J. Membrane Biol. 60, 77–93 (1981)

The Journal of
Membrane Biology

Topical Review

Reconstitution of "Carriers" in Artificial Membranes

Lowell E. Hokin
Department of Pharmacology, University of Wisconsin Medical School, Madison, Wisconsin 53706

In the past decade the "insertion" of "carriers" into artificial bimolecular lipid membranes has become a powerful tool in throwing light on the transport functions of these "carriers," as well as providing proof that the carrier molecules are indeed the transporting entity. In all instances so far studied, these carriers have been proteins. In the transport field, the term "carrier" has a long history dating back to times when the concept of a small mobile carrier was widely held. It now appears that most, if not all, "carriers" are proteins which are fixed in the bilayer and do not rotate. The transport of ligands through the proteins occurs by some gating mechanism involving conformational changes in the proteins which are poorly understood. The technique of reconstitution has not thrown much light on this gating mechanism, and it appears to this reviewer that more sophisticated biophysical techniques will be required in order to do so.

Reconstitution has been approached by two basic techniques: (1) insertion of protein carriers into liposomes and (2) insertion of protein carriers into black lipid membranes (BLM). While the latter technique has the advantage of measurement of electrical parameters, the former technique has proved to be far more successful in reconstituting transport systems, and it is basically simpler to carry out successfully. The studies reviewed here will deal primarily with reconstitution by the formation of proteoliposomes. Reconstitution with BLM's is at a much more primitive stage and will be touched on only briefly.

This review will confine itself to transport proteins in the plasma membrane, the sarcoplasmic reticulum (SR), and, in one instance, the outer membrane of the mitochondrion. The review will also be confined to transport proteins in eucaryotes. The vast literature on the proteins involved in oxidative phosphorylation has been reviewed elsewhere.

Methods of Reconstitution by Formation of Proteoliposomes

Racker (1979) has recently reviewed in detail various methods for reconstitution of transport systems, particularly those involving oxidative phosphorylation, and no attempts will be made here to discuss methodology in detail. Rather, a brief overview will be given.

1) Cholate (or other Detergent)-Dialysis Procedure

This was the first procedure developed for reconstitution (Kagawa & Racker, 1971; Kagawa, Kandrach & Racker, 1973; Racker & Kandrach, 1973). The basic procedure is to mix the transport protein with an excess of phospholipid and detergent, usually cholate, and often at high ionic strengths. The detergent is then removed by dialysis over a period of 1–2 days. During this period, liposomes containing the transport protein "self-assemble." This procedure suffers from several disadvantages. The protein usually suffers serious inactivation by detergent, which is partly offset by addition of excessive amounts of phospholipid and other protective agents such as dithiothreitol.

2) Detergent-Dilution Procedure

This technique is an adaptation of the detergent-dialysis procedure in that the protein-phospholipid-detergent mixture is merely diluted rather than dialyzed. It has the advantage of great rapidity and thus spares some proteins the effects of inactivation by the detergent. However, it has not been successful with many systems in which the cholate-dialysis procedure has worked. An interesting application of the procedure has been the asymmetric insertion of the phage M_{13} coat protein of *E. coli* into "synthetic" liposomes

0022-2631/81/0060-0077 $03.40
© 1981 Springer-Verlag New York Inc.

(Racker, 1979). In the vast majority of reconstitutions by formation of proteoliposomes, the protein is inserted in a roughly 50–50 symmetric fashion.

3) Sonication Procedure

This procedure has proved to be superior to cholate dialysis with some systems, such as bacteriorhodopsin (Racker, 1973). This technique involves sonication of transport protein and lipid over fairly long periods (up to 30 min). Conditions of sonication must be rigorously controlled. A bath sonicator rather than a probe sonicator appears to be essential. Although this technique does not suffer from the disadvantage of detergent inactivation, the prolonged sonication times are often harsh on the transport protein.

4) Freeze-Thaw-Sonication Procedure

This technique was developed by Kasahara and Hinkle (1976, 1977*a, b*) for insertion of the glucose carrier from erythrocytes into liposomes. It consists of first preparing liposomes, then adding transport protein, and freezing at dry-ice temperatures. This is followed by slow thawing at room temperature. Finally, the proteoliposomes are "tightened up" by a brief sonication up to 30 sec. Apparently, the freeze-thaw step, which fuses smaller liposomes into larger liposomes, incorporates the transport protein into the liposome. This technique avoids exposure of the transport protein to detergent and prolonged sonication times. It has been highly successful in reconstituting the purified Na,K-pump from the electroplax of *Electrophorus electricus* and from the rectal gland of *Squalus acanthias* (Hokin, 1979; Racker, 1979; Dixon & Hokin, 1980).

5) Incorporation Procedure

a) With detergents. In this procedure, the preformed liposomes are exposed to the transport protein in the presence of very small amounts of detergent for several hours at 0 °C. Detergents such as cholate and lysolecithin have been used. A particularly useful detergent recently described is octylglucoside (Racker, 1979).

b) Without detergents. This procedure has worked in some instances if liposomes were preformed with appropriate lipids, particularly acidic phospholipids (Eytan, Matheson & Racker, 1976). An important feature of this technique is that it has yielded asymmetrically oriented proteins.

6) Reconstitution in Planar Monomolecular and Bimolecular Membranes

Although this review is not concerned with reconstitution of receptors, the recent reconstitution of the acetylcholine receptor into planar membranes (Schindler & Quast, 1980) should be mentioned. Two compartments of a membrane cell containing suspensions of vesicles rich in acetylcholine receptor and vesicles devoid of receptor were filled to just below the membrane aperture. Monolayers self-assemble at the surfaces of the two solutions. Monolayers are easily combined to planar bilayers by raising the water levels successively above the membrane aperture. Membranes could be formed by placing vesicles containing the acetylcholine receptor only on one side of the chamber. The quantitative aspects of the cation channel, the desensitization, and the ligand binding properties were in close agreement with established whole cell values.

Sarcoplasmic Reticulum Ca^{2+}-ATPase

It is not the purpose here to discuss in detail the structure and function of the SR or the SR Ca^{2+}-ATPase. The interested reader should consult the recent reviews on this subject (Martonosi, 1971; Hasselbach, 1974; MacLennan, Ostwald & Stewart, 1974; MacLennan & Holland, 1975; Fleischer et al., 1979; MacLennan et al., 1979). Briefly, the SR is a specialized membrane network which controls relaxation and contraction of muscle by controlling the Ca^{2+} concentration in the sarcoplasm (Endo, 1977; Tada, Yamamoto & Tonomura, 1978). When muscle is stimulated to contract, the wave of excitation down the plasmalemma of the muscle causes release of Ca^{2+} from the SR, raising the concentration of Ca^{2+} around the myofilaments, and muscle contraction is triggered. Relaxation is brought about by a Ca^{2+}-pump in the SR membrane, which lowers the Ca^{2+} concentration in the sarcoplasm back to the resting level. The pump is capable of concentrating Ca^{2+} in the SR 100,000-fold over that in the sarcoplasm.

The SR contains several proteins which have been isolated and partly characterized (MacLennan, 1970; MacLennan, Seeman, Iles & Yip, 1971; MacLennan & Wong, 1971; MacLennan, Yip, Iles & Seeman, 1972). Those which have been isolated are the 100,000-dalton Ca^{2+}-ATPase, a 55,000-dalton high-affinity calcium-binding protein, the 44,000-dalton calsequestrin, and a 6,000-dalton proteolipid which account for 35–45%, 15–20%, 5–10%, 10–20%, and 2–5% of the SR protein, respectively. Over half of the SR Ca^{2+}-ATPase has been sequenced (MacLennan et al., 1979; Allen, 1980*a, b*; Allen, Bottomley &

Trinnaman, 1980*a*; Allen, Trinnaman & Green, 1980*b*). The 55,000-dalton protein has been resolved by two-dimensional slab electrophoresis into four proteins, the most prominent of which is a diffusely staining 53,000-dalton glycoprotein (MacLennan et al., 1979). The purified SR Ca^{2+}-ATPase is essentially free of glycoprotein, unlike the Na,K-ATPase in which the 100,000-dalton catalytic subunit and the 55,000-glycoprotein cannot be separated without loss of activity (*see* below). Sodium dodecyl sulfate (SDS) electrophoresis of the SR resolves the protein into seven bands (MacLennan et al., 1974). The function of the additional three proteins seen on gels is not known.

In 1968, Martonosi reconstituted Ca^{2+} transport by solubilizing "microsomes" from muscle with deoxycholate and cholate, followed by a 50-fold dilution (Martonosi, 1968). Phospholipase C treatment abolished most of the Ca^{2+}-ATPase activity and Ca^{2+} uptake into the proteoliposomes. Enzyme and transport activity could be restored by addition of lecithin. Attempts to reconstitute Ca^{2+} transport from the purified SR Ca^{2+}-ATPase were unsuccessful until 1972 when Racker reconstituted Ca^{2+} transport from purified Ca^{2+}-ATPase and soybean phospholipids by the cholate-dialysis technique (Racker, 1972). The accumulation of Ca^{2+} into the proteoliposomes was inhibited by ionophores for divalent cations but not by ionophores for monovalent cations. Uptake was sensitive to the neuroleptic drug, chlorpromazine. Initial rates of uptake at 22 °C were approximately 500 nmol/min/mg protein. The system required ATP and Mg^{2+} and was inhibited by addition of EDTA and stimulated by 0.4 M potassium phosphate. The stimulation by phosphate (or oxalate) was due to the formation of insoluble calcium salts in the interior of the proteoliposomes (MacLennan et al., 1974; Meissner & Fleischer, 1974; Knowles & Racker, 1975). In studies of SR vesicles, addition of oxalate or phosphate had long been known to stimulate Ca^{2+} transport due to the formation of insoluble Ca^{2+} salts (Weber, 1969). Addition of oxalate or phosphate to the outside of reconstituted proteoliposomes did not stimulate transport because of the much greater impermeability of proteoliposomes to these anions. These anions had to be added during the dialysis procedure so as to trap them inside the proteoliposome (Knowles & Racker, 1975). Knowles and Racker (1975) found that there was no translocation of phosphate from γ-labeled ATP during Ca^{2+} uptake. This rules out certain models of the Ca^{2+}-pump which had been formulated (Deamer & Baskin, 1972; Makinose, 1973; Martonosi, Lagwinska & Oliver, 1974). Knowles and Racker (1975) further found that if the direction of the Ca^{2+}-pump was reversed by preloading the proteoliposomes with Ca^{2+} and adding ethylene glycol bis (β-aminoethyl ether)-N,N′ tetraacetic acid to the outside, ATP was generated from ADP and Pi. The proteoliposomes also catalyzed ATP-Pi exchange (Racker, 1972; Knowles & Racker, 1975) and ATP-ADP exchange (Martonosi, 1968). Reconstitution by the cholate-dialysis procedure using the SR Ca^{2+}-ATPase purified by the method of MacLennan (1970) required residual deoxycholate for reconstitution. On the other hand, reconstitution of Sephadex-treated enzyme by the sonication procedure did not require deoxycholate (Knowles & Racker, 1975).

In most reconstitution studies, a large excess of lipid is required for optimal transport rates. Fleischer's group (Meissner & Fleischer, 1973, 1974; Wang, Saito & Fleischer, 1979) modified the cholate-dialysis procedure so as to reconstitute at higher temperatures, and were able to prepare proteoliposomes with lipid-to-protein ratios approximating that of the native SR, namely 60% protein and 40% lipid. Proteoliposomes with lower as well as higher lipid-to-protein ratios could also be prepared (Wang et al., 1979).

Detailed electron microscopic studies of reconstituted SR Ca^{2+}-ATPase have been carried out (Jilka, Martonosi & Tillack, 1975; Fleischer et al., 1979; Wang et al., 1979). Freeze-etch electron microscopy showed that the Ca^{2+}-ATPase was incorporated symmetrically into the proteoliposomes (Jilka et al., 1975; Wang et al., 1979). This is in contrast to the asymmetry of the SR Ca^{2+}-ATPase in the native SR vesicles (Saito, Wang & Fleischer, 1967). Using electron microscopic procedures and altering the lipid-to-protein ratios, Wang et al. (1979) were able to show that the smooth surface of the proteoliposome was due to phospholipid and the particles were due to protein. They were also able to correlate the number of particles with the number of pump proteins and arrived at a value which was at least two-to-three pump proteins per particle. Other investigators had arrived at a similar conclusion (Jilka et al., 1975; Scales & Inesi, 1976). Symmetry of particles could be influenced by the ratio of lipid to protein in the reconstituted SR Ca^{2+}-ATPase (Fleischer et al., 1979).

Warren et al. (1974) developed a "double-substitution" technique in which over 98% of the lipid associated with the purified enzyme could be displaced with dioleoyl lecithin. Essentially, this technique involves mixing the purified enzyme with a large excess of dioleoyl lecithin and either deoxycholate or cholate and rapidly centrifuging in a sucrose gradient. The detergent and excess lipid remain at the top of the gradient, and the lipid-substituted enzyme penetrates the gradient. This procedure was repeated

one time. While the purified unsubstituted enzyme could not accumulate Ca^{2+}, the substituted enzyme could. A greater accumulation of Ca^{2+} could be achieved by substituting the dioleoyl lecithin-substituted enzyme with lipids from native SR. The lower accumulation of Ca^{2+} with the dioleoyl lecithin-substituted proteoliposome seems to be due to greater leakiness of oxalate, which is required for maximum accumulation (*see* above). Of about 90 lipid molecules which are associated with one molecule of SR Ca^{2+}-ATPase, an average of less than one lipid molecule from the purified enzyme remained in the "double substituted" reconstituted proteoliposome. This value agrees with a value of 88 lipid molecules at the "transition point," below which the proteoliposomes showed asymmetry of the protein and above which they were much more symmetrical (Fleischer et al., 1979). In a later paper, Bennett et al. (1978) substituted the purified enzyme with a series of defined synthetic dioleoyl phospholipids in order to determine the effect of phospholipid headgroups on SR Ca^{2+}-ATPase activity. Phospholipids with zwitterionic headgroups (dioleoyl phosphatidyl choline and dioleoyl phosphatidyl ethanolamine) supported the highest activity, while phospholipids with two negative charges (dioleoyl phosphatidic acid) supported activity at least twenty times lower. Dioleoyl phospholipids with a single net negative charge supported intermediate SR Ca^{2+}-ATPase activity, which was not affected by the precise chemical structure of the phospholipid headgroup. With respect to Ca^{2+} accumulation, two criteria had to be met: namely, the lipid had to support SR Ca^{2+}-ATPase activity, and it had to form sealed proteoliposomes as determined by electron microscopy. A number of phospholipids met these criteria and they supported transport. This indicates that the lipid specificity of the SR Ca^{2+}-pump is rather broad.

Because of the high permeability of SR vesicles to many ions, including Na^+ and K^+, the vesicles are not suitable for studying the electrogenicity of the SR Ca^{2+}-pump. On the other hand, proteoliposomes reconstituted with purified SR Ca^{2+}-ATPase are highly suited for this purpose (Zimniak & Racker, 1978). The SR Ca^{2+}-pump was reconstituted by the method of Kasahara and Hinkle (1976, 1977*a*, *b*) with egg lecithin as the only phospholipid, and electrogenicity of the SR Ca^{2+}-pump was demonstrated by three independent methods. By varying K^+ concentrations during reconstitution and assay, different K^+ gradients across the membrane were established, permitting the creation of controlled membrane potentials in the presence of valinomycin. Stimulation or inhibition of the rate of ATP-dependent uptake was found to be a function of the electrical potential difference imposed on the vesicular membrane. The potential difference which did not affect the rate of Ca^{2+} transport, i.e., the null point, was $+61 \pm 10$ mV (positive inside the vesicles) and was considered to reflect the membrane potential established by an electrogenic, enzyme-mediated Ca^{2+} uptake into the proteoliposome. With the use of 8-anilino-1-naphthalene-sulfonic acid (ANS) as a fluorescent probe (Azzi, Gherardini & Santato, 1971; Jasaitis, Kuliene & Skulachev, 1971), the potential of actively pumping proteoliposomes was determined and was found to be 51 mV, which is in good agreement with the null-point method. Vanderkooi and Martonosi (1970) found an increase in ANS fluorescence in SR during Ca^{2+} uptake, although they did not interpret their results in terms of a membrane potential. The electrogenicity of the reconstituted system was further confirmed by experiments in which an electrical potential difference across the proteoliposome membrane was induced by addition of lipophilic anions. It should be pointed out that the membrane potential of $+60$ mV obtained by Zimniak and Racker (1978) is not characteristic for the enzyme itself. It is a property of the particular proteoliposomes used and reflects the rate of active Ca^{2+} pumping as well as the rates of leakage of all ions present.

Several authors have studied the effect of the SR fragments, the SR Ca^{2+}-ATPase, and protease-released fragments on passive Ca^{2+} transport and the reconstituted Ca^{2+}-pump. Jilka and Martonosi (1977) measured the uptake and release of Ca^{2+} by SR vesicles and reconstituted Ca^{2+}-ATPase proteoliposomes by stopped-flow fluorescence using chlortetracycline as a Ca^{2+} indicator. Incorporation of SR Ca^{2+}-ATPase into liposomes increased their passive permeability to Ca^{2+} by several orders of magnitude. The fatty acid composition of the liposomes had relatively little effect on permeability. Jilka and Martonosi (1977) interpreted this relative insensitivity to differing fatty acids as indicating that Ca^{2+} channels arise by protein-protein interaction. This hypothesis received some support from the experiments of Vanderkooi, Ierokomas, Nakamura and Martonosi (1977). They labeled covalently the purified SR Ca^{2+}-ATPase with N-iodoacetyl-N′-(5-sulfo-1-naphthyl) ethylenediamine (1,5 IAEDANS) or with iodoacetamidofluorescein (IAF). In proteoliposomes labeled with both fluorescence probes, fluorescence energy transfer was observed from the IAEDANS (donor) to the IAF (acceptor) fluorophore as determined by the ratio of donor and acceptor fluorescence intensities and by nanosecond decay measurements of donor fluorescence in the presence or absence of the acceptor. Up to a 10-fold dilution of the lipid phase of reconstituted proteoliposomes with egg lecithin had no measurable

effect upon the energy transfer, suggesting that random collision between ATPase molecules in the lipid phase is not the principal cause of the observed effect. Addition of unlabeled ATPase in 5 to 10 molar excess over the labeled molecules abolished energy transfer. These studies, along with electron microscopic studies (*see* above), support the existence of ATPase oligomers in the membrane with sufficiently long lifetimes for energy transfer to occur. The Martonosi group has put forward the hypothesis that an equilibrium between monomeric and tetrameric forms of the SR Ca^{2+}-ATPase exists, and these forms are governed by a membrane potential which regulates Ca^{2+} uptake and release by SR membranes during muscle contraction and relaxation.

Insertion into liposomes or into BLM has been extensively studied with the holoenzyme and with tryptic fragments. Brief treatment with trypsin cleaved the 100,000-dalton SR Ca^{2+}-ATPase at two discrete points, yielding 55,000-, 45,000-, 30,000- and 20,000-dalton fragments (Migala, Agostini & Hasselbach, 1973; Thorley-Lawson & Green, 1973; Inesi & Scales, 1974; Stewart & MacLennan, 1974). Shamoo and MacLennan (1974) showed that incorporation of the holoenzyme into a BLM also increased markedly the latter's conductivity to a series of divalent metal ions. When the SR Ca^{2+}-ATPase proteoliposomes were exposed to trypsin for 1 min, the 100,000-dalton SR Ca^{2+}-ATPase was cleaved at one point, yielding a 45,000-dalton and a 55,000-dalton fragment. If the fragments were dissociated with detergents and free detergent was removed, the reconstituted proteoliposomes regained high Ca^{2+} transport activity if they were incubated in SDS solutions at 24 °C for short time periods (Stewart & MacLennan, 1974; Shamoo, Ryan, Stewart & MacLennan, 1976). The "ionophoric site," assayed by its ability to increase divalent metal ion conductance in a BLM or in proteoliposomes, was found in the 55,000-dalton fragment and, after a second cleavage, was found in the 20,000- rather than the 30,000-dalton fragment (Shamoo et al., 1976; Shamoo, Scott & Ryan, 1977; Shamoo, 1978). Using cyanogen bromide, MacLennan et al. (1979) observed divalent cation ionophoric activity in a 13,000-dalton fragment. The order of divalent cation selectivity was different, however, from that of the 20,000-dalton fragment, being $Mn^{2+} > Ca^{2+} > Ba^{2+} > Sr^{2+} > Mg^{2+}$ and $Ba^{2+} > Ca^{2+} > Sr^{2+} > Mg^{2+} > Mn^{2+}$, respectively. The ionophoric activity of the 20,000-dalton fragment has therefore been altered by cleavage to the 13,000-dalton fragment. There has been considerable criticism concerning the physiological significance of the "ionophoric activity" of the SR Ca^{2+}-ATPase fragments, and this problem is yet to be resolved. This criticism is based mainly on the rather low selectivity of the "ionophore" for Ca^{2+} and the different selectivity for divalent cations from that of the SR Ca^{2+}-ATPase. The SR only transports Ca^{2+} and Sr^{2+}, and these are the two ions which activate ATP hydrolysis. Ba^{2+}, Mg^{2+} and Mn^{2+}, which are conducted by the ionophore, have no capacity to stimulate ATP hydrolysis and are not transported by the SR. Therefore, if the ionophore is involved in physiological ion transport, access to it must be stringently controlled by the site of ATP hydrolysis which resides on the 55,000- and 30,000-dalton fragments (Thorley-Lawson & Green, 1973; Stewart & MacLennan, 1974). When Racker (1972) first reconstituted Ca^{2+} transport with purified SR Ca^{2+}-ATPase, he found that phosphate (and later oxalate) had to be inside the proteoliposome in order to trap Ca^{2+} which was transported in. On the other hand, Meissner and Fleischer (1974) were able to reconstitute proteoliposomes containing low amounts of phospholipid from SR or partially purified SR Ca^{2+}-ATPase which retained the ability to transport anions without phosphate or oxalate. These observations raise the possibility that there is a protein required for anion transport in the SR which can be purified away from the SR Ca^{2+}-ATPase but which will reassociate with membranes containing the SR Ca^{2+}-ATPase under the right conditions. The 53,000-dalton glycoprotein recently identified in the 55,000-dalton band seen on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) is a possible candidate for the hypothetical anion carrier (MacLennan et al., 1979).

Miller and Racker (1976) incorporated the SR into BLM by fusion of SR vesicles with the BLM. Fusion was absolutely dependent on three conditions: presence of at least 0.5 mM Ca^{2+}, an acidic phospholipid such as phosphatidyl serine or phosphatidyl glycerol in the BLM, and an osmotic gradient across the SR vesicle. These requirements are identical to conditions under which fusion of phospholipid vesicles occur (Miller, Arvan, Telford & Racker, 1976). In the early phase of fusion, discrete conductance steps were seen which were interpreted as fusion of single SR vesicles with the BLM. Using the same type of system, Miller and Rosenberg (1979) studied the effects of transition metal ions on voltage-gated K^{+} conductance. Potassium conductance was modulated by transition metal ions in two ways. First, the conductance was irreversibly inhibited by certain ions added on either side of the membrane. Second, certain ions caused a reversible three- to fourfold stimulation of K^{+} conductance. This effect was seen only when the transition metal was added to the side of the membrane opposite to the side containing the SR vesicles. These effects were seen only on the monovalent cation conductance channel. Neither effect was

given by alkaline earth cations. Single-channel fluctuation experiments demonstrate that the trans stimulation is an effect upon the probability of the channel's opening rather than on the conductance of the open channel. Miller and Rosenberg (1979) presented a simple model for the channel which involves SH groups in the active channel.

Feigenson (1978) used spin-labeled phosphatidyl choline to detect binding differences between dimyristoyl phosphatidyl choline and egg phosphatidyl choline used in reconstituting the SR Ca^{2+}-pump. The quenching measurements of the Ca^{2+}-pump were found to be sensitive to the fatty acid composition of the immediate environment around the Ca^{2+}-ATPase. These experiments provide a method for determining differences in relative binding strengths of different phospholipids to an intrinsic membrane protein. Other factors, such as phase-transition behavior of a given phospholipid, also affected which phospholipids are bound to the protein.

Na,K-ATPase

Over twenty years ago, Skou (1957) discovered an adenosine triphosphatase in the microsome fraction of the leg nerve of the shore crab which required Mg^{2+}, Na^+ and K^+ for optimal activity. He suggested that this enzyme might be involved in the coupled Na,K-pump (Baker, 1972; Glynn & Karlish, 1975) which serves many important physiological functions such as maintaining osmotic equilibrium in all animal cells, maintaining the gradients of Na^+ and K^+ in excitable tissues such as nerve and muscle, permitting waves of excitation down their membranes, and which effects transepithelial transport of Na^+ and, in some cases, K^+ in such structures as the intestinal epithelium, the renal tubules, and various glandular structures. It is also involved in the transport of certain organic ligands, such as sugars and amino acids, which require Na^+ for co-transport. It has been estimated that in the mammal about 30% of the ATP produced by resting respiration is hydrolyzed via the Na,K-ATPase (Whittam & Ager, 1965). Since Skou's discovery, the Na,K-ATPase has been extensively studied, particularly in the erythrocyte membrane (*see* reviews by Dahl & Hokin, 1974; Schwartz, Lindenmayer & Allen, 1974; Glynn & Karlish, 1975; Jørgensen, 1975; Skou, 1975; Whittam & Chipperfield, 1975), and these studies have provided elaborate detail about the structure, reaction mechanism, and function of the Na,K-ATPase. Space does not permit discussion of these details, and the reader is referred to the above reviews as well as the more recent literature (*see* Skou & Nørby, 1979; and Wallick, Lane & Schwartz, 1979).

The Na,K-ATPase has been purified to homogeneity in a number of laboratories. Essentially two techniques have been used. One technique might be called *negative* purification; i.e., all of the extraneous proteins in the cell membrane fraction are extracted with an anionic detergent, usually deoxycholate or SDS, leaving membrane with the intrinsic NA,K-ATPase behind. Centrifugal fractionations lead to the purified enzyme (Kyte, 1971; Lane, Copenhaver, Lindenmayer & Schwartz, 1973; Jørgensen, 1974). All negative purifications have utilized the mammalian outer renal medulla. The final enzyme varies in degree of denaturation and in specific activity. The other approach for purification might be called *positive* purification in which the Na,K-ATPase is initially solubilized with a non-ionic detergent from cell membrane fractions, followed by somewhat unconventional methods of enzyme purification (Hokin et al., 1973; Dixon & Hokin, 1974; Perrone, Hackney, Dixon & Hokin, 1975, 1978). In all cases of purification, it has been necessary to start with tissues of very high specific activity in Na,K-ATPase, namely, the outer renal medulla of the kidney for all negative purifications and the rectal salt gland of the dogfish, or the electric organ of the electric eel for positive purifications.

Proof that the Na,K-ATPase *is* the Na,K-pump required reconstitution of the pump from purified enzyme. Reconstitution of Na^+ transport was accomplished in 1974 by Goldin and Tong (1974) using the enzyme of Kyte (1971) and by Hilden, Rhee and Hokin (1974) using the enzyme of Hokin et al. (1973). Reconstitution of coupled Na^+ and K^+ transport from pure enzyme from *Squalus acanthias* was accomplished by Hilden and Hokin (1975). Earlier that year, in a brief note, reconstitution of Na^+ and K^+ transport was demonstrated with a highly impure enzyme fraction (microsomes) from brain (Sweadner & Goldin, 1975). In a later paper, Goldin (1977) reconstituted coupled Na^+ and K^+ transport from the purified enzyme of Kyte (1971). Reconstitution of sodium but not potassium transport from a microsomal fraction from the electroplax of *Electrophorus electricus* was reported in a brief note by Racker and Fisher (1975). Anner, Lane, Schwartz and Pitts (1977) reported reconstitution of Na^+ and K^+ transport from Na,K-ATPase purified from the outer medulla of lamb kidney. In all of these earlier studies, with the exception of the paper by Racker and Fisher (1975) who sonicated lipids and enzyme together, reconstitution was done essentially by the cholate-dialysis method. Cholate was rather harsh on the enzyme, and enzyme specific activities of the proteoliposomes containing the purified Na,K-ATPase were only of the order of 100 μmol/Pi/mg protein/hr. Hokin and Dixon (1979) reconstituted Na^+ and K^+ transport from

Na,K-ATPase of *Electrophorus electricus* by the "freeze-thaw-sonication" technique of Kasahara and Hinkle (1977*b*). This enzyme could not be reconstituted with the cholate-dialysis technique because of denaturation by cholate. Proteoliposomes with equal transport rates could also be made from the Na,K-ATPase of *Squalus acanthias*. With this technique, transport rates of at least one order of magnitude higher than the highest rates previously reported with the cholate-dialysis technique were obtained. The liposomes formed by the above methods appear to have single bilamellar membranes (Goldin, 1977; Kasahara & Hinkle, 1977*b*), and the Na,K-ATPase appears to be oriented randomly so that approximately 50% of the substrate sites face outward and 50% face inward (Goldin, 1977). The enzyme molecules with substrate sites facing outward would have their ouabain sites facing inward, and the enzymes with their substrate sites facing inward would have their ouabain sites facing outward. The coupled transports of Na^+ and K^+ have only been successfully demonstrated by adding MgATP to the outside which activates Na^+ transport into the liposome and K^+ transport out of the liposome. This is, of course, opposite to the situation in cells. This means that ouabain will only inhibit if it is introduced inside the liposome since it inhibits only from the outer surface of cells. Vanadate (Cantley, Cantley & Josephson, 1973; Cantley et al., 1977; Josephson & Cantley, 1977; Beauge & Glynn, 1978; Quist & Hokin, 1978; Karlish, Beauge & Glynn, 1979), on the other hand, will only inhibit on the outside of the liposome since it inhibits at or near the ATP site on the inner surface of the membrane. Table 1 shows some of the parameters of the reconstituted Na,K-pump established from earlier work. The fact that the Na,K-pump reconstituted from purified Na,K-ATPase shows the same parameters as the Na,K-pumps in the erythrocyte and the squid axon is convincing evidence that the isolated Na,K-ATPase is the Na,K-pump.

Table 1. Parameters of the Na^+-K^+ pump reconstituted from purified, Na,K-ATPase[a]

1. Ouabain-inhibitable Na^+ and K^+ transport dependent on external MgATP.
2. Transports of Na^+ and K^+ in opposite directions to those in cells.
3. Transports of Na^+ and K^+ coupled (omission of external Na^+ blocks K^+ exit).
4. Stoichiometry of Na^+ transport to K^+ transport 3:2.
5. Ouabain inhibits Na^+ and K^+ transport only from the inside (K^+ side).
6. Gradients approaching the physiological obtained for Na^+ and K^+.
7. Effectiveness of various nucleoside triphosphates for Na,K-ATPase activity and for reconstituted Na^+-K^+ transport parallel each other.
8. Ouabain inhibitable exchange diffusion of Na^+ demonstrable in the presence of ATP and in the absence of K^+.
9. Ouabain inhibitable exchange diffusion of K^+ demonstrable in the presence of ATP and P_i in the absence of Na^+.
10. Na^+ and K^+ transport inhibited by external vanadate.

[a] From Hokin (1979) by permission of Addison-Wesley Publishing Co.

The function of the ~45–55,000-dalton glycoprotein subunit of the Na,K-ATPase is unclear, since the binding sites for most of the ligands involved in Na,K-ATPase reside on the large chain (MgATP site, ouabain site, vanadate site, and possibly the ion-binding sites). It is known that antibody against the glycoprotein inhibits enzyme activity (Rhee & Hokin, 1975) and ouabain binding (Rhee & Hokin, 1979). Wheat germ agglutinin (WGA) also agglutinates and inhibits catalytic activity of the Na,K-ATPase from *Squalus acanthias* (Perrone & Hokin, 1978). There is very little inhibition of the eel enzyme by WGA. This may be due to the fact that the shark enzyme is much richer in N-acetylglucosamine residues attached to the glycoprotein. At low molar ratios of WGA to enzyme, reconstituted Na^+ and K^+ transport are partially inhibited by WGA but "transport" Na,K-ATPase (that component not inhibited by external ouabain) is not (Pennington & Hokin, 1979). This indicates that the transports can be partially uncoupled from catalytic activity and raises interesting questions concerning reaction mechanisms of the Na,K-ATPase in which ion translocations are tightly coupled to partial reactions of the Na,K-ATPase. Interestingly, Na^+ transport was inhibited more than K^+ transport as the concentration of WGA was increased. This indicates that Na^+ transport and K^+ transport can be partially uncoupled by WGA. Uncoupling of Na^+ and K^+ transport has been observed in reconstituted systems under other conditions, e.g., by trypsin treatment (Anner & Jørgensen, 1979). This suggests that there may be separate "channels" or "gates" for Na^+ and K^+. Lectins bind to oligosaccharides on the exterior facing site of the cell membrane. One would therefore expect WGA to inhibit transport in the reconstituted Na,K-ATPase liposomes only when presented to the inside facing surface of the membrane, as is the case for ouabain. This was found to be the case (Pennington & Hokin, 1979). It could be shown by SDS-PAGE that WGA bound specifically to the glycoprotein subunit and not to the catalytic subunit (Pennington & Hokin, 1979). Oligosaccharides rich in N-acetylglucosamine residues, such as β-1,4-di-N-acetylglucosamine and ovomucoid, reversed inhibition of rectal gland Na,K-ATPase by WGA (Perrone & Hokin, 1978) and inhibition of reconstituted Na^+ and K^+ transport (Pennington & Hokin, 1979). The mechanism of inhibition

of reconstituted Na^+ and K^+ transport by WGA is not clear. It is possible that binding of a relatively large molecule such as WGA (molecular weight of dimeric WGA is 35,000 (Nagata & Burger, 1974; Rice & Etzler, 1974)) covers or distorts certain sites on the pump molecule. This may account for the "uncouplings" which have been observed with WGA.

One would predict from the stoichiometry of $3\ Na^+ : 2\ K^+$ that the reconstituted Na,K-pump would be electrogenic with a positive potential from inside to outside. The Na,K-pump in erythrocytes has, in fact, been shown to be electrogenic (Hoffman, Kaplan & Callahan, 1979). One technique for measuring potentials is the use of the lipid-permeant anion, $[^{14}C]SCN^-$, which rapidly equilibrates across biomembranes and distributes itself according to the Gibbs-Donnan potential. If one assumes that the concentration of $[^{14}C]SCN^-$ is the same inside as outside in the absence of ATP, due to diffusional equilibration, one would expect to see a higher concentration of $[^{14}C]SCN^-$ inside than outside when MgATP is added because of the positive potential set up by the Na,K-pump. This was in fact found (Hokin, 1979; Dixon & Hokin, 1980). The $[^{14}C]SCN^-$ reached an equilibrated value in about 7 min with the "plus" ATP value considerably higher than the "minus" ATP value. The calculated potential in the presence of ATP from six experiments was 14 mV. Part of this potential was due to diffusion potential due to the higher K^+ gradient than Na^+ gradient, because when the ionophore, nigericin, was used to collapse the gradients, the ratio of SCN^- dropped from 1.73 to 1.42. Calculation of the potential from the Nernst equation with this latter ratio gave an electrogenic potential of 9 mV, the same found by Hoffman et al. (1979) for the erythrocytes from *Amphiuma* and close to that found for sheep and human erythrocytes. This potential was abolished by either external vanadate or internal ouabain.

If three sodium ions move in for every two potassium ions moving out, either one anion must accompany the three sodium ions or one cation must accompany the two potassium ions to maintain charge equilibrium. This in fact was suggested by the fact that when all Cl^- was substituted with SO_4^{2+}, the stoichiometry of $Na^+ : K^+$ became 1:1 (using $^{35}SO_4^{2+}$, the permeability of sulfate was found to be only 1/200 that of chloride). Thus, in the presence of sulfate, the Na,K-pump can be converted from an electrogenic pump to an electrically neutral Na,K-pump. This strongly suggested that the $3\ Na^+ : 2\ K^+$ stoichiometry was made possible by the co-transport of Cl^- with Na^-. This was shown directly with $^{36}Cl^-$. There was a net movement of $^{36}Cl^-$ into the liposome. This was equal to the difference between the higher Na^+ influx and the lower K^+ outflux so that the net movement of charge in the two directions was the same. These observations also make it highly unlikely that endogenous ions, possibly present in small amounts in the system (H^+, HCO_3^-, etc.), could take the place of Cl^-.

Considerable efforts have been made to look for ionophore activity in liposomes containing the holoenzyme, the glycoprotein (obtained by trypsin treatment in the absence of SDS (Churchill & Hokin, 1976; R. White & L.E. Hokin, *unpublished observations*), and the 12,000-dalton protein seen on SDS-gels of most purified Na,K-ATPase preparations (J. Pennington & L.E. Hokin, *unpublished observations*)). The latter could be somewhat selectively extracted into protein-free liposomes by incubating the holoenzyme with the liposomes. Although these proteins markedly enhanced the permeability of Na^+ and K^+ into the liposomes, they were not selective. It appeared that the permeability of all monovalent ions (Cl^-, Br^-, cations) was enhanced nonspecifically by both the glycoprotein and the 12,000-dalton protein.

Recently, Jørgensen and Anner (1979), using the cholate-dialysis technique for reconstitution from the purified kidney medulla Na,K-ATPase, which had been modified by a single tryptic split, found that after reconstitution the active transport of Na^+ in vesicles containing the trypsinized Na,K-ATPase was reduced to 30–40% of the nontrypsinized preparation. K^+ transport was not affected. Passive Na^+ and K^+ fluxes were unaffected. The residual Na^+ transport was resistant to vanadate, apparently as a result of reduction of vanadate affinity in the trypsinized preparation.

The phospholipid requirements for the Na,K-ATPase and the reconstituted Na^+-K^+ pump have been conflicting (*see* above reviews and Racker & Fisher, 1975). Part of the confusion may be due to the fact that the "boundary lipids," in contrast to the bilayer lipids, have, in most instances, not been removed in studies involving lipid requirements for the enzyme or the pump. Using the double-substitution technique of Warren et al. (1974), Hilden and Hokin (1976) exchanged all boundary lipid in the enzyme with egg phosphatidyl choline, and they reconstituted Na^+-K^+ transport by the cholate-dialysis technique using only egg phosphatidyl choline. Transport was as good or better than transport reconstituted with the native enzyme containing the endogenous lipids. No lipids other than phosphatidyl choline were seen by thin-layer chromatography of the enzyme doubly substituted with phosphatidyl choline.

Amino Acids

The transport of amino acids in animal cells is carried out by several distinct transport systems (Oxender

& Christensen, 1963). The Ehrlich ascites cell has proved to be a useful model in studying amino acid transport in animal cells. In the earlier classification of this system, the transport of neutral amino acids appeared to cluster around two distinct transport systems – an *A* system (alanine preferring) which served to transport amino acids such as alanine, glycine, and serine, and an *L* system (leucine preferring) which served to transport branched chain and aromatic amino acids. These two systems were shown by Oxender and Christensen (1963) to differ in their sensitivity to metabolic inhibitors, pH changes, and external sodium ions, with system *A* being more sensitive to the changes in each case.

Johnstone and Bardin (1976) were the first to reconstitute amino acid transport from animal cells. Purified plasma membranes were dissolved in 2% sodium cholate-4M urea, and reconstitution was carried out by the cholate-dialysis procedure. The resulting proteoliposomes showed uptake of α-aminoisobutyric acid (System *A*) which could be inhibited by L-methionine and much less by L-leucine, as predicted from the known properties of aminoisobutyrate transport in intact cells. In addition, it was possible to show accelerated efflux of intravesicular phenylalanine when phenylalanine was added to the trans side (medium side). The data were consistent with the conclusion that there was carrier-mediated transport in reconstituted proteoliposomes.

Cecchini, Payne and Oxender (1977, 1978) solubilized purified plasma membranes from Ehrlich ascites cells with cholate or Triton X-100 and partially purified the L transport system by following leucine binding with equilibrium dialysis. Leucine binding activity was saturable with high levels of leucine or phenylalanine and was not strongly affected by alanine. These properties are similar to those previously identified as System L (Oxender & Christensen, 1963). Purification procedures which were found useful were: ammonium sulfate fractionation, chromatography on Bio-Gel P-60, and chromatography on DEAE-cellulose. SDS-PAGE showed a major Coomassie-blue staining band at 35,000-daltons and about 14 minor bands. Reconstitution of the cholate-solubilized *A* system was carried out by the cholate-dilution procedure. The Triton-solubilized L system could not be reconstituted. The uptake of alanine or leucine was dependent on a sodium gradient. Furthermore, the uptake of alanine was higher in proteoliposomes when valinomycin was added to vesicles rich in K^+. Presumably, an electrogenic potential generated by the valinomycin-mediated exit of potassium ions produced more alanine uptake than the ion gradients alone.

Nishino et al. (1978) isolated plasma membranes from mouse fibroblast 3TC cells transformed by SV40 virus. The protein was partially purified by treatment with dimethylmaleic anhydride. Reconstitution was carried out by the cholate-Sephadex chromatography technique. The reconstituted proteoliposomes demonstrated sodium-stimulated transport of α-aminoisobutyric acid. The simultaneous addition of NaSCN and α-aminoisobutyric acid produced transient accumulation above the equilibrium level (overshoot, active transport). The authors concluded that purified membrane proteins in mouse fibroblast cells can be reconstituted into proteoliposomes which have the characteristics of the sodium-stimulated and electrochemically sensitive α-aminoisobutyric acid transport system.

Some work has been carried out on reconstitution of the transport of neurotransmitters. This approach could prove useful in connection with studies of the mechanism of psychotropic drugs which affect the transport of neurotransmitters in the central nervous system. Kanner (1978) solubilized and reconstituted the γ-aminobutyric acid (GABA) transporter from rat brain synaptosomes into proteoliposomes. The reconstituted system showed the main features of the native membrane vesicles. These features included sodium and chloride ion dependency, direct driving of GABA transport by artificially imposed ion gradients, and sensitivity to electrogenic potentials and inhibitors. Rudnick and Nelson (1978) disrupted platelet plasma membranes, leaving a high molecular weight aggregate which, when submitted to the cholate-dialysis procedure, formed proteoliposomes which accumulated 5-hydroxytryptamine. The reconstituted transport activity was similar to the native membrane vesicles with respect to its ionic requirements and inhibitor sensitivity.

Facilitated Diffusion of D-Glucose

The stereospecific, saturatable, downhill movement of D-glucose has long been known. It also exhibits a considerable degree of substrate specificity as well as competitive inhibition by structurally related sugars (*see* reviews by LeFevre, 1972, 1975) and Jung (1975).

In recent years the facilitated diffusion of D-glucose has been reconstituted from proteins from the erythrocyte membrane by incorporation into liposomes (Kasahara & Hinkle, 1976, 1977*a*, *b*; Zala & Kahlenberg, 1976; Kahlenberg & Zala, 1977). The proteoliposomes were prepared either by sonication or freeze-thaw sonication. After solubilization with detergents, the transporter was purified to a point where it gave a single diffuse periodic acid Schiff (PAS) positive band on SDS-PAGE, corresponding in position to band 4.5 according to the nomenclature of Steck (1974). Transport activity was purified 15-30-

fold. Calculations indicated that there were about 10^5 copies per erythrocyte. This agrees with calculations made by glucose (Kahlenberg, 1976*a*) or inhibitor binding methods (Lin & Spudich, 1974; Jung & Carlson 1975). Transport was inhibited by agents known to inhibit facilitated D-glucose transport in the erythrocyte such as mercuric ions, cytochalasin B, 1 fluoro-2,4-dinitrobenzene, and *p*-chloromercuribenzene sulfonate. Kahlenberg and Zala (1977) found that phloretin, the well-known inhibitor of facilitated diffusion of D-glucose, partially inhibited transport when it was on both sides of the proteoliposomal membrane. Kasahara and Hinkle (1977*b*) did not find inhibition by phloretin. However, in a recent symposium Hinkle, Sogin, Wheeler and Telford (1979) refer to unpublished observations in which phloretin inhibited reconstituted D-glucose transport with a K_I of about 5 μM. The reconstituted transport of D-glucose was stereospecific (L-glucose was not transported), was saturable (Kahlenberg & Zala, 1977), and was inhibited by the structurally related sugars, maltose, D-mannose, and 3-O-methyl-D-glucose, 2-deoxy-D-glucose, and D-galactose (Kasahara & Hinkle, 1976; Kahlenberg & Zala, 1977). Antibody absorption indicated that the protein was exposed to the inner face of the membrane (Kasahara & Hinkle, 1977*b*).

Goldin and Rhoden (1978) reconstituted stereospecific D-glucose transport from human erythrocytes by hollow-fiber dialysis of a cholate extract of the erythrocyte membrane with added phospholipids and cholesterol. As in the earlier studies, D-glucose transport was inhibited by cytochalasin B, mercuric ions, and phloretin. Fifteen-to-twenty percent of the proteoliposomes contained the transport system. The calculated number of transport copies per red cell agreed with the calculations of the earlier investigators. A simple and ingenious technique was devised and termed "transport specificity fractionation" to purify the glucose carrier. This technique consisted of preloading the proteoliposomes with 0.8 M D-glucose and subsequently incubating in a glucose-free medium so that most of the glucose was transported out only from those proteoliposomes containing the glucose carrier. The reduction in intravesicular density of the sugar-transporting proteoliposomes permitted the separation of this fraction from the rest of the proteoliposome population on density gradients. Only band 4.5 of Steck was enriched, confirming the earlier studies.

Recent electron microscopic studies of reconstituted proteoliposomes containing the D-glucose carrier showed one to a few protein particles per proteoliposome. The mean diameter of the protein, correcting for shadow artifacts, was 62 Å which would be expected of a protein of approximately 100,000 mol wt. Since SDS-PAGE showed a molecular weight of 55,000, these data may indicate that the functional form of the D-glucose carrier may be a dimer.

Edwards (1977) solubilized erythrocyte membranes with deoxycholate; after removal of detergent, he obtained proteoliposomes which demonstrated uptake and release of D-glucose which was sensitive to mercuric ions and phloretin and also demonstrated competitive inhibition by related sugars. The phenomenon of "exchange diffusion," which had been previously demonstrated in the erythrocyte membrane for D-glucose, was also demonstrable in the proteoliposomes; i.e., the uptake or release of D-glucose was increased by increasing the concentration of unlabeled transported sugars on the opposite side of the membrane.

Some work has been done on the incorporation of the glucose carrier into BLM's. Jones and Nickson (1978) incorporated Triton extracts of erythrocyte membranes asymmetrically into lecithin-cholesterol-*n*-decane bilayers and measured the electrical and glucose permeability. The extracts increased electrical conductance and markedly enhanced D-glucose but not L-glucose permeability. The enhanced D-glucose transport was inhibited by monosaccharide transport inhibitors. These data suggest that D-glucose transport had been reconstituted in the BLM. Phutrakul and Jones (1979) obtained similar results. It had earlier been claimed that the glucose carrier resided in band 3 (nomenclature of Steck, 1974) but later studies (Kahlenberg & Zala, 1977; Kasahara & Hinkle, 1977*b*; Goldin & Rhoden, 1978) identified the carrier in band 4.5. Phutrakul and Jones (1979) found that removal of excess Triton from the erythrocyte membrane extracts or prolonged storage facilitated proteolysis of band 3 polypeptides and appearance of enhanced zone 4.5 and low molecular weight material. Incorporation of zone 4.5 polypeptides into the bilayer lipid membranes increased their permeability to D-glucose at 27 and 5 °C. The authors suggested that the components of the monosaccharide system are present in band 3 polypeptides but that they can undergo proteolysis with some retention of transport activity. It seems unlikely that in the experiments of the earlier workers, who eliminated all significant protein except material running in the region of band 4.5 by purification, the band 4.5 proteins had arisen by proteolysis. Certainly, band 4.5 is seen on SDS-PAGE of fresh erythrocyte membranes. More work is required to clarify this problem.

Apart from the erythrocyte membrane, the facilitated diffusion of D-glucose occurs in most mammalian cells and is of considerable importance in fat cells and muscle cells where it controls important metabolic processes and is under the control of insu-

lin. Shanahan and Czech (1977*a*, *b*) treated dimethylmaleic anhydride extracted rat adipocyte membranes with 2% cholate and found in the extract only one major glycoprotein with a molecular weight of 94,000. Reconstitution by the cholate-gel filtration technique gave proteoliposomes which gave specific uptake of D- *vs.* L-glucose uptake. This uptake was inhibited by cytochalasin B, phlorizin, phloretin, dipyridamole, and competitive inhibitors such as 3-O-methylglucose. The authors suggested that the 94,000-dalton glycoprotein was directly involved in fat cell hexose transport.

Sodium-Dependent D-Glucose Transport

In the early 1960's, Crane and his colleagues (Crane, 1960, 1962, 1965; Crane, Miller & Bihler, 1961) formulated the gradient-coupled transport hypothesis for the transport of sugars across the luminal membrane of the brush-border cell of the intestine. According to this hypothesis, the sodium dependence of glucose transport is due to the formation of a ternary complex between sodium, glucose, and membrane transport carrier. This ternary complex was assumed to respond to the electrochemical potential gradient across the membrane. The glucose carrier was specific for D-glucose *vs.* L-glucose, sensitive to phlorizin, Na^+-dependent, Na^+-flux was electrogenic, transport was reversible, and the system showed a fixed stoichiometry.

The field of transport received considerable impetus from the use of vesiculated membrane preparations which were introduced by Kaback (Kaback, 1960; Kaback & Stadtman, 1966). Sugar transport in brush-border membrane vesicles from both the intestine and the kidney has been reviewed by Crane, Malathi and Preiser (1977).

Sodium-dependent D-glucose transport has been reconstituted from brush-border membranes of intestine (Crane, Malathi & Preiser, 1976*a*) and kidney (Crane, Malathi & Preiser, 1976*b*, 1977; Crane, Malathi, Preiser & Fairclough, 1978; Fairclough, Malathi, Preiser & Crane, 1978, 1979; Malathi, Preiser, Fairclough & Crane, 1978). Reconstitution was carried out by brief sonication of brush-border membranes with liposomes prepared by sonication of soybean phospholipids. The intestinal system showed Na^+-dependent uptake of D-glucose, but not L-glucose, and it was inhibited by phlorizin and D-galactose. The lipid-permeant anion, SCN^-, was used to provide a high mobility counterion for Na^+. When Na^+ was replaced with K^+, there was nonspecific low uptake of D- and L-glucose. The kidney system was more suitable for detailed study, and most of the work was carried out with membranes from this organ. Apart from showing the above parameters for the intestine, the proteoliposomes from the kidney showed inhibition of D-glucose by sugars which share the same transport mechanism, viz D-galactose, 6-deoxyglucose and methyl glucoside (Ullrich, 1976). Chloride was not as effective as SCN^- as a counterion for Na^+, presumably because of its lower permeability through the proteoliposome. In brush-border membrane vesicles, the phenomenon of initial "overshoot" was seen with NaSCN outside and KCl inside, or by addition of valinomycin. This rapid rise, followed by a rapid fall in internal glucose, was interpreted as being due to the development of a transient rise in the membrane potential, so as to support momentarily the active transport of the positively charged ternary complex. With the proteoliposomes preloaded with K_2SO_4, an initial "overshoot" was seen with NaSCN outside or with NaCl outside plus valinomycin. Further properties of the reconstituted Na^+-dependent glucose transport system were that it was saturable with a K_m of 0.125–0.135, it was responsive to the volume of the internal liposomal space, and the magnitude of the "overshoot" was proportional to the concentration of protein used in reconstitution. To date, the Na^+-dependent glucose carrier has not been purified, and numerous bands are seen on SDS-PAGE of Triton X-100 solubilized brush-border proteins incorporated into liposomes. It is hoped that the reconstituted system will serve as a useful assay in the purification of the Na^+-dependent glucose carrier. Kinne and Faust (1977) solubilized brush-border membranes with Triton X-100 and found the protein composition of the extract markedly different from brush-border membranes. Proteoliposomes were prepared by sonicating detergent-free protein with natural lipids from kidney. Na^+-stimulated D-glucose, L-alanine, and phosphate transport systems were demonstrated.

ADP, ATP Countertransport in the Mitochondrion

The bulk of most of the ATP utilized by cells is synthesized on the matrix side of the inner mitochondrial membrane. The export of ATP into the cytosol is accomplished by an ADP, ATP carrier which effects rapid exchange between ADP and ATP. This carrier has been isolated and its properties studied in considerable detail by Klingenberg and his collaborators. They have shown that the transport rates of the carrier are compatible with those required for ATP-synthesis by oxidative phosphorylation. The transport system has high specificity for ADP and ATP (Pfaff & Klingenberg, 1968). An important feature of the exchange reaction is its regulation by mem-

brane potential. In uncoupled mitochondria, the exchange is essentially symmetric. However, in coupled mitochondria. the exchange is highly asymmetric. The ATP uptake step is largely inhibited so that ADP is preferred to ATP and in the release mechanism, ATP is largely preferred to ADP. The driving force of this regulation is the membrane potential (Pfaff & Klingenberg, 1968). Highly specific inhibitors of the ADP, ATP translocator, such as atractylate and bongkrekate, have been useful in isolating and characterizing the carrier. Using these inhibitors, the number of binding sites, as well as the affinities for substrates, have been determined (Klingenberg, 1976). It appears that there are two sites per respiratory chain or ATPase complex. Atractylate and bongkrekate do not interact with ADP and ATP binding sites in the same manner. Whereas atractylate displaces ADP, bongkrekate increases ADP binding (Erdelt, Weidemann, Buchholz & Klingenberg, 1972). Detailed studies with these inhibitors suggested that a single binding site turned either inside or outside and was not in direct equilibrium with the added ligands (Klingenberg et al., 1973). A crucial finding was that atractylate binds to the carrier from the outside but bongkrekate binds only from the inside. The site was shown to reorient across the membrane only when loaded with substrate, ADP or ATP. Both inhibitors then displace the substrates either towards the outside or inside. Thus the single binding site drastically changes its specificity by switching from the cytosolic side (*c*-state) with a high affinity for atractylate, to the matrix side (*m*-state) with a high affinity for bongkrekate. The altering affinity for ADP and ATP incorporates the "reorienting binding site hypothesis" (Klingenberg & Buchholz, 1973) and the gated-pore" hypothesis of the ADP, ATP carrier (Klingenberg et al., 1976).

By using non-ionic detergents and bringing the carrier into the *c*-state by binding carboxyatractylate (CAT), it was possible to isolate large amounts of the ADP, ATP carrier protein in its undenatured form as a CAT-protein complex (Riccio, Aquila & Klingenberg, 1975*a, b*; Klingenberg, Riccio & Aquila, 1978). The carrier could also be isolated as a more unstable bongkrekate-protein complex (Aquila, Eirmann, Babel & Klingenberg, 1978). The protein isolated from beef heart and rat liver was found to be a dimer containing two subunits with a molecular weight of 30,000. There is one binding site for CAT or bongkrekate. This is consistent with the "gated-pore model" which predicts a single binding site in a channel between two proteins. It was calculated that the carrier accounts for 12% of the total protein in beef heart mitochondria, making it the most abundant protein in these organelles. The existence of two conformations, the *c*- and *m*-state, and the substrate-activated transition could be demonstrated with the isolated protein. Thus, the isolated protein exchanged bongkrekate against atractylate, or *vice versa,* only in the presence of ADP (Aquila et al., 1978)

The ADP, ATP transport system has been reconstituted from purified carrier (Kramer & Klingenberg, 1977*a, b*, 1979, 1980; Kramer, Aquila & Klingenberg, 1977; Shertzer, Kanner, Banerjee & Racker, 1977). The reconstituted system has the same inhibitory properties and follows a 1:1 exchange. Reconstitution allowed determination of the separate rate constants of forward- and back-flux of ADP and ATP. Reconstitution also proved that the isolated 60,000 mol wt protein formed the complete transport system and that there are no cofactors involved. The molecular weight was further confirmed by gel filtration, sedimentation in sucrose gradients, and analytical ultracentrifugation (Hackenberg & Klingenberg, 1980). These studies showed that in a Triton X-100 solution, the protein is completely enveloped in an ellipsoid micelle of about 150-Triton molecules and 16 phospholipid molecules. The exchange of ADP for ATP involves a net movement of one negative charge in the direction of ATP movement because of the net negative charge of 1 for ATP. The exchange might thus be predicted to be influenced by a membrane potential in the reconstituted system. This was in fact shown with proteoliposomes made from egg lecithin and the purified ADP-ATP carrier. Membrane potentials were set up by K^+ gradients in the presence of valinomycin. With a net potential outside, the exchange of external ADP against internal ATP was strongly favored as compared to ATP uptake against ADP efflux. Membrane potential in the opposite direction stimulated the reverse direction of exchange.

Plasma Membrane Ca^{2+}-ATPase

The most extensively studied plasma membrane Ca^{2+}-ATPase is the ($Ca^{2+}+Mg^{2+}$)-ATPase from the erythrocyte membrane. The successful physiological functioning of the human erythrocyte depends in part on its ability to generate a low intracellular Ca^{2+} concentration of 10^{-7} M (Weed & Chailley, 1973; Schatzmann, 1975). Maintenance of this Ca^{2+} level is partly due to the low passive Ca^{2+} permeability of this system (Schatzmann & Vincenzi, 1969), but it is also caused by an active Ca^{2+} transport system which is present in the membrane of red blood cells (Schatzmann, 1966; Schatzmann & Vincenzi, 1969). Erythrocyte plasma membranes contain a ($Ca^{2+}+Mg^{2+}$)-dependent ATPase (Wins & Schoffeniels, 1966; Schatzmann & Rossi, 1971), and evidence suggests that this enzyme is the transport protein itself (Wolk, 1972; Schatzmann, 1975). Peterson, Ronner and Carafoli (1978) partially purified

the ($Ca^{2+}+Mg^{2+}$)-ATPase from human erythrocyte membranes by solubilizing with Triton X-100 or with isoelectric focusing after Triton X-100 solubilization. The ($Ca^{2+}+Mg^{2+}$)-ATPase was selectively incorporated into liposomes of defined lipid content. With ($Ca^{2+}+Mg^{2+}$)-ATPase purified by either procedure, reactivation of enzyme activity occurred when the proteoliposomes consisted of acidic lipids, especially phosphatidyl serine. By comparing enzyme activity in the isoelectrically focused reconstituted system with that for a control detergent solubilized system, it was estimated that the ($Ca^{2+}+Mg^{2+}$)-ATPase was purified approximately 60-fold. Electron micrographs of the reconstituted enzyme system showed closed vesicles of varying sizes with diameters approaching a maximum of 1,000 Å. No Ca^{2+} transport studies were carried out. Haaker and Racker (1979) isolated the ($Ca^{2+}+Mg^{2+}$)-ATPase from the plasma membranes of pig erythrocytes. The enzyme was activated by high concentrations of Tween 20 or by an appropriate mixture of Triton X-100 and phospholipids. The enzyme was highly unstable in the absence of Ca^{2+} and an activator protein. The enzyme was incorporated into liposomes by the freeze-thaw-sonication method of Kasahara and Hinkle (1976, 1977*a, b*). After removal of the non-ionic detergent by passage through a phenyl Sepharose 4B column, the reconstituted proteoliposomes catalyzed rapid ATP-dependent uptake of Ca^{2+}. A "modulator protein" from brain could be substituted for the natural activator protein and stimulated Ca^{2+} uptake in reconstituted proteoliposomes. Galloway and Furlong (1979) reconstituted ATP-dependent Ca^{2+} transport from pigeon erythrocyte membranes by solubilizing in Triton X-100, mixing with phospholipid and cholesterol, and removing the Triton X-100 by addition of bovine high density lipoprotein. SDS-PAGE of the reconstituted proteoliposomes showed the same protein pattern as the original membranes. The reconstituted proteoliposomes showed the same ATP-dependent Ca^{2+} accumulation as the original membrane vesicles.

Kurebe (1978) prepared proteoliposomes from intestinal brush-border membranes and found ATP-independent and ATP-dependent Ca^{2+} uptake and ($Ca^{2+}+Mg^{2+}$)-ATPase activity. High dibucaine concentrations (1 mM) inhibited Ca^{2+} uptake and ($Ca^{2+}+Mg^{2+}$)-ATPase activity, but low dibucaine (0.2 mM) stimulated both activities. Kinetic analysis of the data obtained with the lower concentration of dibucaine suggested that this compound competes with Ca^{2+} for binding sites on the outer surface of the membranes and that its interaction with Ca^{2+} induces a conformational change in the membranes accompanied by an increase in the passive permeability to Ca^{2+} and activation of the ATP-mediated Ca^{2+}-pump. Papazian, Rahamimoff and Goldin (1979) incorporated synaptosomal vesicles into liposomes by the cholate-dialysis technique using an 80-fold excess of the soybean phospholipid mixture, Asolectin. They claimed that most of the vesicles would be expected to have only one or, at most, a few proteins. The proteoliposomes containing the ATP-dependent Ca^{2+} transport system were purified from the bulk of the preparation by "transport specificity fractionation," i.e., their density was increased by precipitation of Ca^{2+} oxalate due to the ATP-dependent transport of Ca^{2+} into the proteoliposomes and this was followed by density-gradient fractionation. A 100-fold purification resulted. On SDS gels, two proteins with molecular weights of 94,000 and 140,000 were seen, and it was claimed that these two proteins are responsible for the reconstituted Ca^{2+} transport. Although reconstitution was obviously achieved, the purity of the system depends on the assumption that the majority of the proteoliposomes sedimenting with the proteoliposomes containing the Ca^{2+}-pump contained only one protein per liposome; a larger number of proteins per liposome would bring down extraneous proteins along with the Ca^{2+}-pump protein. The SDS gels are an insufficient criterion of purity, nor is there any direct evidence that both bands on the SDS gels belong to the Ca^{2+}-pump. It should be pointed out that most transport proteins in the membrane contain a subunit with a molecular weight around 100,000 (*see* above), so that more than one protein could be in this band.

In many excitable tissues, a Na^{+}/Ca^{2+} antiport mechanism operates in the plasma membrane to maintain a low intracellular Ca^{2+} concentration (Baker, 1976; Blaustein, 1976; Mullins, 1976; Requena, DiPolo, Brinley & Mullins, 1977). The Na^{+}/Ca^{2+} antiport system is electrogenic with 3–4 Na^{+} exchanged for each Ca^{2+}. Vesicles have been isolated from the heart of rabbits (Reeves & Sutko, 1979) and dogs (Pitts, 1979). Miyamoto and Racker (1980) isolated similar vesicles from bovine heart muscle and partially purified the Ca^{2+}/Na^{+} antiporter with sodium cholate at high ionic strength (0.5 M NaCl) in the presence of 2.4% soybean phospholipids. Appropriate dilution and sedimentation yielded a preparation of the transporter which could be reconstituted into liposomes. The proteoliposomes catalyzed Ca^{2+} uptake with a specific activity about five times higher than native vesicles. External Na^{+} inhibited Ca^{2+} uptake with a K_i of 50 mM. The uptake of Ca^{2+} was also inhibited when the Na^{+} gradient was collapsed with nigericin. Ca^{2+} uptake was stimulated when either valinomycin or dinitrophenol was added to the assay, in line with the known electrogenic nature of

the transport system. This reconstituted system serves as an assay for further purification of the antiport protein.

Anion Transport Protein

Affinity labeling with site-directed alkylating compounds has identified the anion carrier protein in the erythrocyte membrane (Cabantchik & Rothstein, 1974*a, b*; Ho & Guidotti, 1975). This protein is present in band 3 of Steck (Steck, 1974). Several methods for isolating band 3 have been reported (Findlay, (1974; Yu & Steck, 1975; Furthmayr, Kahane & Marchesi, 1976; Kahlenberg 1976*b*). Anion transport was reconstituted from a crude erythrocyte membrane extract by Rothstein, Cabantchik, Balshin and Juliano (1975). Ross and McConnell (1977) purified band 3 to "near homogeneity" by concanavalin A affinity-column chromatography of dodecyl trimethyl ammonium bromide (DTAB) extracts of erythrocyte membranes. Proteoliposomes were formed by mixing the purified band 3 with egg lecithin, erythrocyte membrane lipids, glycophorin, cholesterol, and DTAB and dialyzing away the DTAB. The proteoliposomes transported sulfate much faster than either sucrose or Na^+. The halftime for sulfate uptake was 25–30 min. After long incubations, the fraction of radioactivity retained by the proteoliposomes was the same for sucrose and sulfate, indicating uptake into the internal volume of the proteoliposomes, since it is unlikely that sulfate and sucrose would bind nonspecifically to the membrane to the same degree. The anion transport protein in erythrocytes is sensitive to treatment with pyridoxal phosphate-$NaBH_4$ (Cabantchik, Blashin, Breuer & Rothstein, 1975). Ross and McConnell (1977) found that reconstituted sulfate transport was also sensitive to pyridoxal phosphate-$NaBH_4$ treatment. In a later paper, Ross and McConnell (1978) found by isoelectric gel separation that the anion transport protein "purified" by affinity-column chromatography could be resolved into two proteins in roughly equal amounts and a small amount of protein at the top of the gel and a poorly defined set of bands in the pH 5.0–6.0 region. Triglycerides, diglycerides, cholesteryl esters, cholesterol, and phosphatidyl choline co-purified with band 3. Twenty-nine thousand lipid molecules were present for every band 3 molecule in the proteoliposomes. Antibodies prepared against the purified band 3 protein appeared to react only with the cytoplasmic face of band 3. This is reminiscent of antibody interaction with the Na,K-ATPase in kidney microsomal vesicles (Kyte, 1974). Reconstituted sulfate transport was inhibited by the potent inhibitor of erythrocyte anion transport, 2-(4′-aminophenyl)-6-methyl benzene thiazo-3′7-disulfonic acid (APMB). Band 3 was spin-labeled with N-(oxyl-2,2,-6,6-tetramethyl-4-piperidinyl) maleimide (MSL). One MSL molecule reacted with one band 3 molecule. It had been previously reported that band 3 had only one reactive sulfhydryl group which was not part of the active site (Steck, Weinstein, Straus & Wallach, 1970; Ho & Guidotti, 1975; Lepke & Passow, 1976). The main component of the paramagnetic resonance spectrum of spin-labeled band 3 in the reconstituted proteoliposomes was consistent with a spin label rigidly fixed to a very slowly rotating protein (McCalley, Shimshick & McConnell, 1972). Reconstituted transport was not inhibited by MSL.

Concluding Remarks. Perhaps the most important question which the reconstitution technique has answered is the following: "Is the isolated protein the molecular machine which drives the movements of the ligands in question?" When the isolated protein is homogeneous, this question can be answered unequivocally; examples of this are given here, especially in those cases where the transport protein exhibits an enzymatic activity such as an ATPase. Where the transport protein does not exhibit enzymatic activity, the reconstitution technique has provided a means of purifying the protein by following transport activity. Reconstitution has also thrown some light on the interaction of phospholipids with the transport protein, but this area is far from clear. In general, there does not appear to be a high degree of specificity *vis-a-vis* the lipids forming the bilayer or the boundary lipids. Reconstitution has been useful in defining stoichiometries in a pure system, and it is gratifying that in general the stoichiometries have confirmed those already determined in intact cells, vesicles, and ghosts. Electrogenicity has been determined in some reconstituted systems, and here, too, the electrical properties have agreed rather well with cellular systems. In a few systems, reconstitution has provided important evidence for a major theory. For example, the synthesis of ATP from light-driven proton transport in reconstituted bacteriorhodopsin has provided strong evidence for the chemiosmotic hypothesis of Mitchell (*see* Racker, 1979).

In general, however, the reconstitution technique has not led to elucidation of the molecular mechanism of translocation of transported ligands. Answers to this basic question await new and sophisticated techniques which will elucidate the three-dimensional structure of the transport protein in different transport modes, much as X-ray crystallography and sequencing have done for small water-soluble proteins.

This work was supported by grants from the National Institutes of Health (GM-26000, HL-16318) and the National Science Foundation (PCM 76-20602).

References

Allen, G. 1980*a*. *Biochem. J.* **187**:545

Allen, G. 1980*b*. *Biochem. J.* **187**:565

Allen, G., Bottomley, R.C., Trinnaman, B.J. 1980*a*. *Biochem. J.* **187**:577

Allen, G., Trinnaman, B.J., Green, N.M. 1980*b*. *Biochem. J.* **187**:591

Anner, B.M., Jørgensen, P.L. 1979. *In:* NA,K-ATPase: Structure and Kinetics. J.C. Skou and J.G. Nørby, editors. p. 87. Academic Press, London

Anner, B.M., Lane, L.K., Schwartz, A., Pitts, B.J.R. 1977. *Biochim. Biophys. Acta* **467**:340

Aquila, H., Eirmann, W., Babel, W., Klingenberg, M. 1978. *Eur. J. Biochem.* **85**:549

Azzi, A., Gherardini, P., Santato, M. 1971. *J. Biol. Chem.* **246**:2035

Baker, P.F. 1972. *In:* Metabolic Pathways. L.E. Hokin, editor. Vol. VI, p. 243. Academic Press, New York

Baker, P.F. 1976. *Fed. Proc.* **35**:2589

Beauge, L.A., Glynn, I.M. 1978. *Nature (London)* **272**:551

Bennett, J.P., Smith, G.A., Houslay, M.D., Hesketh, T.R., Metcalfe, J.C., Warren, G.B. 1978. *Biochim. Biophys. Acta* **513**:310

Blaustein, M.P. 1976. *Fed. Proc.* **35**:2574

Cabantchik, I.Z., Balshin, M., Breuer, W., Rothstein, A. 1975. *J. Biol. Chem.* **250**:5130

Cabantchik, I.Z., Rothstein, A. 1974*a*. *J. Membrane Biol.* **15**:207

Cabantchik, I.Z., Rothstein, A. 1974*b*. *J. Membrane Biol.* **15**:227

Cantley, L.C., Jr., Cantley, L.G., Josephson, L. 1973. *J. Biol. Chem.* **253**:7361

Cantley, L.C., Jr., Josephson, L., Warner, R., Yanagisawa, M., Lechene, C., Guidotti, G. 1977. *J. Biol. Chem.* **252**:7421

Cecchini, G., Payne, G.S., Oxender, D.L. 1977. *J. Supramol. Struct.* **7**:481

Cecchini, G., Payne, G.S., Oxender, D.L. 1978. *Membrane Biochem.* **1**:269

Churchill, L., Hokin, L.E. 1976. *Biochim. Biophys. Acta* **434**:258

Crane, R.K. 1960. *Physiol. Rev.* **40**:789

Crane R.K. 1962. *Fed. Proc.* **21**:891

Crane, R.K. 1965. *Fed. Proc.* **24**:1000

Crane, R.K., Malathi, P., Preiser, H. 1976*a*. *Biochem. Biophys. Res. Commun.* **71**:1010

Crane, R.K., Malathi, P., Preiser, H. 1976*b*. *FEBS Lett.* **67**:214

Crane, R.K., Malathi, P., Preiser, H. 1977. *In:* Biochemistry of Membrane Transport. FEBS-Symposium No. 42. G. Semenza and E. Carafoli, editors. p. 261. Springer-Verlag, Berlin

Crane, R.K., Malathi, P., Preiser, H., Fairclough, P. 1978. *Am. J. Physiol.* **234**:E1

Crane, R.K., Miller, D., Bihler, I. 1961. *In:* Membrane Transport and Metabolism. A. Kleinzeller and A. Kotyl, editors. p. 439. Czech Acad. Sci. Press, Prague

Dahl, J., Hokin, L.E. 1974. *Annu. Rev. Biochem.* **43**:327

Deamer, D.W., Baskin, R.J. 1972. *Arch. Biochem. Biophys.* **153**:47

Dixon, J.F., Hokin, L.E. 1974. *Arch. Biochem. Biophys.* **163**:789

Dixon, J.F., Hokin, L.E. 1978. *Anal. Biochem.* **86**:378

Dixon, J.F., Hokin, L.E. 1980. *J. Biol. Chem.* **255**:10681

Edwards, P.A.W. 1977. *Biochem. J.* **164**:125

Endo, M. 1977. *Physiol. Rev.* **57**:71

Erdelt, H., Weidemann, M.K., Buchholz, M., Klingenberg, M. 1972. *Eur. J. Biochem.* **30**:107

Eytan, G.D., Matheson, M.J., Racker E. 1976. *J. Biol. Chem.* **251**:6831

Fairclough, P.D., Malathi, P., Preiser, H., Crane, R.K. 1978. *In:* Proceedings of the VIIth International Congress of Nephrology. p. 61. S. Karger, Basel

Fairclough, P.D., Malathi, P., Preiser, H., Crane, R.K. 1979. *Biochim. Biophys. Acta* **553**:295

Feigenson, G.W. 1978. *FEBS Lett.* **96**:51

Findlay, J.B.C. 1974. *J. Biol. Chem.* **249**:4398

Fleischer, S., Wang, C.T., Hymel, L., Seelig, J., Brown, M.R., Herbette, L., Scarpa, A., McLaughlin, A.C., Blasie, J.K. 1979. *In:* Function and Molecular Aspects of Biomembrane Transport. E. Quagliariello et al., editors. p. 465. Elsevier/North-Holland, Amsterdam

Furthmayr, H., Kahane, I., Marchesi, V.T. 1976. *J. Membrane Biol.* **26**:173

Galloway, D.R., Furlong, C.E. 1979. *Arch. Biochem. Biophys.* **197**:158

Glynn, I.M., Karlish, S.J.D. 1975. *Annu. Rev. Physiol.* **37**:13

Goldin, S.M. 1977. *J. Biol. Chem* **252**:5630

Goldin, S.M., Rhoden, V. 1978. *J. Biol. Chem.* **253**:2575

Goldin, S.M., Tong, S.W. 1974. *J. Biol. Chem.* **249**:5907

Haaker, H., Racker, E. 1979. *J. Biol. Chem.* **254**:6598

Hackenberg, H., Klingenberg, M. 1980. *Biochemistry* **19**:548

Hasselbach, W. 1974. *In:* The Enzymes. (3rd Ed.) P.D. Boyer, editor. Vol. 10, p. 431. Academic Press, New York

Hilden, S., Hokin, L.E. 1975. *J. Biol. Chem.* **250**:6296

Hilden, S., Hokin, L.E. 1976. *Biochem. Biophys. Res. Commun.* **69**:521

Hilden, S., Rhee, H.M., Hokin, L.E. 1974. *J. Biol. Chem.* **249**:7432

Hinkle, P.C., Sogin, D.C., Wheeler, T.J., Telford, J.N. 1979. *In:* Function and Molecular Aspects of Biomembrane Transport. E. Quagliariello, F. Palmieri, S. Papa, and M. Klingenberg, editors. p. 487. Elsevier/North-Holland, Amsterdam

Ho, M.K., Guidotti, G. 1975. *J. Biol. Chem.* **250**:675

Hoffman, J.F., Kaplan, J.H., Callahan, T.J. 1979. *Fed. Proc.* **38**:2440

Hokin, L.E. 1979. *In:* Membrane Bioenergetics. C.P. Lee, G. Schatz and L. Ernster, editors. p. 281. Addison-Wesley, Reading, Mass.

Hokin, L.E., Dahl, J.D., Deupree, J.D., Dixon, J.F., Hackney, J.F., Perdue, J.R. 1973. *J. Biol. Chem.* **248**:2593

Hokin, L.E., Dixon, J.F. 1979. *In:* Na,K-ATPase: Structure and Kinetics. J.C. Skou and J.G. Nørby, editors. p. 47. Academic Press, London

Inesi, G., Scales, D. 1974. *Biochemistry* **13**:3298

Jasaitis, A.A., Kuliene, V.V., Skulachev, V.P. 1971. *Biochim. Biophys. Acta* **234**:177

Jilka, R.L., Martonosi, A.N. 1977. *Biochim. Biophys. Acta* **466**:57

Jilka, R.L., Martonosi, A.N., Tillack, T.W. 1975. *J. Biol. Chem.* **250**:7511

Johnstone, R.M., Bardin, C. 1976. *J. Cell. Physiol.* **89**:801

Jones, M.N., Nickson, J.K. 1978. *Biochim. Biophys. Acta* **509**:260

Jørgensen, P.L. 1974. *Biochim. Biophys. Acta* **356**:36

Jørgensen, P.L. 1975. *Q. Rev. Biophys.* **7**:239

Jørgensen, P.L., Anner, B.M. 1979. *Biochim. Biophys. Acta* **555**:485

Josephson, L., Cantley, L.C., Jr. 1977. *Biochemistry* **16**:4572

Jung, C.Y. 1975. *In:* The Red Blood Cell. D.M. Surgenor, editor. Vol. II, p. 705. Academic Press, New York

Jung, C.Y., Carlson, L.M. 1975. *J. Biol. Chem.* **250**:3217

Kaback, H.R. 1960. *Fed. Proc.* **19**:130

Kaback, H.R., Stadtman, E.R. 1966. *Proc. Natl. Acad. Sci. USA* **55**:920

Kagawa, Y., Kandrach, A., Racker, E. 1973. *J. Biol. Chem.* **248**:676

Kagawa, Y., Racker, E. 1971. *J. Biol. Chem.* **246**:5477

Kahlenberg, A. 1976*a*. *J. Biol. Chem.* **251**:1582

Kahlenberg, A. 1976*b*. *Anal. Biochem.* **74**:337

Kahlenberg, A., Zala, C.A. 1977. *J. Supramol. Struct.* **7**:287

Kanner, B.I. 1978. *FEBS Lett.* **89**:47

Karlish, S.J.D., Beauge, L.A., Glynn, I.M. 1979. *Nature (London)* **282**:335

Kasahara, M., Hinkle, P.C. 1976. *Proc. Natl. Acad. Sci. USA* **73**:396

Kasahara, M., Hinkle, P.C. 1977*a*. *In:* Biochemistry of Membrane

Transport. G. Semenza and E. Carafoli, editors. p. 346. Springer-Verlag, Berlin
Kasahara, M., Hinkle, P.C. 1977*b*. *J. Biol. Chem.* **252**:7384
Kinne, R., Faust, R.G. 1977. *Biochem. J.* **168**:311
Klingenberg, M. 1976. *In:* The Enzymes of Biological Membranes: Membrane Transport. A.N. Martonosi, editor. Vol. 3, p. 383. Plenum, New York
Klingenberg, M., Buchholz, M. 1973. *Eur. J. Biochem.* **38**:346
Klingenberg, M., Riccio, P., Aquila, H. 1978. *Biochim. Biophys. Acta* **503**:193
Klingenberg, M., Riccio, P., Aquila, H., Buchanan, B.B., Grebe, K. 1976. *In:* The Structural Basis of Membrane Function. Y. Hatefi and L. Djavadi-ohaniance, editors. p. 293. Academic Press, London
Klingenberg, M., Scherer, B., Stengel-Rutkowski, L., Buchholz, M., Grebe, K. 1973. *In:* Mechanisms in Bioenergetics. G.F. Azzone et al., editors. p. 257. Academic Press, New York
Knowles, A.F., Racker, E. 1975. *J. Biol. Chem.* **250**:3538
Kramer, R., Aquila, H., Klingenberg, M. 1977. *Biochemistry* **16**:4949
Kramer, R., Klingenberg, M. 1977*a*. *Biochemistry* **16**:4954
Kramer, R., Klingenberg, M. 1977*b*. *FEBS Lett.* **82**:363
Kramer, R., Klingenberg, M. 1979. *Biochemistry* **18**:4209
Kramer, R., Klingenberg, M. 1980. *Biochemistry* **19**:556
Kurebe, M. 1978. *Mol. Pharmacol.* **14**:138
Kyte, J. 1971. *J. Biol. Chem.* **246**:4156
Kyte, J. 1974. *J. Biol. Chem.* **249**:3652
Lane, K., Copenhaver, J.H., Jr., Lindenmayer, G.E., Schwartz, A. 1973. *J. Biol. Chem.* **248**:7197
LeFevre, P.G. 1972. *In:* Metabolic Pathways. L.E. Hokin, editor. Vol. VII, p. 438. Academic Press, New York
LeFevre, P.G. 1975. *In:* Current Topics in Membranes and Transport. F. Bronner and A. Kleinzeller, editors. Vol. 7, p. 109. Academic Press, New York
Lepke, S., Passow, H. 1976. *Biochim. Biophys. Acta* **455**:353
Lin, S., Spudich, J.A. 1974. *Biochem. Biophys. Res. Commun.* **61**:1471
MacLennan, D.H. 1970. *J. Biol. Chem.* **245**:4508
MacLennan, D.H., Holland, P.C. 1975. *Annu. Rev. Biophys. Bioeng.* **4**:377
MacLennan, D.H., Klip, A., Reithmeier, R., Michalak, M., Campbell, K.P. 1979. *In:* Membrane Bioenergetics. C.P. Lee, G. Schatz and L. Ernster, editors. p. 255. Addison-Wesley, Reading, Mass.
MacLennan, D.H., Ostwald, T.J., Stewart, P.S. 1974. *Ann. N. Y. Acad. Sci.* **227**:527
MacLennan, D.H., Seeman, P., Iles, G.H., Yip, C.C. 1971. *J. Biol. Chem.* **246**:2702
MacLennan, D.H., Wong, P.T.S. 1971. *Proc. Natl. Acad. Sci. USA* **68**:1231
MacLennan, D.H., Yip, C.C., Iles, G.H., Seeman, P. 1972. *Cold Spring Harbor Symp. Quant. Biol.* **37**:469
Makinose, M. 1973. *FEBS Lett.* **37**:140
Malathi, P., Preiser, H., Fairclough, P., Crane, R.K. 1978. *In:* Cell Membrane Receptors for Drugs and Hormones: A Multidisciplinary Approach. R.W. Straub and L. Bolis, editors. Raven, New York
Martonosi, A. 1968. *J. Biol. Chem.* **243**:71
Martonosi, A. 1971. *In:* Biomembranes. L.A. Manson, editor. Vol. 1, p. 191. Plenum, New York
Martonosi, A., Lagwinska, E., Oliver, M. 1974. *Ann. N. Y. Acad. Sci.* **227**:549
McCalley, R.C., Shimshick, E.J., McConnell, H.M. 1972. *Chem. Phys. Lett.* **13**:115
Meissner, G., Fleischer, S. 1973. *Biochem. Biophys. Res. Commun.* **52**:913
Meissner, G., Fleischer, S. 1974. *J. Biol. Chem.* **249**:302
Migala, A., Agostini, B., Hasselbach, W. 1973. *Z. Naturforsch.* **28**:178
Miller, C., Arvan, P., Telford, J.N., Racker, E. 1976. *J. Membrane Biol.* **30**:271
Miller, C., Racker, E. 1976. *J. Membrane Biol.* **30**:283
Miller, C., Rosenberg, R.L. 1979. *Biochemistry* **18**:1138
Miyamoto, H., Racker, E. 1980. *J. Biol. Chem.* **255**:2656
Mullins, L.J. 1976. *Fed. Proc.* **35**:2583
Nagata, Y., Burger, M.M. 1974. *J. Biol. Chem.* **249**:3116
Nishino, H., Tillotson, L.G., Schiller, R.M., Inui, K.-I., Isselbacher, K.J. 1978. *Proc. Natl. Acad. Sci. USA* **75**:3856
Oxender, D.L., Christensen, H.N. 1963. *J. Biol. Chem.* **238**:3686
Papazian, D., Rahamimoff, H., Goldin, S.M. 1979. *Proc. Natl. Acad. Sci. USA* **76**:3708
Pennington, J., Hokin, L.E. 1979. *J. Biol. Chem.* **254**:9754
Perrone, J.R., Hackney, J.F., Dixon, J.D., Hokin, L.E. 1975. *J. Biol. Chem.* **250**:4178
Perrone, J.R., Hokin, L.E. 1978. *Biochim. Biophys. Acta* **525**:446
Peterson, S.W., Ronner, P., Carafoli, E. 1978. *Arch. Biochem. Biophys.* **186**:202
Pfaff, E., Klingenberg, M. 1968. *Eur. J. Biochem.* **6**:66
Phutrakul, S., Jones, M.N. 1979. *Biochim. Biophys. Acta* **550**:188
Pitts, B.J.R. 1979. *J. Biol. Chem.* **254**:6232
Quist, E.E., Hokin, L.E. 1978. *Biochim. Biophys. Acta* **511**:202
Racker, E. 1972. *J. Biol. Chem.* **247**:8198
Racker, E. 1973. *Biochem. Biophys. Res. Commun.* **55**:224
Racker, E. 1979. *Methods Enzymol.* **55**:699
Racker, E., Fisher, L.W. 1975. *Biochem. Biophys. Res. Commun.* **67**:1144
Racker, E., Kandrach, A. 1973. *J. Biol. Chem.* **248**:5841
Reeves, J.P., Sutko, J.L. 1979. *Proc. Natl. Acad. Sci. USA* **76**:590
Requena, J., DiPolo, R., Brinley, F.J., Jr., Mullins, L.J. 1977. *J. Gen. Physiol.* **70**:329
Rhee, H.M., Hokin, L.E. 1975. *Biochem. Biophys. Res. Commun.* **63**:1139
Rhee, H.M., Hokin, L.E. 1979. *Biochim. Biophys. Acta* **558**:108
Riccio, P., Aquila, H., Klingenberg, M. 1975*a*. *FEBS Lett.* **56**:129
Riccio, P., Aquila, H., Klingenberg, M. 1975*b*. *FEBS Lett.* **56**:133
Rice, R.H., Etzler, M.E. 1974. *Biochem. Biophys. Res. Commun.* **59**:414
Ross, A.H., McConnell, H.M. 1977. *Biochem. Biophys. Res. Commun.* **74**:1318
Ross, A.H., McConnell, H.M. 1978. *J. Biol. Chem.* **253**:4777
Rothstein, A., Cabantchik, Z.T., Balshin, M., Juliano, R. 1975. *Biochem. Biophys. Res. Commun.* **64**:144
Rudnick, G., Nelson, P.J. 1978. *Biochemistry* **17**:5300
Saito, A., Wang, C.-T., Fleischer, S. 1967. *J. Cell Biol.* **79**:601
Scales, D., Inesi, G. 1976. *Biophys. J.* **16**:735
Schatzmann, H.J. 1966. *Experientia* **22**:364
Schatzmann, H.J. 1975. *In:* Current Topics in Membranes and Transport. F. Bonner and A. Kleinzeller, editors. Vol. 6, p. 125. Academic Press, New York
Schatzmann, H.J., Rossi, G.L. 1971. *Biochim. Biophys. Acta* **241**:379
Schatzmann, H.J., Vincenzi, F.F. 1969. *J. Physiol.* (*London*) **201**:359
Schindler, H., Quast, U. 1980. *Proc. Natl. Acad. Sci. USA* **77**:3052
Schwartz, A., Lindenmayer, G.E., Allen, J.C. 1974. *Pharmacol. Rev.* **24**:3
Shamoo, A.E. 1978. *J. Membrane Biol.* **43**:227
Shamoo, A.E., MacLennan, D.H. 1974. *Proc. Natl. Acad. Sci. USA* **71**:3522
Shamoo, A.E., Ryan, T.E., Stewart, P.S., MacLennan, D.H. 1976. *J. Biol. Chem.* **251**:4147
Shamoo, A.E., Scott, T.L., Ryan, T.E. 1977. *J. Supramol. Struct.* **6**:345

Shanahan, M.F., Czech, M.P. 1977*a*. *J. Biol. Chem.* **252**:6554
Shanahan, M.F., Czech, M.P. 1977*b*. *J. Biol. Chem.* **252**:8341
Shertzer, H.G., Kanner, B.I., Banerjee, R.K., Racker, E. 1977. *Biochem. Biophys. Res. Commun.* **75**:779
Skou, J.C. 1957. *Biochim. Biophys. Acta* **23**:394
Skou, J.C. 1975. *Q. Rev. Biophys.* **7**:401
Skou, J.C., Nørby, J.G. (editors). 1979. Na,K-ATPase: Structure and Kinetics. p. 1. Academic Press, London
Steck, T.L. 1974. *J. Cell Biol.* **62**:1
Steck, T.L., Weinstein, R.S., Straus, J.H., Wallach, D.F.H. 1970. *Science* **168**:255
Stewart, P.S., MacLennan, D.H. 1974. *J. Biol. Chem.* **249**:985
Sweadner, K.J., Goldin, S.M. 1975. *J. Biol. Chem.* **250**:4022
Tada, M., Yamamoto, T., Tonomura, Y. 1978. *Physiol. Rev.* **58**:1
Thorley-Lawson, D.A., Green, N.M. 1973. *Eur. J. Biochem.* **40**:403
Ullrich, K.J. 1976. *Kidney Int.* **9**:134
Vanderkooi, J.M., Ierokomas, A., Nakamura, H., Martonosi, A. 1977. *Biochemistry* **16**:1262
Vanderkooi, J.M., Martonosi, A. 1970. *Fed. Proc.* **29**:936
Wallick, E.T., Lane, L.K., Schwartz, A. 1979. *Annu. Rev. Physiol.* **41**:397
Wang, C.-T., Saito, A., Fleischer, S. 1979. *J. Biol. Chem.* **254**:9209
Warren, G.B., Toon, P.A., Birdsall, N.J.M., Lee, A.G., Metcalfe, J.C. 1974. *Proc. Natl. Acad. Sci. USA* **71**:622
Weber, A. 1969. *Curr. Top. Bioenerg.* **1**:203
Weed, R.I., Chailley, B. 1973. *In:* Red Cell Shape. M. Bessis, R.I. Weed, and P.F. Leblond, editors. p. 55. Springer-Verlag, Berlin
Whittam, R., Ager, M.E. 1965. *Biochem. J.* **97**:214
Whittam, R., Chipperfield, A.R. 1975. *Biochim. Biophys. Acta* **415**:149
Wins, T., Schoffeniels, E. 1966. *Biochim. Biophys. Acta* **120**:341
Wolk, H.U. 1972. *Biochim. Biophys. Acta* **266**:361
Yu, J., Steck, T.L. 1975. *J. Biol. Chem.* **250**:9170
Zala, C.A., Kahlenberg, A. 1976. *Biochem. Biophys. Res. Commun.* **72**:866
Zimniak, P., Racker, E. 1978. *J. Biol. Chem.* **253**:4631

Received 22 September 1980