\text{COO}^- + \text{Na}^+ \rightleftharpoons \text{R}^+\text{COONa}^+ + \text{H}^+. \quad (3)$$
Under steady-state conditions, the pump will establish a dynamic equilibrium across the membrane. If more Na\(^+\) is now added continuously to the inner solution phosphorylation is stimulated. New thioesters form as the concentration of R\(^1\)COOP\(^2-\) rises. The resulting ATP-induced contraction exposes to the outer solution Na\(^+\) previously adsorbed by the inner, expanded springs, and as the contracting springs assume the K\(^+\) configuration the Na\(^+\) is expelled in exchange for outer K\(^+\). Under the stimulus of the inner Na\(^+\) addition, some of the thioesters hydrolyse to provide further R\(^1\)COOH for participation in reaction (3) with the result that K\(^+\), previously adsorbed from the outer solution, now becomes exposed to the inner solution and exchanges for Na\(^+\) as the springs expand. Eventually a new steady-state condition is established.

As has been described in Part I, such a system involves a contractile–dilation equilibrium in which the free energy changes of reactions at the ion-exchange sites interact with those of the crosslinking reactions and a change in either the ion concentration gradients, or of the phosphorylation potential, changes the equilibrium of the system.

(b) *Evidence for Thioacylation*

It has been observed, in (Na\(^+\)+K\(^+\))-induced ATPase prepared from electric organs, that the addition of Na\(^+\) stimulates an ADP–ATP transphosphorylation reaction which increases with increasing amounts of Na\(^+\) to a constant value at concentrations in excess of c. 0·1–0·2m Na\(^+\) (Fahn, Koval, and Albers 1966). A parallel increase in labelled phosphate incorporation is also observed (Fahn, Koval, and Albers 1968). Many other workers have observed Na\(^+\) stimulation of phosphorylation (see Glynn 1968; Post 1968). Such observations are interpreted as being due to the effect of Na\(^+\) on reactions (1) and (3) together with facilitated expansion of the springs.

If the influence of the springs is removed by blocking thioacylation with a thiol inhibitor, then Na\(^+\) can only influence phosphorylation in the model through reaction (3). Since the extent of phosphorylation [reaction (1)] will be enhanced in the forward direction by increasing amounts of Na\(^+\), but the reversed reaction will show a contrary dependence, ADP–ATP transphosphorylation, in the presence of a thiol inhibitor and of increasing amounts of Na\(^+\), should show a maximum. Such an effect has been observed at a concentration of c. 0·02m Na\(^+\) in electric organ (Na\(^+\)+K\(^+\))-induced ATPase inhibited by \(N\)-ethylmaleimide (Fahn, Hurley, Koval, and Albers 1966). The inhibition of (Na\(^+\)+K\(^+\))-induced ATPase by \(N\)-ethylmaleimide is reduced in the presence of ATP (Skou 1965; Fahn, Hurley, Koval, and Albers 1966).

Pig kidney (Na\(^+\)+K\(^+\))-induced ATPase has been divided into a fraction which shows a small Na\(^+\)-stimulated labelling with \(^{32}\)P but which is not dephosphorylated by K\(^+\) (Rendi 1966). Whereas a (Na\(^+\)+K\(^+\))-induced ATPase microsomal preparation from ox brain is inactivated by exposure to 2–8m urea, c. 2·5 mM concentrations of ATP give partial or complete protection to the ATPase (Skou 1965; Cooper and McIlwain 1967). ATP and alkali metals exhibit a stabilizing influence on the purified enzyme (Medzhiradsky, Kline, and Hokin 1967; Yoshida et al. 1969). These
observations are consistent with an ATP-induced thioacylation reaction crosslinking
two polymer units which could otherwise be separated.

The concept of thioacylation producing a crosslink is consistent also with the
inhibitory effect of the cardiac glycosides, and strophanthidin-3-haloacetates, referred
to in Section III(f).

Oligomycin does not inhibit phosphorylation, but behaves somewhat like
ouabain in inhibiting dephosphorylation (Whittam, Wheeler, and Blake 1964; Hokin et al. 1965; Fahn, Koval, and Albers 1968). There is evidence suggesting that
some ATP ("effector ATP") is adsorbed on the phosphorylating enzyme so as to
induce a conformation essential for crosslinking (Heinz and Hoffman 1965; Garrahan
and Glynn 1967; Askari and Koyal 1968). It is suggested that oligomycin inhibits
crosslinking, but not phosphorylation, by either preventing adsorption of effector
ATP, or by mechanically impeding the contraction required for crosslinking (see
Askari and Koyal 1968).

The postulate of effector ATP accounts also for "phosphatase" activity (see
Israel and Titus 1967; Askari and Koyal 1968). In its absence, crosslinking is
prevented and the expanded enzyme can react with acetyl phosphate, and the like,
in a Na\(^+\)-stimulated phosphorylation reaction. Addition of K\(^+\) is considered to favour
the reaction by contracting the spring sites and thereby increasing access to R\(^1\)COO\(^-\).
The reduced thermal effect associated with phosphatase activity is consistent also
with such a mechanism (see below).

The fact that N-ethylmaleimide does not inhibit phosphorylation, that it
enhances and enables a maximum to be observed in the ADP-ATP transphosphoryl-
at reaction, and that ATP protects against thiol and urea inhibition strongly
supports the concept of thioester crosslinking.

(c) Histidine

The reversible formation, and hydrolysis, of the postulated thioester crosslinks
in the model requires the presence of a catalyst, which could be histidine.

Acyladenosine monophosphate and acetylphosphate form thioesters slowly
with glutathione or coenzyme A, but the rate of acyl transfer is increased greatly by
low concentrations of imidazole (Jencks 1957). The imidazole in N-propyl-\(\gamma\)-(4-imidazolium)thiobutyrate, where the thioester is in a favourable configuration with
respect to the imidazole, catalyses the hydrolysis of the thiobutyrate at a rate at least
a million times that for the hydrolysis of the unbound thioester in water at neutrality
and at room temperature (Bruce 1959). The catalytic reaction shows specificity for
thioesters since neither the ester nor the amide of \(\gamma\)-(4-imidazoyl)butyric acid
hydrolyses at room temperature. Histidine might account for the pH maximum at
pH 7.2-7.6 in the pH–activity curves of (Na\(^+\)+K\(^+\))-induced ATPase (Charnock and
Post 1963; Skou 1965). A maximum at pH 7.4 is observed with acetate kinase
(Hokin, Mokotoff, and Kupchan 1966) and at pH 8.0 for acetylcholinesterase
(Oosterbaan and Jansz 1964), an enzyme which contains active histidine and serine
residues.

It is concluded that histidine could catalyse the thioacylation reaction in either
direction and that its presence with glutamate and serine residues would be consistent
with other known enzymatic groupings in hydrolases.
(d) Evidence for a Configurational Change

The model requires that there be a configurational change following thioacylation.

Vibratory movements have been observed as changes in refractive index under a phase-contrast microscope in erythrocyte membranes (see Kavanau 1965). A close relationship between membrane shimmering and the active transport of sugars has been demonstrated in these membranes (Kavanau 1965). The ATP for the sodium pump in erythrocytes is derived from glycolysis rather than from respiration; both the active transport of Na⁺ and the membrane shimmering of the erythrocyte membrane are inhibited by agents inhibiting glycolysis, such as fluoride and non-iodoacetate (Kavanau 1965), but the fluoride may have a direct inhibitory effect on the sodium pump (Opit, Potter, and Charnock 1966). Harris et al. (1968) have commented recently on extensive configurational changes which can be observed in erythrocyte ghosts. ATP shrinks the de-energized vesicular spherical configuration; this evidence was cited in favour of a conformational theory of ion-transport, but it is not established that it is connected with the sodium pump. Robinson (1967a) has shown, by light scattering, that ATP induces a structural change in membrane fragments, and has obtained kinetic data for (Na⁺+K⁺)-induced ATPase which he suggests indicates configurational changes associated with allosteric processes (Robinson 1967b, 1968), but the interpretation has been challenged by Priestland and Whittam (1968). The observation that binding of cardiac glycosides by (Na⁺+K⁺)-induced ATPase is stimulated by ATP, Mg²⁺, and Na⁺, but is depressed by K⁺, also suggests a configurational change (Matsui and Schwartz 1968).

It is concluded that the configurational change in the model is a reasonable postulate in harmony with the above observations and with the effects of crosslinking on the ion selectivity of ion-exchange resins (see Part I).

V. Exchange Diffusion

R¹COOP₂⁻ is required for thioacylation under physiological conditions of active transport to energize crosslinking.

If, after establishing a steady state corresponding to item 1, Table 1, external K⁺ is then removed from the model, active transport of Na⁺ out of the membrane becomes difficult because external K⁺ is not available to facilitate contraction and expulsion of Na⁺. If the external Na⁺ concentration is sufficiently high it will contract the springs by reducing the electrostatic repulsive forces and be adsorbed as the unhydrated ion. Owing to its adverse hydration energy, such accommodation of Na⁺ is possible only in the absence of sufficient K⁺ to saturate the exchange sites.

If, in addition to removing external K⁺, the internal concentration of Na⁺ and of ATP is also reduced, and the concentration of P₁ greatly increased, the sign of the free energy change now alters (item 2, Table 1) and the pump reverses. Such changes so lower the phosphorylation potential that the tendency for ATP to produce cross-links can no longer oppose the tendency for Na⁺ to move inwardly by contraction and subsequent hydrolysis of the springs. Such contraction by external Na⁺ can synthesize thioesters by direct reaction between R¹COOH and R₂SH, provided effector ATP is present to induce a favourable conformation for crosslinking. The high concentration
of $P_1$ facilitates formation of acyl phosphate by attacking the thioesters (see Bruice 1959) and reversing reaction (2), and the high concentration of ADP and low internal concentrations of Na$^+$ and of ATP facilitate the synthesis of ATP by reversing equations (1) and (3).

The ensuing expansion and contraction cycles enables Na$^+$ in the outer and inner solutions to exchange with the Na$^+$ in the springs on either side of the membrane and so permit exchange diffusion. Such an exchange mechanism is inhibited by ouabain or oligomycin being adsorbed so as to prevent contraction or crosslinking.

**Table 1**

<table>
<thead>
<tr>
<th>Item</th>
<th>$Na_i$</th>
<th>$Na_o$</th>
<th>$K_i$</th>
<th>$K_o$</th>
<th>$10^3 \times$ Molar Concentration of:</th>
<th>$\Delta F$ (kcal/mole)</th>
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<td></td>
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<td>5</td>
<td>1·5</td>
<td>0·32</td>
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<tr>
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<td>147</td>
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<td>0·67</td>
<td>0·33</td>
</tr>
<tr>
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<td>150</td>
<td>0·1</td>
<td>1·5</td>
<td>0·32</td>
</tr>
<tr>
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<td>10</td>
<td>1</td>
<td>150</td>
<td>0·1</td>
<td>0·67</td>
<td>0·33</td>
</tr>
</tbody>
</table>

* Data from Garrahan and Glynn (1967c).

If conditions on the inner side of the membrane remain approximately constant, reducing the external Na$^+$ concentration will reduce Na$^+$-exchange diffusion and ATP synthesis as Na$^+$-induced contraction becomes increasingly difficult and the concentration of the thioester falls. The exchange will cease at a sufficiently low concentration of external Na$^+$. As shown by items 3 and 4, Table 1, when there is virtually no external K$^+$, and very little external Na$^+$, active transport of Na$^+$ out from the membrane is strongly hindered, even when the phosphorylation potential is substantial. However, at these low external cation concentrations a new factor—spring hydrolysis—becomes important and has not been taken into account in the thermodynamic calculations of Table 1.

The exchange sites of the springs are anions $R_s^-$ of weak acids which would hydrolyse in dilute solutions:

$$R_sNa + H_2O \iff R_sH + NaOH.$$  \hspace{1cm} (4)

Such a reaction can be substantial in solutions containing few exchangeable cations. For example, the titration curve of a carboxyl ion-exchange resin shifts by two pH units when compared in the presence of water and in a $0·09M$ solution of Na$^+$ (Weiss et al. 1966). Higher concentrations of Na$^+$ have comparatively little effect on the titration curve. The degree of hydrolysis increases with decreasing acidic strength of the exchange sites. The acidity of a carboxyl ion-exchange resin decreases with increasing crosslinking (see Weiss et al. 1966).
The contracted spring sites will therefore be weaker acids than the expanded sites because of the lower dielectric constant of the medium adjacent to the sites and because of the greater crosslinking. Consequently, when an expanded Na$^+$ spring contracts there will be a tendency for increased protonation, and some Na$^+$ will tend to be rejected and replaced by H$^+$ in the contracted sites. At the higher Na$^+$ concentrations corresponding to items 1 and 2, Table 1, the hydrolysis will be small but will increase rapidly with decreasing amounts of Na$^+$ in the region of very low Na$^+$ concentrations corresponding to items 3 and 4 in Table 1.

As a consequence of the replacement of unhydrated cations by covalently bound H$^+$ on contraction, the osmotic and electrostatic forces tending to expand the springs will be substantially reduced and will thereby facilitate operation of the pump. Protons will then be carried inwards, as well as Na$^+$, at the contracted sites, and hydrated Na$^+$ will move out with the expanded sites owing to the increased acidity of the latter. The essentially 1 : 1 Na$^+$ exchange characteristic of the operation at high salt concentrations therefore becomes replaced to an increasing extent by a H$^+$–Na$^+$ exchange reaction in very dilute solutions. Consequently the Na$^+$ efflux decreases at first with decreasing Na$^+$ concentration to a minimum value and then increases with further decreasing amounts of Na$^+$ at very low Na$^+$ concentrations.

The addition of external K$^+$, at Na$^+$ concentrations above those corresponding to the reversal point, will facilitate contraction. The sign of the free energy change will reverse (see item 1, Table 1), active transport of Na$^+$ out of the cell will now be possible, and the Na$^+$ efflux will therefore increase. The addition of K$^+$ at the low external Na$^+$ concentrations below the reversal point will tend to reduce the Na$^+$ efflux as K$^+$ replaces H$^+$ and Na$^+$. K$^+$ will be more effective than Na$^+$ at the contracted sites in reversing equation (4) and the consequent reduction in the number of protonated sites on the addition of K$^+$ will make contraction more difficult, the thioester concentration will decrease, and the Na$^+$ efflux across the membrane will be reduced.

Such behaviour of the model is consistent with the recent extensive study of Na$^+$ exchange diffusion by Garrahan and Glynn (1967a, 1967b, 1967c, 1967d, 1967e).

VI. STOICHEIOMETRY

Because of the reduced acidity of the contracted rather than the expanded exchange sites, more Na$^+$ will leave the springs than K$^+$ enters. There will be a range of salt concentrations above which there will be comparatively little change in the hydrolysis of the spring sites, so that the stoichiometry in this region will be determined only by the differing acidity of the contracted and expanded sites. Consequently pump stoichiometry bears little relationship to the work being performed and a roughly constant number of ions will be exchanged for each molecule of ATP utilized when the external salt concentration is high.

Such behaviour is consistent with the experimental observations, which have usually been performed at high salt concentrations (see Glynn 1968; Post 1968). Values ranging from two to four Na$^+$ ions being pumped out for each molecule of ATP hydrolysed, in exchange for rather fewer K$^+$ ions, have been repeatedly reported. This could be accounted for if there were four exchange sites for each crosslink. Under normal conditions, where about three Na$^+$ ions need to be carried out, this
would imply that an average of one in four expanded sites was protonated. Partial protonation should apply also to the springs in the action potential model (Part II), where the effect of alkali in triggering off a nerve was attributed to increased dissociation of the contracted sites.

VII. AMINO ACID EFFLUX

The principal anions within the squid axon are aspartate and glutamate. If, as postulated above, undissociated contracted spring sites in the model can be produced readily in the absence of external exchangeable cations, under such conditions it is reasonable to postulate that subsequent expansion of the contracted sites and their contact with the inner solution would enable some aspartate, or glutamate, anions to enter the expanded springs if present in the inner solution; normally they would be repelled by the anionic exchange sites. The amino acids could then be adsorbed at the undissociated sites by protonation of their amino groups, and be subsequently exposed and ejected into the external solution during the next contraction cycle as a result of the reduced acidity. ATP would be consumed. Being anions, the amino acids would carry with them Na\(^+\) and K\(^+\) from the inner solution. The addition of increasing amounts of K\(^+\) to the external solution would rapidly reduce the concentration of protonated spring sites and so would inhibit the amino acid efflux, but Na\(^+\), being much less strongly adsorbed, would inhibit less strongly. Internal Na\(^+\) would be required to stimulate thioester hydrolysis and phosphorylation. Such an efflux of amino acids in Na\(^+\)-loaded squid nerves has been observed by Baker (1964).

VIII. LIPID AS A CONTROL POINT

Since lipid is essential for sodium pump activity [see Section III(b)] the possibility needs to be considered that spring elasticity involving bilipid hydrocarbons influences ion selectivity and pump activity. There is evidence for such behaviour.

The pump is inhibited by the detergents dodecyl sulphate (Nagano et al. 1967) and deoxycholate (Järnefelt 1962), and also by deoxycholic (Conway and Hingerty 1953). The local anaesthetics, chlorpromazine and amytal, inhibit the pump (Järnefelt 1962), as do general anaesthetics (Gottlieb 1968; Mullins 1968). Reducing the temperature from 37 to 6°C results in progressive sodium pump inhibition, and activity falls off sharply to a low value below 6°C (Gruener and Avi-Dor 1966). Ouabain is only active above 6°C, and, at fixed concentrations, its inhibitory activity increases with temperature (Gruener and Avi-Dor 1966). The amount of oligomycin required to effect a given degree of inhibition increases with temperature (Gruener and Avi-Dor 1966). The temperature sensitivity of phosphatase activity is also less than when ATP is the substrate (Gruener and Avi-Dor 1966). Such results strongly support the concept of spring elasticity and suggest a glass transition temperature at 6°C.

Recent evidence suggests that the photoreceptors in the ventral eye of Limulus contain a light-sensitive, electrogenic, sodium pump (Smith et al. 1968). Photo-isomerization of 11-cis-retinal in the photoreceptors transforms it from a rigid, strongly sterically hindered structure, to a mobile all-trans structure (Wald 1968). If the sodium pump springs were attached in bilipids with the retinal hydrocarbons, the effect of light on changing the retinal from what might well be a glass to a rubber
would change the contraction–dilation equilibrium of the pump and would alter its ion selectivity and the membrane potential.

IX. Discussion

The pump model provides one specific example of the more general principles proposed in Part I. It has been shown how the dilation–contractile equilibrium of a phospholipopptide, with its associated Na⁺–K⁺ selectivity characteristics, might be utilized for active transport of Na⁺. This necessitates the following changes in the simple action potential spring. The conformational change must block, or open, appropriate channels across the membrane in order to provide the requisite vectorial action for a pump. A mechanism must be provided for energizing contraction of the spring under adverse conditions where otherwise it would remain expanded. It has been shown how ATP-energized crosslinking can provide such a mechanism. The pump model also has an elastic contractile mechanism, associated with bilipid hydrocarbons, which enables it to be influenced by anaesthetics, steroids, and retinal photoisomerization, and so provides a control point. Allosteric control might be accomplished by hormonal-regulated release of AMP which may be preferred, rather than ATP, for inducing the requisite conformation for crosslinking.

The success of such a model in accounting for the wealth of data relating to the sodium pump suggests that different crosslinking mechanisms may be implicated in other processes of active transport utilizing the same basic principles. Many such processes are energized by ion concentration gradients (see Albers 1967) and these fall into three distinct categories.

A glycine pump in pigeon red cells is driven by a Na⁺ gradient (Vidaver 1964). Its dependence on Cl⁻ ions, and their influence on pump affinity, suggests that the ion gradient drives a reversible crosslinking reaction, involving salt links BA between a base B⁺ and a Na⁺-selective anion A⁻, which changes substrate affinity:

\[ \text{BA} + \text{NaCl} \rightleftharpoons \text{BCl} + \text{ANa}. \]  \hspace{2cm} (5)

A sodium pump maintaining a low Na⁺ concentration inside a membrane could indirectly drive such a pump, since the low Na⁺ concentration would favour cross-linking.

Some other pumps seem only to involve a Na⁺ gradient and one such alanine pump in cell nuclei exhibits a pH maximum (Alfrey 1961). The following crosslinking mechanism of such a pump could account for the pH maximum if it involved salt-link formation between a weak-base component W and a Na⁺-selective weak-acid anion A⁻:

\[ \text{AHW} + \text{Na}^+ \rightleftharpoons \text{ANa} + \text{H}^+ + \text{W}. \]  \hspace{2cm} (6)

Only Na⁺ ions need promote dissociation if a buffer is present to remove protons, and if the ruptured basic site becomes less basic as a result of retracting into a less polar environment.

Some other sugar and amino acid pumps utilize both Na⁺ and K⁺ concentration gradients (Crane, Forstner, and Eichholz 1965; Albers 1967). If these contained membrane springs which increased the specificity of a substrate receptor on dilation, K⁺ ions could contract the springs to enable substrate to actively desorb. Na⁺ ions would have an opposing dilatory influence.
Yet another type of ion pump will be discussed in Part IV of this series (Weiss 1969c) in connection with mitochondrial oxidative phosphorylation. It may be considered as a variant of the mechanism whereby Na\(^+\) promotes hydrolysis of thioester crosslinks in the sodium-pump model. In mitochondria, a contraction induced by reduction of the respiratory carriers will be postulated to synthesize thioesters in a mechanico-chemical reaction and energize the release, under appropriate conditions, of alkali:

$$\text{R}^1\text{COOM} + \text{R}^2\text{SH} \Rightarrow \text{R}^1\text{COSR}^2 + \text{MOH}. \quad (7)$$

The intramitochondrial alkali then releases substrate anions held at protonated weak-base membrane sites, across which substrate passes, so that the overall pumping effect is energized accumulation of substrate anions and cations.

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