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The Binding of Saxitoxin and Tetrodotoxin to Excitable Tissue

J. M. RITCHIE and R. B. ROGART*

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I. Introduction

The formulation of the ionic hypothesis of nervous conduction by HODGKIN and HUXLEY (1952; see also HODGKIN, 1964), especially with its emphasis on the voltage-clamping technique, made it clear that the action potential in excitable membrane is generated by two ionic currents: an early sodium current and a late potassium current. The nervous membrane, however, is not uniformly permeable to the two cations, sodium and potassium; instead, the currents flow at certain specialized patches or sites on the membrane rather than across all parts of it. Furthermore, pharmacological evidence shows that the two currents flow across the membrane at two different types of sites. For example, the tetraethylammonium ion completely blocks the late potassium current both in the squid giant axon (by internal application, ARMSTRONG and BINSTOCK, 1965) and in frog myelinated nerve fibers (by application, externally, HILLE, 1967; and internally, ARMSTRONG and HILLE, 1972). Similarly — and more germanely for the present review — the early sodium current can be prevented either by saxitoxin or by tetrodotoxin. The toxin has to be applied externally, for example, to lobster giant axons and frog myelinated nerve (NARAHASHI et al., 1964; HILLE, 1968a), for it is quite ineffective when applied internally to the squid giant axon (NARAHASHI et al., 1966). These two toxins are important, not so much because they are active in extremely low concentrations—they are in fact among the most potent nonprotein poisons known; rather it is because their action is so highly specific. Unlike the local anesthetic agents, for example, which block the sodium current in low concentrations but which in higher concentrations also block the potassium current, the two toxins, saxitoxin and tetrodotoxin, even in relatively high concentrations, have only a single known pharmacological action, namely the specific blocking of sodium channels. It is because of this specificity that the extent to which these toxins are bound to conducting tissue can be used as a measure of the number of sodium channels. They are in fact used as specific chemical markers for this important membrane component of excitable tissue (see RITCHIE, 1975a).

The binding of saxitoxin and tetrodotoxin to excitable membrane is characterized by two parameters that provide information about the sodium channel. The first parameter, the maximum saturable uptake of toxin per unit weight of tissue, M, provides a measure of density of sodium channels in a conducting tissue. If the conductance per unit surface area (or the number of ions crossing this unit surface area per action potential) is known, the conductance per sodium channel (or the ionic flux per sodium channel per action potential) can be determined from the M value. The second parameter, the equilibrium dissociation constant of the toxin-sodium channel interaction, K, provides a measure of the affinity of saxitoxin or tetrodotoxin for the sodium channel. By examining the effects of competitive inhibitors of toxin binding, of changes in environment of the sodium channel, and of chemical alteration of the sodium channel, on the apparent value of K_t for saxitoxin or tetrodotoxin binding, one can infer a great deal about the molecular structure and function of the toxin binding site.

The present review will begin by summarizing, in Sections II and III, the structural and electrophysiological basis for the action of saxitoxin and tetrodotoxin. Early binding studies with both unlabeled and labeled toxin will then be discussed in light of their experimental significance (Sect. IV). Studies with unlabeled toxins first indicated the sparsity of sodium channels in nerve membrane. The densities of channels obtained, however, served only as preliminary estimates because of lack of precision of bioassay for measuring toxin uptake. Early studies with radioactive toxins established that the toxins could be labeled without destroying activity. Complete binding curves were obtained, showing that a saturable maximum component of uptake of radioactivity per unit area of membrane surface corresponding to uptake by sodium channels could be determined and that the dissociation constant, K_t, for this binding could also be assessed. However, results obtained by different laboratories on the same preparation for maximum saturable uptake of toxin per unit area varied greatly. It soon became clear that despite the fact that biochemical tests indicated high radiochemical purity, labeled toxin did contain impurities; errors thus arose in the assessment of the amount of toxin uptake. Methods of toxin labeling and determination of purity will be discussed next (Sect. V). In the light of improved methods for determining the purity of labeled toxin, the binding of toxin to nerve and muscle membranes will be discussed next (Sect. VI and VII). Finally, studies characterizing the sodium channel from measurements of toxin channel affinity under a variety of conditions will be discussed (Sect. VIII to XII).

II. Saxitoxin and Tetrodotoxin

A. Source of the Toxins

To date no satisfactory synthetic substitute for the toxins is available, even though both are rather low molecular weight substances (300–319) of known molecular structure (tetrodotoxin, WOODWARD, 1964; saxitoxin, SCHANTZ et al., 1975). Furthermore, even though the complete synthesis of tetrodotoxin

(KISHI et al., 1972), and of saxitoxin (TANINO et al., 1977) is now possible, synthetic toxin, at the moment, is not generally available to replace the natural product. The toxins currently used are thus of biological origin, and their potency must be verified by bioassay since no chemical tests are available for assaying and identifying them in the concentrations usually used in biological experiments.

Saxitoxin is produced by a marine dinoflagellate *Gonyaulax catenella* (see SCHANTZ, 1969, 1973; SCHANTZ, et al., 1975). When the conditions of temperature and light are right, this organism reproduces itself (blooms) so rapidly as to discolor the sea, hence the term red tide. Shellfish, especially clams and mussels, feeding on the dinoflagellates at this time become poisonous to man causing paralytic shellfish poisoning. The symptoms of paralytic shellfish poisoning are virtually identical with those of *Fugu* fish poisoning (see below). They are due to the saxitoxin ingested by the shellfish, which is stored for weeks (mussels) or many months (clams), especially in the hepatopancreas or, in the case of clams, also in the siphon. One small mussel may contain 50 human lethal doses.

Tetrodotoxin, the poison classicaly associated with the Japanese *Fugu* fish, occurs in the tissues of at least 40 species of Puffer fish, mostly belonging to the family *Tetraodontidae* (FUHRMAN, 1967). It is most highly concentrated in the ovaries and liver, smaller amounts being found in the intestines and skin. Surprisingly, the identical toxin, originally called tarichatoxin, also occurs in the eggs of various species of Western American newts of the genus *Taricha* (BUCHWALD et al., 1964; MOSHER et al., 1964), in a Pacific goby (NOGUCHI and HASHIMOTO, 1973), and in the skin of some Atelopid Central American frogs (KIM et al., 1975). The biological significance of the toxin in these widely different groups of animals is unknown.

Symptoms of both paralytic shellfish poisoning and *Fugu* fish poisoning reflect the progressive paralysis of the excitability mechanism of nerve and muscle. They begin with numbness in the lips, tongue, and fingertips that may be apparent within a few minutes after eating. This is followed by feelings of numbness and weakness in the legs, arms, and neck, which progress to a general muscular incoordination. In severe poisoning, death occurs from respiratory paralysis. Artificial respiration is the only known antidote. If the patient survives, 24 h prognosis is good with rapid, complete recovery.

B. Structure of the Toxins

Figure 1 shows the structures of saxitoxin (SCHANTZ et al., 1975) and of tetrodotoxin (WOODWARD, 1964). Although the two toxins differ in their chemical structure and one is a monovalent cation while the other is a divalent cation, a certain similarity between the two toxins does exist. For example, tetrodotoxin contains one guanidinium group, and saxitoxin contains two. Guanidinium is an organic cation that is known to be able to pass through the sodium channel, and in sodium-free solution, it can actually substitute for sodium in sustaining excitability (for references, see HILLE, 1971). The presence of the guanidinium moiety seems critical for the pharmacological action of the toxins. In fact, in 1965, KAO and NISHIYAMA were the first to make the now generally accepted suggestion that the

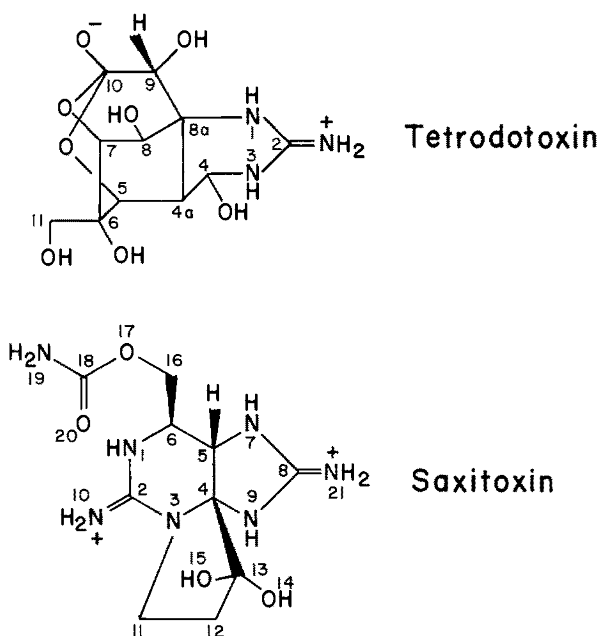

Fig. 1. The structure of saxitoxin (SCHANTZ et al., 1975) and of tetrodotoxin (WOODWARD, 1964)

guanidinium group found in both toxin molecules enters the sodium channel, where it then becomes stuck because the rest of the molecule is too bulky to pass. SMYTHIES et al. (1971) have suggested that tetrodotoxin ties together in a highly efficient manner various parts of the sodium channel. As a result, the passage of sodium ions through the channel is physically prevented and the consequence is a block in conduction. From an analysis of the permeability of various organic cations that pass through the sodium channel, HILLE (1971) proposed that the part of the channel where the toxins finally stick is near a narrow ionic selectivity filter that is approached through a pore 3 Å × 5 Å. About one-quarter of the way into the pore is an anionic group, probably a carboxyl group, that is responsible both for binding the cationic toxins and for binding a variety of metal cations, including sodium. The pore is lined by six oxygen atoms, which hydrogen bond to the toxin molecule stabilizing it in position. According to this model, the sodium channel would function by first binding one of a variety of inorganic or organic cations to the same cationic binding site, each with a different affinity. The selectivity filter then presents another energy barrier, through which the cations pass with a rate determined by the energy requirement for each particular ion. Using this model and studying the permeability of a variety of organic and inorganic cations with voltage-clamp measurements, HILLE (1975a) was able to determine approximate dissociation constants for the cationic binding site and heights of energy barriers for the selectivity filter.

The recent determination of the structure of saxitoxin, first by SCHANTZ et al. (1975) and subsequently by BORDNER et al. (1975), has allowed HILLE (1975b)

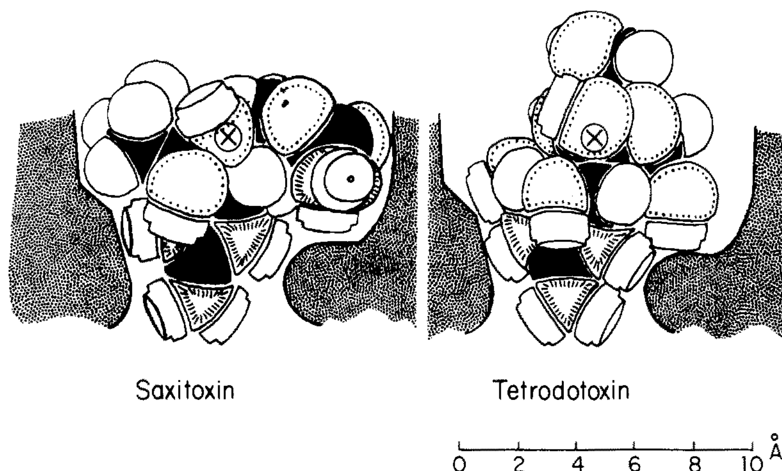

Fig. 2. Saxitoxin and tetrodotoxin on their receptors. The shading on the atoms of toxin represent: carbon, black; hydrogen, white; oxygens, dotted margins; nitrogen, dashed margins. The stippled areas represent the receptor in the sagittal section with the narrow selectivity filter below. Most of the receptor is hydrogen bond accepting, and there is a negative charge associated with the selectivity filter. A circled X has been drawn in the same position with respect to the receptor in two cases. The X falls on a hydroxyl group attached to an unusually electropositive carbon

to refine further the structure of the channels. According to this refinement, the 3 Å × 5 Å opening to the selectivity filter is approached through an antechamber of larger molecular dimensions, about 9 Å × 10 Å. This structure allows the guanidinium moiety of tetrodotoxin or the guanidinium moiety of saxitoxin around the 8-carbon atom to be inserted into the selectivity filter of the pore; in the case of saxitoxin, the second, planar guanidinium group around the 2-carbon remains resting against the pore (Fig. 2, taken from HILLE, 1975b). Such a model helps to explain why even minor changes in the structure of either saxitoxin or tetrodotoxin lead to almost complete loss of biological activity; for example, the low biological activity of alkyl, deoxy, and anhydro derivatives of tetrodotoxin are readily accounted for (HILLE, 1975b) by the loss of hydrogen bonds between modified toxin and sodium channels.

C. Structure and Activity Relationships in Saxitoxin and Tetrodotoxin Derivatives

1. The Effect of pH on Saxitoxin and Tetrodotoxin

Tetrodotoxin can exist in solution either as a zwitterion or as a cation in two forms, all three being in equilibrium with each other (WOODWARD, 1964). The pKa for the equilibrium between the cationic and zwitterionic forms is about 8.8. Similarly, the 2-nitrogen of saxitoxin will be in its cationic form at acidic values of pH. Studies on the effect of varying the pH of the bathing solution on the action of

both saxitoxin and tetrodotoxin have indicated that the cationic form is the one that seems to be important in producing nerve block (CAMOUGIS et al., 1967; OGURA and MORI, 1968; HILLE, 1968a; NARAHASHI et al., 1969).

2. Relatively Inactive Saxitoxin and Tetrodotoxin Derivatives

Most modifications of saxitoxin and tetrodotoxin, except those described in Section B. III. 3 below, are relatively inactive and so only a few examples will be mentioned, rather than discussing the toxin derivatives comprehensively. For instance, in the dihydro derivative of saxitoxin formed by reduction of saxitoxin with hydrogen at 1 atmosphere pressure over Adams platinum catalyst, the two hydroxyl groups at the 13-carbon atom are replaced by hydrogens: dihydrosaxitoxin is not toxic (MOLD et al., 1957). Furthermore, eight derivatives of tetrodotoxin, prepared by degradation of the parent toxin, have been studied both chemically (TSUDA et al., 1964) and pharmacologically (DEGUCHI, 1967; NARAHASHI et al., 1967; OGURA and MORI, 1968). The least active of these degradation products is tetrodonic acid. The most active, deoxytetrodotoxin, is one to five orders of magnitude less potent than tetrodotoxin. Even then, it is not clear how active these derivatives truly are, because although the purity of the crystalline samples of the compounds was verified by infrared spectra, NMR, and mass spectra, etc., the possibility of contamination with trace amounts of tetrodotoxin, which would not be detected by any chemical or physical means, could hardly be eliminated.

3. Pharmacological Active Derivatives of Saxitoxin and Tetrodotoxin

Until recently, all attempts to make structural alterations to either saxitoxin or tetrodotoxin that retained biological activity have been unsuccessful. Such active derivatives would seem to be essential to provide a functional organic group on the molecule that would allow preparation of radioactively or fluorescently labeled toxins. Despite the widespread use of both toxins to block sodium currents in excitable membrane, this lack of active derivatives has impeded progress in characterizing and isolating the sodium channel. For example, the activities of the tetrodotoxin derivatives referred to in Section II. C. 2. were so low that they might well have been due to traces of unreacted tetrodotoxin. Within the last 2 years, however, two extremely exciting findings have been made of active derivatives both of tetrodotoxin (TSIEN et al., 1975) and of saxitoxin (GHAZAROSSIAN et al., 1976). Furthermore, GUILLORY et al. (1977) have just reported the first covalent binding of a tetrodotoxin analogue to sodium channels using an aryl azido photoaffinity label.

TSIEN et al. (1975) have modified tetrodotoxin to give two derivatives, one of which retains considerable pharmacological activity. The first compound, which is virtually devoid of biological activity, is nortetrodotoxin in which the hydroxy and methoxy groups attached to the 6-carbon atom (Fig. 1) are replaced by a ketone group. Activity is, however, restored when the nortetrodotoxin is treated with methoxamine. The resultant compound, the methoxamine of nortetrodotoxin or a tetrahydryl intermediate, is about one-third as potent as tetrodotoxin

itself; such potency cannot be explained by contamination with unreacted tetrodotoxin because the product is formed through the intermediate nortetrodotoxin that contains the biological activity of not more than 0.5% tetrodotoxin. This finding of TSIEN et al. (1975), that an active methoxamine product can be formed from a relatively inactive compound suggests that the 11-end of the tetrodotoxin molecule (Fig. 1) has a significant role to play in the binding to the membrane receptor. Previous speculation had attached all the importance to the guanidinium moiety and to the cationic head at the 2-position. Other carbonyl group reactions based on nortetrodotoxin might enable a more detailed mapping of the structure activity of relation of this portion of the molecule. The six position would thus seem to be a reasonable starting point for attaching ligands irreversibly with retention of reasonable pharmacological activity.

GHAZAROSSIAN et al. (1976) have similarly been successful in their work with saxitoxin. They have now prepared and described the biological properties of an acid hydrolysis product of saxitoxin that retains biological activity. In this compound, the carbamyl group attached at the 17-oxygen of the saxitoxin molecule (Fig. 1) has been replaced by hydrogen, yielding decarbamyl saxitoxin. This has about the same order of potency as saxitoxin itself — which is perhaps not unexpected since the guanidinium moiety that is thought to insert into the selectivity filter is intact, as also are 12-carbon atom and its environment which remain available for the proposed nucleophilic attack by the receptor. The importance of this first biologically active hydrolysis product of saxitoxin, as with the active derivative of tetrodotoxin of TSIEN et al. (1975), is that it opens up the possibility of developing a radioimmunoassay for the toxins as well as affinity chromatographic and affinity labeling techniques for isolating the toxin receptor.

III. Electrophysiological Properties of Saxitoxin and Tetrodotoxin

It has been known for many years that both toxins exert powerful effects on the excitable membranes of nerve and muscle (for reviews see: KAO, 1966; EVANS, 1972; BLANKENSHIP, 1976). In the mid-1960's voltage-clamp experiments made it clear that these toxins selectively abolish the sodium currents of excitable membrane (squid giant axon, NAKAMURA et al., 1965a; MOORE et al., 1967a; lobster giant axon, NARAHASHI et al., 1964; TAKATA et al., 1966; electric eel electroplaque, NAKAMURA et al., 1965b; and the node of Ranvier myelinated nerve, HILLE, 1966, 1968a). Figure 3, which is taken from HILLE (1968a), illustrates the fundamental electrophysiological action of the two toxins. Figure 3 shows the voltage-clamp currents in a single myelinated nerve fiber. In the absence of toxin (left-hand record), two components are seen: an early downward deflection due to current in the sodium channels, and a later upward deflection due to current in the potassium channels. In the presence of saxitoxin, the early current is completely abolished while the late current remains unaffected (right-hand record). The effect is rapidly reversed on removing the toxin. This figure also illustrates another important point, namely that the action of the toxin depends on an action on the channel, and does not represent an interaction

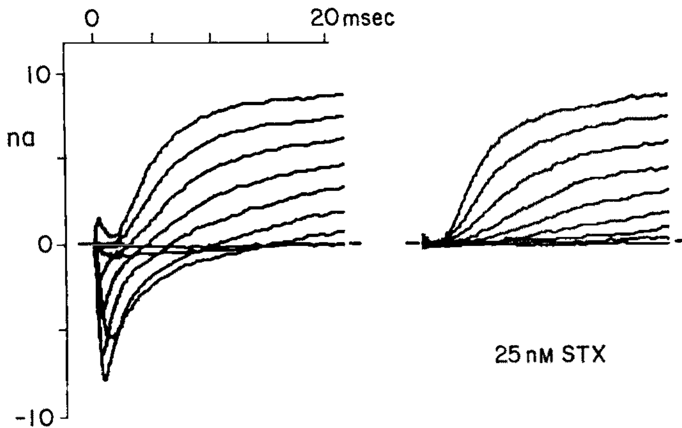

Fig. 3. The effect of saxitoxin on the ionic currents in a voltage-clamped single frog node of Ranvier. A downward deflection indicates an inward current. The records, which were drawn by computer, represent the voltage-clamped currents minus the leakage currents in sodium-free lithium Ringer solution before (left-hand records) and during (right-hand records) treatment with 25 nM saxitoxin. The temperature was 6.5° C. (Taken from HILLE, 1968)

specifically with the sodium ion; for in this experiment the sodium of the Ringer solution had been replaced by lithium. The lithium ion, since it is able to replace the sodium ion as far as the conduction of the spike is concerned, is, like sodium, unable to pass through the channel that has been blocked by the toxin. Other experiments (for references, see HILLE, 1970) again emphasize that it is the blockage of the channel itself that is involved, and neither the particular ion nor its direction of flow through the channel is important. For example, under the appropriate conditions, the sodium currents flowing during a voltage-clamp experiment may be entirely outward rather than inward as normally occurs during an action potential. Even these outward currents, however, are blocked by tetrodotoxin.

Since the action of the toxins is on the time- and voltage-dependent sodium channels, it is not surprising that the toxins have no marked effect on resting potential. For in normal resting preparations these channels are almost entirely closed, $\bar{g}_{Na}$ being close to zero at rest. However, the sodium channels are not *entirely* closed at rest. For example, HODGKIN and KATZ (1947) showed that the resting sodium permeability accounts for 2–4 mV of the resting potential, and FREEMAN (1971) has demonstrated that application of 300 nM tetrodotoxin to the bathing medium hyperpolarizes squid axons by 5 mV. These observations can be readily accounted for if, because of the presence of a drug like veratridine (see below) or for some other reason, some fraction of the channels remains open at rest. In that case, blocking the channels by saxitoxin or tetrodotoxin will lead to a hyperpolarization.

One of the more important findings in HILLE's (1968a) study of the two toxins is illustrated in Figure 4 which shows the dose-response curve of saxitoxin. As HILLE pointed out, this curve is quite consistent with the idea that it is the combination of a single toxin molecule with a single sodium channel that results in the blocking action. Had, for example, two toxin molecules been required

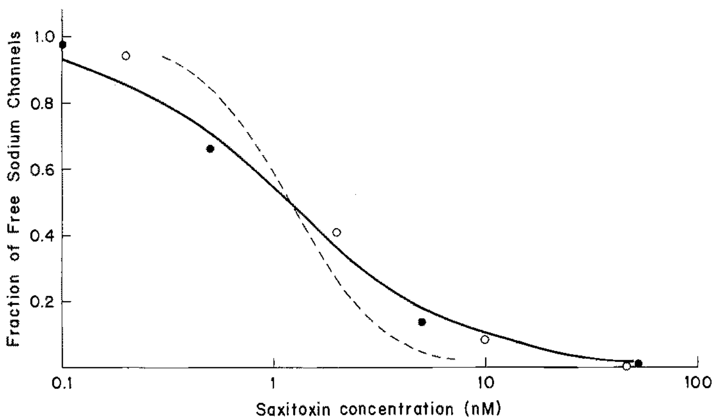

Fig. 4. The dose-response relationship for saxitoxin in frog single myelinated nodes of Ranvier. The ordinate is the maximum sodium conductance at various concentrations of saxitoxin relative to that in normal Ringer solution. The full circles are from experiments in which the potassium currents have been eliminated by 5 mM TEA. The solid line is the theoretical dose-response relationship of a system in which one saxitoxin molecule binds reversibly to its receptor to produce its effect; the broken line shows a similar theoretical relationship if two saxitoxin molecules are required

for each channel, the slope of the dose-response curve (interrupted line, Fig. 4) would have been much steeper at its midpoint than found by HILLE (1968a). Furthermore, in squid giant axons, CUERVO and ADELMAN (1970) showed that the dose-response curve for tetrodotoxin is again fitted quite well by a Langmuir-type dissociation curve.

Subsequent experiments with other tissues have in general confirmed this notion. For example, in systems of small nonmyelinated fibers voltage-clamping is difficult, if not impossible, and so it is difficult to determine how the sodium conductance, $\bar{g}_{Na}$, is progressively decreased by increasing concentrations of toxins. Yet it is important to estimate K_t electrophysiologically in these tissue since many of the chemical studies of toxin-channel interaction depend on the use of small diameter fibers because they contain a huge area of membrane surface. For this reason, COLQUHOUN and RITCHIE (1972a) used a model, based on arguments involving the independence principle and numerical solutions of the Hodgkin-Huxley equations, to show that the effects of various concentrations of tetrodotoxin on the curves relating the height of the action potential or the conduction velocity of the compound action potential to the logarithm of the external sodium concentration are consistent with the idea that one tetrodotoxin molecule binds to, and blocks, a single sodium channel.

All the electrophysiological experiments, therefore, are consistent with the idea that binding or uptake of toxin is Langmuir, hyperbolic, in character. The uptake, U_t, from a given bathing concentration of toxin, $[T]$, is thus given by:

$$U_t = M/(1 + K_t/[T]), \tag{1}$$

where M is a constant equal to the maximum binding capacity of the tissue and K_t is the equilibrium dissociation constant. The value of K_t for the various tissues,

i.e., the external concentration at which half the sites are saturated, is very small, being 0.1–10 nM.

Whether or not binding is Langmuir or whether more than one toxin molecule binds to a single channel are critical questions deserving future study. For all estimates of channel density made so far rely on the assumption of a one-to-one correspondence; and although the evidence for this is reasonable (HILLE, 1968a; CUERVO and ADELMAN, 1970; COLQUHOUN and RITCHIE, 1972a), it is certainly not absolute.

IV. Early Binding Studies

A. Unlabeled Toxin

The earliest attempt to determine the sodium channel density in nerve membrane was made by MOORE et al. (1967b). They used an elegant technique, first used about 50 years earlier to determine the density of glycoside binding sites in heart muscle (see CLARK, 1933). They estimated the amount of tetrodotoxin taken up by a nerve membrane at the time of block and hence determined the sodium channel density. Up to seven lobster nerve trunks were dipped successively in a small volume of artificial sea water containing 300 nM tetrodotoxin. Each nerve trunk bound some of the toxin in the solution, and because of cumulative uptake by the successive nerves dipped into it, the solution was eventually left with an insufficient concentration of toxin to block conduction. After allowing for dilution of the initial toxin-containing solution by the extracellular spaces of the lobster nerve trunks, MOORE et al. (1967b) argued that there were probably fewer than 13 sodium channels per μm^2 of axon in lobster nerve, assuming that each adsorbed tetrodotoxin molecule blocks no more than one sodium channel. Subsequently, using a similar bioassay procedure, KEYNES et al. (1971) confirmed the sparseness of tetrodotoxin binding sites (i.e., sodium channels) in the nonmyelinated nerves of lobster and also of crab and rabbit. For the walking leg nerves of lobster (a different species from that studied by MOORE et al., 1967b) KEYNES et al. (1971) found a channel density of $36/\mu m^2$. For crab it was $49/\mu m^2$ and for the rabbit vagus nerve $75/\mu m^2$. Later studies (see Sect. VI.A.1.) on the density of sodium channels in these nonmyelinated preparations using saxitoxin that had been highly specifically labeled suggests that the true density is higher than that obtained either by MOORE et al. (1967b) or by KEYNES et al. (1971).

Although more reliable numbers for the channel densities in these and other tissues are now available from the later work, these earlier studies and the techniques involved should not be ignored. For it is important to realize that although radioactive studies have largely replaced, because of convenience, the earlier studies based solely on bioassay, all such radioactive studies ultimately depend on bioassay for their reliability. In the absence of any unique chemical identification of either saxitoxin or tetrodotoxin, the radioactive experiments necessarily depend on a bioassay uptake experiment of the kind just described so that the amount

of toxin bound to the preparation can be related to the amount of radioactivity bound, hence yielding the specific radioactivity and radiochemical purity of the toxin.

B. Labeled Toxin

To be quite sure that the toxin taken up by the preparation is bound to the sodium channels and not to some other component requires that the character of such uptake be identical with that of the known electrophysiological interaction between the toxin and the sodium channel derived from voltage-clamp experiments. Thus, one should be able to demonstrate that the binding is Langmuir in type, i.e., the uptake is a hyperbolic function of the bathing concentration of toxin, and the concentration at which sites (M) become half saturated (K_t) should be of the order of a few nanomolar. The uptake, thus, is given by Equation 1. Such complete binding curves are difficult to construct in bioassay experiments of the type described above because each individual determination takes too long for more than a few points to be determined on a single preparation. Furthermore, the bioassay method is limited at both extremes of concentration. On the one hand, at low concentrations, it is difficult to determine the amount of toxin removed by the preparation because low concentrations of toxin (less than a few nanomolar) are extremely difficult to bioassay accurately. And on the other hand, at high concentrations, the bioassay again becomes inaccurate because the limited amount of toxin removed results in very little difference between the final and initial bathing concentrations (which is difficult to determine given the errors inherent in any bioassay). A major advance, therefore, was made when HAFEMANN (1972) showed that it was possible to use the WILZBACH method to label the toxins radioactively. Following this demonstration, COLQUHOUN et al. (1972) determined the complete binding curves of such a tritium-labeled tetrodotoxin to a variety of nonmyelinated nerve fibers (rabbit vagus, lobster walking leg, garfish olfactory nerves). Two components of uptake of labeled compound were always seen: (1) there was a linear nonspecific component of binding, and (2) there was a hyperbolic saturable component of binding that was half-saturated at concentrations of toxin of a few nanomolar, i.e., the same order as the corresponding value determined directly by voltage-clamp experiments in squid giant axons (CUERVO and ADELMAN, 1970) and in myelinated nerve fibers (HILLE, 1968a), and indirectly in small nonmyelinated nerve fibers (COLQUHOUN and RITCHIE, 1972a). Subsequent experiments (see Sect. VI.A.1.) have shown that the absolute sodium channel densities obtained in these initial experiments were too low — presumably because of undetected radioactive impurity in the preparation (see Sect. V.B.). Nevertheless, the fact that a component of the binding curve that saturated at concentrations of a few nanomolar was found strongly suggested that the component being studied was indeed the sodium channels. It should be noted that in subsequent experiments where the labeled toxin was used solely as an indicator of the interaction of various other drugs and cations with the sodium channel (as, for example, in HENDERSON et al., 1974), the fact that the preparation may have been impure does not affect the validity of the conclusions reached

that the toxins seem to act at a metal cation binding site in the sodium channel, nor does it affect the correctness of the calculations of the dissociation constants for the competing cations.

V. Methods of Labeling

A. The Wilzbach Method

In the Wilzbach method (WILZBACH, 1957) of labeling, the toxin is exposed to a highly radioactive tritium gaseous atmosphere for several weeks (HAFEMANN, 1972); or in the modified WILZBACH method (DORFMAN and WILZBACH, 1959), it is exposed for only a few hours but in the presence of an electric discharge (COLQUHOUN et al., 1972). The labeling produced is fairly nonspecific, and considerable breakdown of the irradiated toxin is likely, so that extensive subsequent purification is necessary. COLQUHOUN et al. (1972, 1975) and HENDERSON et al. (1973) pointed out the danger of either the WILZBACH or the modified Wilzbach method of labeling, namely, the possibility that the final preparation may contain radioactivity not just in the toxin but in some other closely related compound produced in the labeling process. These impurities may be difficult to separate by the standard biochemical procedures available.

B. Errors Due to Impurity, Their Assessment, and Their Elimination

The errors that may arise as the result of impurities may perhaps be best seen by considering a hypothetical example. Imagine, for example, that a binding curve is obtained for a milligram of nerve bathed in a variety of concentrations of a labeled, impure toxin solution. Suppose biochemical tests fail to detect any impurity and so the solution is taken to be radiochemically pure; and suppose, further, that bioassay and scintillation counting of this toxin solution show that the radioactivity of the bathing solution corresponds with 90 dpm/f-mol. The preparation shows a maximum saturable uptake of radioactivity of 9000 dpm/mg. If all the radioacitivity is truly associated with toxin, this would correspond with a toxin uptake (hence channel density) of 100 f-mol/mg wet tissue.

However, suppose that two-thirds of the radioactivity in the bathing solution is not in the toxin but in a closely related impurity not detected by biochemical tests (so that the true specific activity of the tetrodotoxin is only 30 dpm/f-mol and not the apparent 90 dpm/f-mol); an uptake of 6000 dpm/mg would response with a channel density of 300 f-mol/mg, i.e., three times the first estimate. Clearly, the first calculation would represent a drastic underestimation of binding sites. It is important to assess directly the fraction of the radioactivity actually associated with the toxin rather than relying on indirect biochemical means to assure the quantitative accuracy of binding experiments.

LEVINSON (1975) first pointed out the problem in assessing the purity of WILZBACH-labeled tetrodotoxin, even when it seems to be radiochemically pure

judged by its electrophoretic migration as a single radioactive peak at a variety of pH's, and by the appearance of a single distinct peak on thin layer chromatography (see also Colquhoun et al., 1972; Henderson et al., 1973). Greater sensitivity in bioassay technique allowed him to determine more directly the radiochemical purity of labeled tetrodotoxin by comparing the saturable uptake of toxin by an eel electroplaque preparation (determined by bioassay) with the corresponding saturable uptake of radioactivity. From the estimated value of the true specific activity so obtained, s_{est}, and the apparent specific activity, s_{app} (i.e., the *total* radioactivity in the preparation divided by the biological activity), the purity can be determined as s_{est}/s_{app}. The purity of his preparation determined in this way was in fact only about 0.30.

However, even the criteria used by Levinson (1975) for determining the true specific activity of labeled toxin may not be sufficiently stringent because the specific activity of tritiated tetrodotoxin estimated in this way is unreliable, particularly when it is not clear what (or how many) different labeled compounds might be in the solution and what their affinity for the nerve membrane might be. For example, in Figure 3 of Levinson (1975), only part of the "saturable" labeled component of binding is abolished by adding a large excess of unlabeled toxin.

It is difficult to treat analytically the most general case in which there are saturable and linear components of uptake of toxin (representing specific binding to sodium channels, nonspecific accumulation near charged membranes as in Section IX, etc.) together with saturable and linear components of uptake of impurity, these uptakes being associated with corresponding components of uptake of radioactivity. For the sake of simplicity, therefore, consider to begin with the case, perhaps unrealistic, of just two saturable components of uptake, one of toxin and the other of impurity (the latter could also represent linear binding of impurity if N and K_i, defined below, are very large). Imagine, for example, a solution containing a concentration a_t of toxin and a concentration a_i of an impurity. The specific activities of these two components are s_t and s_i, respectively. Assume that this has already been brought to equilibrium with a negligible amount of tissue that contains a single homogeneous population of M toxin binding sites (equilibrium dissociation constant K_t) and a single homogeneous population of N impurity binding sites (equilibrium dissociation constant K_i). Then uptakes of toxin and impurity (U_t and U_i, respectively) are given by:

$$U_t = M/(1+K_t/a_t) \tag{2}$$

$$U_i = N/(1+K_i/a_i) \tag{3}$$

The radioactivity associated with these two uptakes (T* and I*, respectively) are thus given by:

$$R^* = T^* + I^*. = U_t \cdot s_t + U_i \cdot s_i \tag{4}$$

By the method of estimation of purity used by Levinson (1975), the estimated true specific activity of the toxin (s_{est}) would be given by the ratio of the saturable components of radioactivity and of biological activity disappearing from the solution when equilibrated with the preparation, i.e., R^*/U_t. From the above equations, therefore:

$$s_{est} = s_t\left(1+\frac{U_i \cdot s_i}{U_t \cdot s_t}\right) = s_t\left(1+\frac{N}{M}\cdot\frac{s_i}{s_t}\cdot\frac{(1+K_t/a_t)}{(1+K_i/a_i)}\right) = s_t\,(1+g) \tag{5}$$

The second term, g, (which incidentally would be a constant if $K_t/K_i = a_t/a_i$) can be neglected only if the binding capacity N for the impurity is zero, if the specific activity of the impurity s_i is zero, or if the experiment is carried out at relatively low toxin concentrations and the ratio of binding

constants K_i/K_t is very much greater than the ratio $N \cdot a_i \cdot s_i/a_t \cdot s_t \cdot M$. No information is available on any of these terms. Thus, it would be dangerous (and unwarranted) to assume as correct even the most likely possibility (i.e., the third possibility) If g is not zero at all concentrations of toxin, the estimates of the true specific activity s_{est} will be higher than the correct value s_t. At low concentrations of toxin, the true specific activity of the toxin will be estimated by the experimental procedure as:

$$s_{est} = s_t\,(1 + N \cdot K_t \cdot a_i \cdot s_i/M \cdot K_i \cdot a_t \cdot s_t) \tag{6}$$

At high concentrations it will be estimated as:

$$s_{est} = s_t\,(1 + N \cdot s_i/M \cdot s_t) \tag{7}$$

In both cases, estimates of the purity based on s_{est}/s_{app} will be erroneously high.

One way of avoiding this danger of overestimation of the purity would be to relate the loss of biological activity from the bathing medium only to that fraction of the radioactivity that disappears on initial equilibration from a test solution but which *re*appears on adding excess cold tetrodotoxin to the medium. This procedure could prevent the overestimation of true specific activity that results from the fact that some of the radioactivity that initially disappears represents uptake of the radioactive impurity, and it could yield the correct value for s_t.

However, the above calculation would be vitiated if biological activity (and its associated radioactivity) were taken up not just into a saturable component, as assumed above, but also into a nonsaturable linear component. The radioactivity associated with this linear uptake of true toxin would not be displaced by addition of the excess of cold toxin, and the value of s_{est} determined from the ratio of the reappearing radioactivity and disappearing biological activity would be erroneously low. A linear component of radioactive uptake is indeed found in all labeled binding experiments (HAFEMANN, 1972; COLQUHOUN et al., 1972; HENDERSON et al., 1973; ALMERS and LEVINSON, 1975; RITCHIE et al., 1976). LEVINSON (1975) has suggested that this linear component represents binding of impurity rather than true toxin. However, both theory and experiment (COLQUHOUN et al., 1975; RITCHIE and ROGART, unpublished observations) show that under appropriate circumstances the linear component of binding can represent almost exclusively nonspecific uptake of true toxin and not of impurity. It would seem, therefore, that although one might avoid the danger of overestimating the purity, one might be doing so only at the expense of underestimating it.

In the light of the above discussion, it would seem that the best way of determining the purity of a labeled toxin is to do so at low concentrations where the amount of nonspecific uptake is low relative to the specific uptake. Furthermore, the determination should be made in a preparation where the coefficient b for the linear component of uptake is small. In lobster nerve, for example, virtually all the radioactivity taken up from low toxin concentrations can be shown experimentally to be displaceable by tetrodotoxin. The purity of the toxin determined in lobster nerve by simultaneous determination of the uptake of biological activity by bioassay and of the associated radioactivity can thus be reliably used to convert radioactive uptake to toxin uptake in the other preparations.

Although the Wilzbach method is very useful, it *must* be accompanied by detailed and painstaking purification and even then reservations and ambiguities remain. Even the fact that the estimate made may in the end be correct is no justification for an improper procedure. Clearly, the ultimate answer to possible criticisms of the results obtained by various authors would be to develop a simple clean method of labeling that anyone can use without difficulty. The method recently used by RITCHIE et al. (1976) and described in Section V.C is a step in this direction.

C. Specific Tritium Exchange Method

The method utilizes exchange of tritium from carrier-free tritiated water to particular methylene hydrogens on the saxitoxin molecule (those attached to the

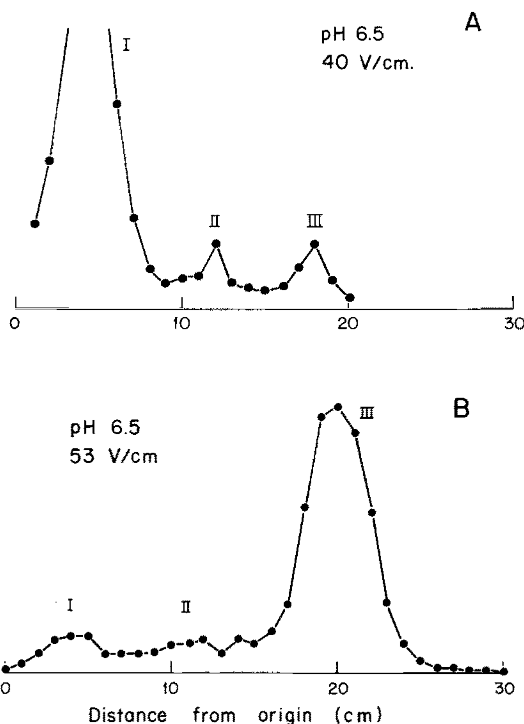

Fig. 5. Radioactivity profiles from high-voltage paper electrophoresis of tritiated saxitoxin. Profile A: saxitoxin labeled by modified Wilzbach method. Peak I contains about 95% of the radioactivity, but all the biological activity is located under Peak III. Profile B: exchange labeled saxitoxin. Peak III contains 75% of the radioactivity and all the biological activity. The material was dissolved in 0.1 M sodium acetate, pH 6.0, 0–4° C, and then put directly on the electrophoresis paper. The positions of the corresponding peaks for the two profiles are not identical because slightly different physical conditions were used for the two electrophoresis runs. (Taken from RITCHIE et al., 1976)

12-carbon in Fig. 1) that are susceptible to exchange with solvent hydrogen. The resultant labeled compound (which has to be stored frozen and used experimentally at low temperature, about 4° C) is much more highly specifically labeled than by the Wilzbach method; on the average each saxitoxin molecule contains one tritium atom (theoretical maximum, 2) — a 300-fold improvement over the Wilzbach method. What is much more important is that the radiochemical purity is much greater, being about 70–80%. Much of the reason for the greater purity lies in the specificity of the labeling. As Figure 5 shows, most of the label in the crude toxin preparation labeled by the Wilzbach method is not associated with toxin. In contrast, in preparations labeled by the new method, nearly all of the radioactivity is in the saxitoxin and relatively little further purification is necessary. Thus, all the biological activity is in peak III in both electrophoresis profiles in Figure 5. However, only 5% of the radioactivity appears here in Wilzbach-labeled toxin, whereas 75% of it appears in the toxin labeled by the new method.

VI. Binding of Toxin to Nerve Membranes

A. Intact Nerve

1. Small Nonmyelinated Nerve Fibers

In their original experiments with unlabeled tetrodotoxin, MOORE et al. (1967) demonstrated the extreme sparsity of sodium channels in the nerve membrane, concluding that there were less than $13/\mu m^2$ in lobster nonmyelinated axons. Shortly afterward, KEYNES et al. (1971) confirmed that the sodium sites were very thinly distributed. The early experiments with radioactively labeled tetrodotoxin (HAFEMANN, 1972; COLQUHOUN et al., 1972; HENDERSON et al., 1973) also confirmed the scanty distribution of sodium channels, although for reasons discussed earlier, the lack of exact knowledge on the purity of the toxins gave too low a value for their number.

Use of purer, more specifically labeled preparations of saxitoxin (Sect. V.C.) gives more reliable estimates for the sodium channel density in these various preparations of small nonmyelinated fibers. Figure 6 shows that the uptake of labeled saxitoxin by the nonmyelinated fibers for the rabbit vagus nerve (●) is clearly the sum of two components: a saturable, hyperbolic component of binding and a linear component representing nonspecific uptake. Only this latter component remains in the presence of an added excess of unlabeled toxin (○). All the saturable component, therefore, is sensitive to displacement by tetrodotoxin

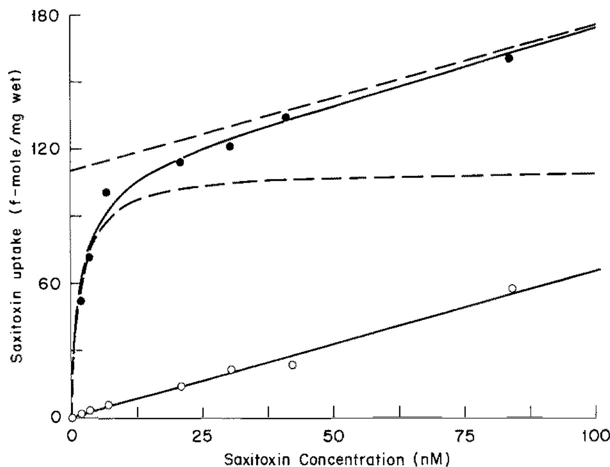

Fig. 6. The uptake of labeled STX by the nonmyelinated fibers of rabbit desheathed vagus nerve at different external concentrations of toxin. The nerves were equilibrated for 8 h at 2–4° C with the STX. Each point is the mean of determination of five different nerves. The interrupted line is the asymptote of the binding curve. The total binding curve is the relation:

$$U = \text{STX bound} = 0.65\,[\text{STX}] + 110\,[\text{STX}]/(1.8 + [\text{STX}]),$$

where U is given in f-mol/mg and [STX] is given in nM. The linear and saturable components are drawn separately. The binding curves shown are least squares fits to the points; ●, in the presence of varied concentrations of labeled STX; ○, in the presence of varied concentrations of labeled STX and 10 μM unlabeled TTX.

Table 1

Preparation	Average fiber diameter (μm)	Membrane surface per unit area (cm^2/mg)	Equilibrium dissociation constant (nM)	Maximum binding capacity (f-mol/mg)	Sites per unit area (μm^{-2})	Reference
1. Nonmyelinated nerve						
Rabbit vagus	0.7	6.0	1.8	100	100	RITCHIE et al. (1976)
Lobster walking leg	—	7.0	8.5	94	90	RITCHIE et al. (1976)
Garfish olfactory	0.24	65	9.8	377	35	RITCHIE et al. (1976)
Squid giant axon						
Loligo forbest		0.1	—	—	553	LEVINSON and MEVES (1975)
Loligo pealii	454	0.1	3.8	—	170	RITCHIE et al. (unpublished)
2. Myelinated nerve						
Rabbit sciatic	8	8[a]	1.3	17.1[b]	12,000	RITCHIE and ROGART (1977a)
3. Muscle, sartorius						
Rana temporaria	80	0.35	5	22	380	ALMERS and LEVINSON (1975)
Rana pipiens	55					JAIMOVICH et al. (1976)
Rana pipiens, (Southern)	58	0.45	4.7	25.3	341	RITCHIE and ROGART (1977b)
Rana pipiens, (Northern)	61	0.45	2.7	13.8	195	RITCHIE and ROGART (1977b)
Rana temporaria (detubulated)	—	—	3	16	—	JAIMOVICH et al. (1976)
4. Muscle, diaphragm						
rat	31	0.70	3.8	24.5	206	RITCHIE and ROGART (1977b)
Rat denervated	—	—	4.1	16.2	—	RITCHIE and ROGART (1977b)

[a] Estimated nodal area.
[b] Corrected for estimated binding by nonmyelinated fibers.

and so truly represents binding to sodium channels. The linear component obtained with the new purer saxitoxin is about the same (relative to the saturable component) as that obtained previously with less pure, Wilzbach-labeled toxin (see Section VI.A.4).

Table 1 summarizes the results with the various preparations of small nonmyelinated fibers. For garfish nerve, the sodium channel density is $35/\mu m^2$; for lobster and rabbit nerve, it is 90 and $110/\mu m^2$, respectively.

2. Squid Giant Axon

The earliest experiments were done on the small nonmyelinated fibers of the garfish (average diameter 0.24 μm) and rabbit (average diameter 0.7 μm). These fibers were chosen because they contain a relatively higher proportion of nerve membrane than do large fibers; for example, garfish olfactory nerve contains 65,000 cm^2/g nerve (EASTON, 1971) compared with only 80 cm^2/g for a squid giant axon that is 500 μm in diameter. A preparation with a large surface area is necessary because of the low density of channels and the relatively low specific radioactivity of the toxin.

There is relatively little membrane per unit weight of squid giant axon, so direct measurement of binding capacity seemed difficult. However, an analysis of the time course of tetrodotoxin action on squid giant axons suggested that the number of binding sites might be considerably higher than in other nerves, being $300–600/\mu m^2$ (KEYNES et al., 1973; KEYNES et al., 1974). Indeed, LEVINSON and MEVES (1975) were able to determine directly the binding of labeled tetrodotoxin to giant axons of the squid *Loligo forbesi*. The data they obtained were somewhat scattered. Nevertheless, it seems clear that in addition to a large nonspecific linear component, there is indeed a saturable component of binding to squid giant axons. Although it was not possible to measure the hyperbolic component of saturable binding, they were able to estimate roughly the maximum saturable uptake by measuring the intercept of the linear component determined at "high concentrations" of toxin, i.e., approximating the asymptote to the total binding curve. From this estimate of maximum saturable binding capacity and of the diameters of the axons used, they estimated that the site density was about $550/\mu m^2$, which is substantially higher than the site densities found in the small nonmyelinated fibers previously studied. This estimate is consistent with the estimate of sodium channels in the squid giant axon based on the maximum charge movement during the asymmetric displacement gating currents (KEYNES and ROJAS, 1974). The larger number may represent an adaptation for high nervous conduction velocity (HODGKIN, 1975).

More recently RITCHIE, ROGART and STRICHARTZ (unpublished observation) have examined the binding of labeled saxitoxin to giant axons of the squid *Loligo pealii*. Unfortunately, relatively few points (41) were determined so there is a good deal of statistical uncertainty on the precise values, particularly of the value of 3.8 nM for K_T. The maximum saturable binding capacity was, however, fairly well determined, being 170 ± 20 (SE) binding sites per μm^2 axonal membrane. This number is lower than that found by LEVINSON and MEVES (1975). But whether this represents a real difference in sodium channel density between the

axonal membrane of the two species of squid cannot be determined at the moment, given the statistical uncertainty of the observations.

The absolute value of the saturable binding component in squid axons is of critical importance for comparison with the results of gating current and noise experiments. It is unfortunate, therefore, that the degree of confidence that can be placed in the estimates of MEVES and LEVINSON (1975) for *Loligo forbesi* or in the corresponding numbers obtained for *Loligo pealii* is rather small. Further study of these preparations will clearly be rewarding.

3. Myelinated Fibers

Myelinated nerve conducts by transmission of electric excitation from node to node through local electric circuits. This "saltatory" mode of conduction results from a discontinuity in the excitability properties of the axon; excitable regions (nodes) alternate with nonexcitable passive core conductors (myelinated internodes). Although definitive proof of saltatory conduction has been long available (TASAKI and TAKEUCHI, 1941, 1942; HUXLEY and STÄMPFLI, 1949), it has remained unlcear whether the inexcitability of the internode is primarily a result of its being insulated by the myelin sheath or whether it reflects an inherent inexcitability of the internodal axonal membrane itself. Essentially, the critical question is whether or not the membrane in myelinated nerve is inhomogeneous in the sense that the sodium channels necessary for nerve excitation are present only at the nodes of Ranvier and not in the internodal membrane. The answer to this question, apart from its intrinsic interest, is crucial for an understanding of the pathophysiology of demyelinating diseases such as multiple sclerosis.

COLQUHOUN et al. (1972) and BENZER and RAFTERY (1972) examined the binding of labeled tetrodotoxin to the trigeminal nerve of the garfish, which consists solely of myelinated fibers. They were unable to detect with certainty any saturable binding component that might represent the specific binding at the nodes of Ranvier. More recently, STRONG (1974) was also unable to detect any measurable binding of labeled tetrodotoxin to homogenized frog myelinated fibers, and this finding has been subsequently confirmed by RITCHIE and ROGART (1977a).

However, it is now clear that myelinated nerve fibers of the rabbit do indeed contain a large saturable component of binding, which is inhibited by large concentrations of unlabeled toxin (RITCHIE and ROGART, 1977a). In both intact and homogenized rabbit sciatic nerve, the value of M, the maximum saturable binding capacity, is 19.9 f-mol · mg wet^{-1}. The values of K_t in the intact and homogenized nerves are 3.4 and 1.3 nM, respectively.

Since the sciatic nerve contains nonmyelinated as well as myelinated fibers, a critical question in these mammalian experiments concerns the extent to which the saturable component represents binding to the myelinated rather than just to nonmyelinated fibers. Fortunately, YATES et al. (1976) have recently completed an extensive study of the fiber distribution of the sciatic nerve of the 20-week-old rabbit (used in the binding experiments). From their data, RITCHIE and ROGART (1977a) calculated that less than 14% of the saturable component could be attributed to binding to nonmyelinated fibers.

Having thus settled that the bulk of the binding was in fact to the myelinated

fibers, RITCHIE and ROGART (1977a) examined where on the myelinated fiber the saxitoxin binds. Since the internodal membrane is sealed from the extracellular compartment by the extremely close paranodal contact made by the myelin sheath, the saxitoxin could have had access only to the axonal membrane at the nodes of Ranvier. That being so, the nodal density of sodium channels can be estimated on the basis of assumptions similar to those of RUSHTON (1951) concerning the relationship betwen the size of the node and the amount of nodal membrane. On this basis, there is an extremely high density of sodium channels at the mammalian node, about $12{,}000/\mu m^2$. By contrast, the internodal membrane has practically none (less than $25/\mu m^2$).

The high density of sodium channels at the node revealed by binding studies (RITCHIE and ROGART, 1977a) agrees excellently with the results of electrophysiological experiments. For example, in squid axon, where there are about 580 binding sites/μm^2 (LEVINSON and MEVES, 1975) the peak inward current density is about 1 mA/cm^2 (HODGKIN and HUXLEY, 1952); in the node of Ranvier, however, the peak current is much greater, being about 100 mA/cm^2 (NONNER et al., 1975a.b) Furthermore, independent estimates for channel density in frog nodes of Ranvier from noise measurements (CONTI et al., 1976a.b) and from gating current measurements (NONNER et al., 1975a.b) give values of $2000/\mu m^2$ and $5000/\mu m^2$, respectively.

The value of 12,000 sodium channels per μm^2 for the mammalian fiber represents an extremely close packing — about one channel for every 8000 Å^2 of membrane compared with one sodium channel every 10^6Å^2 in the rabbit vagus nerve. If the channel is associated with a spherical protein 80 Å in diameter, with a maximum cross-sectional area of 5000 Å^2 (LEVINSON and ELLORY, 1973) there is clearly relatively little room on the nodal membrane for its other components such as sodium pumps. However, it should be emphasized that these estimates of channel density are average values that depend on a large number of assumptions that are difficult to test at the moment. Nevertheless, in spite of the inherent uncertainties, it is clear that the channel density at the node is extremely high.

From the fact that homogenization did not increase the binding capacity for saxitoxin, RITCHIE and ROGART (1977a) concluded that there were relatively few sodium channels, if any, on the internodal axonal membrane under the myelin. There would thus seem to be, as QUICK and WAXMAN (1976) have recently speculated, structural differences between nodal and internodal axonal membrane that may have special relevance in the context of demyelinating disease. The fact that nerve fibers that have been demyelinated, by experiment or by disease, fail to conduct impulses may simply result from an electrophysiological mismatch of the myelinated and demyelinated regions. For example, the safety factor might be too low to allow the last intact node to excite the demyelinated region beyond. The binding experiments, however, favor a different and sufficient explanation, namely that there are just not enough sodium channels in the axonal membrane under the myelin to support conduction so that the internodal membrane is inexcitable electrically. Such a scheme would account for the block of conduction in demyelinated nerve; it also suggests a mechanism for the return of function that is commonly observed clinically in demyelinating disease (see ROGART and RITCHIE, 1977b). Multiple sclerosis, for example, is a primary

demyelinating disease of the central nervous system in which myelin is destroyed while the axon remains relatively intact. There is, however, a poor correlation of the signs and symptoms of the disease with the anatomical presence of plaques containing demyelination (see ROGART and RITCHIE, 1977a, 1977b). Function may recover in nerve tracts in which the myelin loss is sufficiently gross to leave lengths of 3–4 mm of bare axon exposed. The suggestion was made (RITCHIE and ROGART, 1977a) that the explanation might be that sufficient sodium channels may in time spread into the internodal region after demyelination to support electric conduction. The postulated spread of channels in the internodal membrane would be analogous to the spread of acetylcholine receptors over the muscle membrane from the endplate region that occurs after denervation.

4. The Linear Component of Binding

A consistent feature of all labeled experiments is the existence of a linear nonsaturable component of binding. Does this represent uptake of true toxin, of impurity, or of both? In experiments on the binding of tetrodotoxin to muscle, ALMERS and LEVINSON (1975) and JAIMOVICH et al. (1976) have suggested that this component is due to uptake of impurity since in bioassay experiments, no linear component of toxin uptake was detected. However, this explanation can apply, at best, only partially to the experiments with the exchange-labeled saxitoxin; for in homogenates of rabbit brain, RITCHIE and ROGART (unpublished experiments) have shown that the linear component of uptake of exchange-labeled saxitoxin largely represents true biological activity and not the uptake of impurity.

The extent to which the linear component of the previously used labeled tetrodotoxin represents uptake of true toxin or of impurity remains unclear. COLQUHOUN et al. (1972) and HENDERSON et al. (1973) were originally satisfied with the densities of sodium channels they obtained using Wilzbach-labeled toxin because their values for the maximal saturable uptake of labeled toxin agreed so well with the total uptake of unlabeled toxin obtained by KEYNES et al. (1971) for rabbit vagus nerve, and by MOORE et al. (1967b) for lobster nerve. Since it is now clear that these densities were originally underestimated, this agreement must have been fortuitous. If in the case of labeled tetrodotoxin the linear component of radioactive uptake is indeed due to uptake of impurity, one should expect the *total* uptake of unlabeled toxin to agree with the *specific* (and not *total*) uptake of labeled toxin. In fact, the unlabeled *total* uptake found by KEYNES et al. (1971) for rabbit vagus nerve is about three times the *specific* uptake obtained in the experiments of COLQUHOUN et al. (1972). This finding agrees reasonably well with the *specific* uptake in Figure 6.

The presently available evidence seems to suggest that the linear component can reflect uptake either of an impurity or of toxin itself, depending, for example, on the toxin preparation being used. Linear uptake could result from its uptake (rather than its binding) into some intracellular compartment; for example, tritiated water in the case of exchange-labeled saxitoxin would distribute in the intracellular space. It could also result from the fact that any fixed negative charges in the membrane will lead to higher concentrations of cation

(the sodium of the bathing medium, the toxins, or charged impurities) in the few angstroms just outside the membrane compared with those in the bathing solution (see Sect. IX). With the large membrane surface area of the biological preparations used in the binding studies, this effect is not neglibible, particularly in the case of Wilzbach-labeled toxin where about 70% of the preparation is impure (LEVINSON, 1975) and charged.

B. Homogenized Nerve

Binding experiments with intact nerve preparations suffer from the disadvantage that passive diffusion of a drug into it may be extremely slow, especially when the external concentration is small (see, for example. COLQUHOUN and RITCHIE, 1972b). Furthermore, biological variation in preparations often leads to a great deal of uncertainty. These particular difficulties can be overcome by using homogenized preparations (HAFEMANN, 1972; BENZER and RAFTERY, 1972; HAFEMANN and UNSWORTH, 1973; BARNOLA et al., 1973; CHACKO et al., 1974; BALERNA et al., 1975; REED and RAFTERY, 1976; RITCHIE and ROGART, 1977a) but other difficulties remain. Firstly, as with intact nerve, homogenization experiments cannot be as selective as the electrophysiological experiments. They use many or all components of a particular tissue, whereas the electrophysiological experiments can select only part of it. For example, studies have been made on homogenates of nerve trunks, or of nerve bundles mostly or entirely composed of large numbers of small fibers from various animals (crayfish, lobster, and squid). However, the best corresponding electrophysiological data have been obtained from *single* giant axons of the same animals (crayfish, SHRAGER, 1974; lobster, JULIAN et al., 1962; and squid, HODGKIN and HUXLEY, 1952). The electrophysiology of these giant axons may well differ from that of the very small fibers which usually predominate in the homogenization experiments and for which, unfortunately, quantitative electrophysiology is largely lacking. Secondly, there are other technical problems peculiar only to homogenized preparations. For example, in sucrose density gradient differential centrifugation, one cannot be absolutely sure what particular components of membrane are selected in any given fraction. In addition, vesicle formation of the homogenate may seal off important sites from contact with the bathing medium. In the following paragraphs, binding studies to homogenized tissue will be examined with three related questions in mind. Firstly, how successful have the procedures used been at isolating relatively pure axolemmal preparations? Secondly, are there sodium channels in nonaxonal membranes such as Schwann cells, or can saxitoxin and tetrodotoxin be considered as true markers for membrane excitability? Thirdly, how do studies on homogenized tissue compare with those on intact nerve?

One advantage of homogenization is that it can be used along with differential centrifugation to yield well-characterized membrane fragments, and, it is hoped, even homogeneous axolemmal preparations (CHACKO et al., 1974; BARNOLA et al., 1973; BALERNA et al., 1975). For example, BARNOLA et al. (1973), studying the binding of tritiated tetrodotoxin to different fractions of homogenized lobster walking leg nerve, found an uptake of tetrodotoxin of 2.1

p-mol/mg protein for a pellet of the nerve homogenate preparation, 5.1 p-mol/mg protein for the total nerve plasma membrane, 9.5 p-mol/mg protein for a plasma membrane fraction I (which they believe to correspond with axolemma), and 3.4 p-mol/mg protein for fraction II (which they believe to correspond with plasma membrane of periaxonal cells, primarily Schwann cells, but see below). Similar results were obtained for homogenized garfish olfactory nerve by CHACKO et al. (1974) (namely, 3.7 p-mol/mg protein for fraction I and 1.6 p-mol/mg protein for fraction II) and for homogenized crab nerve by BALERNA et al (1975) (12 p-mol/mg protein and 13.2 p-mol/mg protein, respectively, for two axonal membrane fractions). Their fraction I contained 0.3% of the protein from the original membrane and 4.5% of the terodotoxin binding sites; their fraction II contained 1.3% of the total protein and 22.4% of the total tetrodotoxin binding sites. Uptake by their original homogenate was 0.9 p-mol/mg protein. It is interesting that the sodium-potassium-dependent ATPase content of the tissue in the experiments of BALERNA et al. (1975) paralleled the tetrodotoxin binding activity at the various stages of the purification procedure. This fact, together with the finding that other markers not specifically localized to axonal membrane (such as membrane bound acetylcholine esterase and other subcellular markers) decreased during the course of purification, is probably good evidence for a true purification of plasma membrane, either axolemma or Schwannlemma or both.

REED and RAFTERY (1976) have carried out similar experiments on plasma membrane isolated from *Electrophorus electricus* (see Sect. VIII.D).

The most intriguing question raised so far in the homogenization experiments concerns the specificity of the action of saxitoxin and tetrodotoxin. BARNOLA et al. (1973) believe that their fraction II is derived mainly from plasma membranes of periaxonal cells, such as Schwann cells. Yet, fraction II binds tetrodotoxin. Furthermore, VILLEGAS et al. (1976), studying electrophysiologically the Schwann cells of the giant axon of the squid, *Sepioteuthis sepioidea*, identified "sodium pathways" that are opened by veratrine and by batrachotoxin and which can then subsequently be blocked by tetrodotoxin; these channels are electricly inexcitable and show no voltage-dependent conductance. Putting these two observations together, VILLEGAS et al. (1976) propose that saxitoxin and tetrodotoxin also bind to sites that are not the voltage-dependent sodium channels that underlie the generation of the action potential.

Resolution of this question is clearly critical for the ultimate understanding of the membranal protein and phospholipid components involved in the control of ionic movement. The proposal of VILLEGAS et al. (1976) relies heavily on the ability of the differential centrifugation techniques to yield a preparation of periaxonal plasma membranes, uncontaminated by axolemmal membrane. However, the question of whether the purification is selective, preferring either axolemmal or Schwann cell membrane, remains open. Their finding that fraction II binds tetrodotoxin could indeed mean that periaxonal cells bind the toxin — and this is their interpretation. Alternatively, it could be taken as good evidence that fraction II contains a considerable amount of axolemmal membrane. Since the suggestion they put forward is quite new, and extremely important if true, it would seem that the burden of proof is on VILLEGAS et al. (1976) to show that the preparations of fraction II are indeed free of axolemmal contaminants.

Clearly, it would be interesting to compare the density of sodium channels per unit area obtained on homogenized tissue with the corresponding values in intact tissue. For if the estimated density of sodium channels is the same both in the intact nerve and in the purified axolemmal fraction of the homogenized nerve, it would imply that all of the sodium channels are located on the axolemma. Unfortunately, it is often difficult to do this because the amount of protein per unit weight of original tissue — which is required for the comparison — is not always clearly stated. And without this value, no accurate comparison can be made between the intact and homogenized preparations because of problems of toxin impurity posed above. BARNOLA et al.(1973) and CHACKO et al. (1974) did, however, calculate the surface area per unit weight of protein from electron-micrographs, and they obtained values for the sodium channel density of $36/\mu m^2$ and $6/\mu m^2$ for lobster walking leg nerve and garfish olfactory nerve, respectively. These values are a good deal lower than the parent intact tissue (Table 1). Furthermore, CHACKO et al (1974) give values for the protein content of their fraction I and the yield of fraction I from the original intact tissue, from which an uptake of toxin by fraction I of 25 p-mol/mg wet can be calculated for garfish olfactory nerve, which again is quite low (compared with the 377 p-mol/mg wet of Table 1). One explanation for the low estimates is that the toxin in these studies was quite impure and so the specific uptake was much underestimated (see Sect. V.B.).

C. Solubilized Nerve

One of the most exciting findings of HENDERSON and WANG (1972), subsequently confirmed by BENZER and RAFTERY (1973), was that it is possible to solubilize the protein that binds tetrodotoxin by using the detergent Triton-X 100. Such solubilized preparations from garfish olfactory nerve fibers seem to retain the full binding properties of the original nerves.

The ultimate aim of such experiments is to isolate the sites of toxin binding, thus making the sodium channel, or at least this part of the sodium channel, available for study by the wide variety of physical and biochemical techniques for protein analysis. HENDERSON and WANG (1972) estimated that the apparent molecular weight of this tetrodotoxin binding component was 500,000 or more using the gel filtration technique. This rather high molecular weight for a membrane protein was confirmed by BENZER and RAFTERY (1973) also using the gel filtration technique and by LEVINSON and ELLORY (1973) using an alternative approach, namely, the estimation of the molecular size of the binding site by radiation inactivation. These latter authors calculated a molecular weight for the binding site of about 230,000. This corresponds with a spherical protein about 80 Å in diameter, which is certainly large enough to extend right across the nerve membrane. The molecular size obtained by the gel filtration techniques, although of the same order, is larger than that obtained using the irradiation inactivation method. This may well be caused, at least in part, either by the detergent used in the gel filtration method being bound to the protein or to the aggregation of protein during the solubilization procedure. It would be interesting to use the irradiation inactivation method to study not just the protein in

homogenized preparation as LEVINSON and ELLORY (1973) have already done, but to repeat these experiments during the solubilization of membrane proteins. Such studies might well give evidence for the existence of some of these proteins *in situ* as polymers.

The procedure developed by HENDERSON and WANG (1972) for solubilizing the sodium channel also solubilizes a variety of other proteins. The best material so far obtained after gel filtration is at most 1% pure, representing about a 20-fold purification relative to the total membrane protein. The solubilization and gel filtration procedures are, at best, nonspecific, and could not be expected to select for the sodium channel in preference to other membrane proteins of similar size. The successes with purification of other membrane proteins, for example, the acetylcholine receptor (for review see RANG, 1975) and sodium-potassium ATPase pump (for review, see SCHWARTZ et al., 1975) have depended on use of an active compound with high affinity for the membrane receptor attached by a ligand to a column. Unfortunately, such a column-bound ligand does not yet exist for the sodium channel, and all subsequent attempts to purify the material further than HENDERSON and WANG (1972) have led to loss of tetrodotoxin binding activity.

VII. Binding to Muscle Membrane

A. Frog Muscle

1. Normal Muscle

Mainly because preparations with a large amount of membrane per unit weight of tissue seemed desirable, the earliest investigations of saxitoxin and tetrodotoxin binding were conducted on preparations of small nonmyelinated nerve fibers, where electrical information is difficult, perhaps impossible, to obtain. Muscle, being composed of much larger diameter fibers, has a comparatively small amount of membrane per unit weight; for example, frog sartorius muscle has a surface membrane area of about 450 cm^2/g, which is one to two orders of magnitude less than in nonmyelinated nerves. This is unfortunate because most of our knowledge about sodium channels has come from electrical studies with the voltage-clamp techniques. A substantial advance was, therefore, made by ALMERS and LEVINSON (1975) when they determined the extent of tetrodotoxin binding to frog muscle. By combining their results with the electrical data of ADRIAN et al. (1970) and of ILDEFONSE and ROY (1972), they could then estimate the conductance of a single sodium channel.

By comparing the radioactivity and the biological activity uptakes in eel electroplaque particulate fractions as functions of concentration, ALMERS and LEVINSON (1975) estimated that the actual purity of the tetrodotoxin used in their experiments was about 30%. Correcting for this, they showed that there was a saturable component of tetrodotoxin binding to frog sartorius muscle of about

22 p-mol/g wet weight, the dissociation constant being about 5 nM. With labeled tetrodotoxin there was also a linear, nonspecific component of binding. In experiments where the uptake of unlabeled toxin was determined by bioassay, only the saturable component was observed; the linear component was absent. A similar observation was made subsequently by JAIMOVICH et al. (1976). Assuming that the average muscle fiber of the kind used in their experiments is a cylinder 80 μm in diameter and ignoring for the moment the density of sodium channels in the *T*-tubules, ALMERS and LEVINSON (1975) calculated that the channel density is about 380 channels/μm^2 of muscle fiber surface membrane.

2. Variety or Species Differences

Using the exchange-labeled saxitoxin described previously, RITCHIE and ROGART (1976b) examined the binding of labeled toxin to both amphibian and mammalian muscle. The binding of labeled saxitoxin to sartorius muscles of *Rana pipiens* was found to be of the same order as that previously found by ALMERS and LEVINSON (1975) for *Rana temporaria* and by JAIMOVICH et al. (1976) for *Rana pipiens*. RITCHIE and ROGART (1977b) made the further interesting observation that different varieties of the same species of frog (*Rana pipiens*) have different densities of sodium channels. One, a Southern variety, was larger and adapted to a warmer ambient temperature; the other, a Northern variety, was smaller and had to be kept in the cold. Histological examination showed no difference in the average fiber size, both being on average about 60 μm in diameter. Nevertheless, the uptake of labeled toxin by the Northern variety is only about 14 p-mol/g wet, which is about half the value of 25 p-mol/g wet found for the Southern variety. This finding is important because it means that variety (and hence, presumably, species) differences must exist so that *close* agreement between the results of one laboratory and the next are not necessarily to be expected. It would clearly be extremely interesting to obtain electrophysiological data to determine whether or not the membrane conductances of the two types of fiber were also different.

3. Single Channel Conductance

Techniques have become increasingly available in recent years for estimating the density of sodium channels in excitable tissue: amount of saxitoxin or tetrodotoxin bound, gating currents, noise measurements. In preparations like the frog sartorius muscle, it is possible to estimate the single channel conductance based on these estimates of channel density together with experimental values for the electric conductance of the membrane.

It is clear from the above finding of different channel densities in two varieties of the same species of frog and from the finding of different membrane conductances in one frog, *Rana pipiens* (HILLE and CAMPBELL, 1976), compared with *Rana temporaria* (ADRIAN et al., 1970) and *Rana esculenta* (ILDEFONSE and ROY, 1972), that such comparisons, and such estimations of the single channel con-

ductances are only valid if all experimental observations are made in the same species and group of animals.

Based on the range of values in the literature for sodium channel density of $180/\mu m^2$ (RITCHIE and ROGART 1977b, Northern *Rana pipiens*) to $380/\mu m^2$ (*Rana temporaria*, ALMERS and LEVINSON, 1975; Southern *Rana pipiens*, RITCHIE and ROGART 1977b; *Rana pipiens*, JAIMOVICH et al. 1976) and on the range of membrane conductances of 22 mS/cm^2 (*Rana esculenta*, ILDEFONSE and ROY, 1972) to 328 mS/cm^2 (*Rana pipiens*, Northern and Southern, HILLE and CAMPBELL, 1976), the single channel conductance can be estimated as 1.5–15.0 pS, but this must be regarded as a tentative estimate that gives only the order of size. For comparison, the conductances of the channels formed by the antibiotic gramicidin A and of the acetylcholine channel at the neuromuscular junction are 10–30 pS (see ALMERS and LEVINSON, 1975).

4. Detubulated Muscle

The value of 380 sites/μm^2 of surface membrane calculated above does not take into account the fact that sodium channels may not be distributed just on the surface membrane of the fiber; they also certainly occur on the membrane of the transverse tubular system. Unfortunately, there is as yet no direct electrophysiological evidence concerning the relative distribution. However, ADRIAN and PEACHEY (1973) and HILLE and CAMPBELL (1976) infer from their electrophysiological experiments that perhaps 0.15–0.25 of sodium channels are in the tubules, although it has six times the amount of membrane as does the cell surface. This, at most, would reduce the channel density in the surface membrane to about 280 sites/μm^2, and there would be $16/\mu m^2$ on the tubular membrane.

JAIMOVICH et al (1976) have estimated the fraction of tetrodotoxin binding sites that are in the surface membrane more directly by examining in bioassay experiments with unlabeled tetrodotoxin the binding of toxin to detubulated frog muscle. Detubulation was accomplished by glycerol-induced shock (HOWELL and JENDEN, 1967; EISENBERG and GAGE, 1969). After this treatment, fibers retain the ability to conduct action potentials along the surface membrane, but fail to contract, presumably because the T system is no longer activated. In normal muscle, JAIMOVICH et al. (1976) found that the maximum binding capacity was 35 p-mol/g wet, which is somewhat higher than that reported by ALMERS and LEVINSON (1975). This difference, however, seems fully accounted for by the fact that the average fiber diameter in the sartorius muscles used in the experiments of JAIMOVICH et al. (1976) was about 55 μm, whereas that in the experiments of ALMERS and LEVINSON (1975) was 80 μm. Referred to surface area (and neglecting the T system), the same channel density, $380/\mu m^2$, is indicated by the results of both groups. In detubulated muscle, more than 50% of the tetrodotoxin binding sites are inaccessible to toxin. They again become accessible to toxin on homogenization. JAIMOVICH et al. (1976), therefore, concluded that somewhat more than half the sodium channels in frog muscle are in the T system. These measurements would suggest a value of 175 sites/μm^2 for the surface membrane of muscle and 40–50 sites/μm^2 for the tubular membrane.

B. Mammalian Muscle and the Effect of Denervation

One well-known effect of denervation on mammalian muscle fibers is to cause them to become sensitive to acetylcholine at regions away from the motor end-plates (MILEDI, 1960) — an effect that is well-correlated with the appearance of binding sites for α-bungarotoxin at nonjunctional regions of the fiber (MILEDI and POTTER, 1971; HARTZELL and FAMBROUGH, 1972; BERG et al., 1972; CHANG et al., 1973). Furthermore, the action potential in the mammalian denervated muscle becomes relatively resistant to the blocking action of saxitoxin and tetrodotoxin (REDFERN and THESLEFF, 1971a, 1971b); HARRIS and THESLEFF, 1971; ALBUQUERQUE and WARNICK, 1972). Are these two effects related, and what is the nature of the toxin-resistance?

All action potentials that are blocked by tetrodotoxin or saxitoxin are sodium-dependent. However, the converse is not true; many sodium-dependent action potentials are tetrodotoxin resistant. Toxin-resistance seems to be generally found in mammalian muscle whose innervation either is immature or is absent. For example, the action potentials of embryonic and newborn rat muscles are toxin-resistant (HARRIS and MARSHALL, 1973), as is that of the L6 myotubes of rat skeletal muscle (SASTRE and PODLESKI, 1976); and in the adult, denervation of rat muscle leads to the development of toxin-resistant action potentials (REDFERN and THESLEFF, 1971b; HARRIS and THESLEFF, 1971; COLQUHOUN et al., 1974; MARSHALL and WARD, 1974). Resistance occurs to both toxins, but perhaps somewhat less towards saxitoxin than tetrodotoxin (HARRIS and THESLEFF, 1971; COLQUHOUN, D., 1976, unpublished observations). In such denervated muscle, the depolarization produced by batrachotoxin, which is normally antagonized by tetrodotoxin, also becomes tetrodotoxin-resistant (ALBUQUERQUE and WARNICK, 1972).

Does the development of tetrodotoxin-resistance mean that a change in the affinity for the toxin of the normal channels has occurred, indicating perhaps that the structure of preexisting channels has been altered as HARRIS and THESLEFF (1971) have suggested; have entirely new channels appeared; and is this related to the appearance of acetylcholine-sensitive sites? These questions have been examined by COLQUHOUN et al. (1974) and by RITCHIE and ROGART (1977b).

1. Normal Mammalian Muscle

In their original experiments, COLQUHOUN et al. (1974) showed that the uptake of labeled tetrodotoxin by normal rat diaphragm, as in nerve, is described by the usual equation:

$$U_t = b\,[T] + M/(1 + K_t/[T] \tag{8}$$

Perhaps because the preparation of toxin they used was relatively impure, the value of b relative to M was extremely large. As a result, an accurate estimate of K_t (6 nM) was obtained with difficulty, and the absolute value of M was only a fraction of its true value.

RITCHIE and ROGART (1977b) obtained a value for K_t of 3.9 mM and a value for M of 23.9 f-mol/mg for binding of exchange-labeled saxitoxin to rat diaphragm muscle (●, Fig. 7). With what densities do these toxin uptakes correspond?

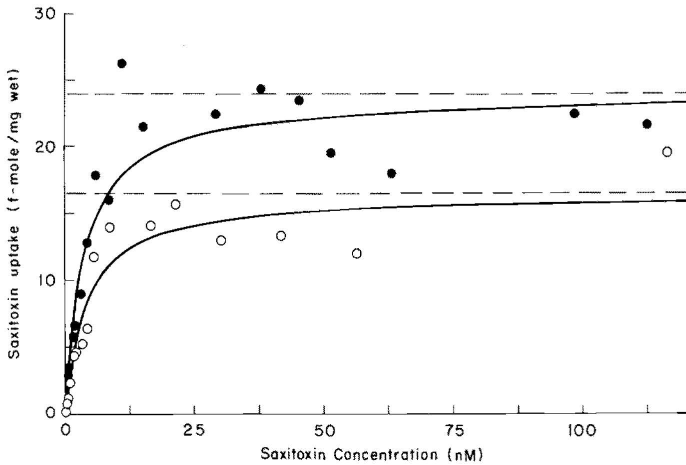

Fig. 7. The saturable component of uptake of labeled saxitoxin by normal (●) and denervated (○) rat diaphragm muscle. The linear component was determined in the presence of 10 μM tetrodotoxin and subtracted from the experimental points. The muscles were equilibrated for 8 h at 2–4° C with the saxitoxin. Each point is the mean of determinations in five different preparations. The interrupted lines are the asymptotes of the binding curves. The saturable binding curve for the normal muscle (●) and denervated (○) muscles ($U_{\text{innervated}}$ and $U_{\text{denervated}}$, respectively) are given by the relations: $U_{\text{innervated}}$ = STX bound = 24.5 [STX]/(3.8 + [STX]), $U_{\text{denervated}}$ = STX bound = 12.2 [STX]/(4.1 + [STX], where U is given in f-mol/mg wet and [STX] is given in nM. The binding curves shown are least squares fits to the points

Unfortunately, an exact answer depends on information on the extent of membrane in the T-tubule system, which is as yet incompletely known. One can, however, calculate an upper limit by assuming that all the toxin-binding sites occur on the external surface membrane. Measurement of phase contrast micrographs of the rat diaphragm fibers (kindly supplied by Dr. D.M. FAMBROUGH) showed that the average cross-sectional area (determined by planimetry) was $951 \pm 28\ \mu m^2$ and that the average circumference was 130.4 μm, which corresponds with an average diameter of 31.2 μm (see also FAMBROUGH, 1974). The upper limit for the sodium channel density on normal rat diaphragm, then, is $206/\mu m^2$.

2. Denervated Mammalian Muscle

The effects of denervation on the specific saturable component was still more difficult to determine. COLQUHOUN et al. (1974), studying [^{3}H]tetrodotoxin binding to denervated rat diaphragm, found that denervation reduced the ratio M/K (i.e., the value of the maximum binding capacity, M, divided by the equlibrium dissociation constant, K_t). But they were unable to demonstrate whether this reflected a fall in M or an increase in K_t, partly because of the low specific activity of their labeled preparation and partly because the linear nonspecific component of uptake was large compared with the specific component of uptake. On balance, COLQUHOUN et al. (1974) felt that it resulted from a decrease in M.

Using exchange-labeled saxitoxin, RITCHIE and ROGART (1977b) compared binding to normal and denervated rat diaphragm. Their experiments show that,

indeed, denervation has little, if any, effect on K_t, which changes from 3.9 mM in normal rat diaphragm to 4.2 nM in denervated diaphragm. The value of M, however, the maximal binding capacity, definitely decreases, from 23.9 p-mol/g wet to about 16.5 p-mol/g wet (○, Fig. 7).

The decrease in saxitoxin binding noted in these experiments might perhaps reflect the natural death of normal sodium channels; these channels would then be replaced by tetrodotoxin-resistant channels. A more likely explanation at present, however, seems to be that denervation produces little or no change in the density of sodium channels in the muscle membrane. For an early effect of denervation is swelling of the muscle (SOLA and MARTIN, 1952; MILEDI and SLATER, 1969), without any change in the muscle membrane area. The decrease in the density of sodium channels per unit *weight* of muscle would thus reflect the increase in weight rather than a change in density of muscle sodium channels per unit *area* of membrane.

Thus, denervation may change neither the dissociation constant, K_t, nor the density of normal sodium channels per unit area in muscle membrane. The toxin-resistant action potentials presumably reflect the appearance of an entirely new population of toxin-resistant sodium channels. This is consistent with the finding of GRAMPP et al. (1971) that resistance does not develop in the presence of protein synthesis inhibitors.

VIII. Chemical Characterization of the Sodium Channel in Terms of Its Ability to Interact with Various Compounds

Labeled tetrodotoxin and saxitoxin, because they bind highly specifically to the sodium channel — in intact nerve preparations, in homogenized preparations, and in solubilized preparations of membrane proteins — can be used as a tool to study the interaction of other chemical agents with the sodium channel. The principle is quite simple. A toxin molecule (T) reacts with the sodium channel (R) monomolecularly according to the reaction:

$$T+R \overset{K_t}{\rightleftharpoons} T\cdot R, \qquad (9)$$

where K_t is the equilibrium dissociation constant for the toxin. If another agent (I) is also present that reacts with the same receptor (with an equilibrium dissociation constant K_i) according to the relation:

$$I+R \overset{K_i}{\rightleftharpoons} I\cdot R \qquad (10)$$

then the uptake of toxin by the preparation, which is normally given by:

$$U_t=M/(1+K_t/[T]) \qquad (11)$$

willbe reduced to a new value U_t' which is given by:

$$U_t'=M/(1+K_t\,(1+[I]/K_i)/[T]) \qquad (12)$$

The value of K_i can be calculated then from the size of the fraction U_t'/U_t since K_t, $[T]$, and $[I]$ are all known. Substances with a high affinity for the site will yield low values for K_i, and substances with a low affinity for the site will yield high values for K_i. If the foreign substance has no affinity whatsoever for the site, there will be no diminution of toxin uptake, K_i being infinite.

A. Veratrine Alkaloids and Batrachotoxin

The most interesting substances to test are those for which electrophysiological data indicate that the substances interact with at least some site on the sodium channel. Two such substances are veratridine and batrachotoxin. When a frog nerve fiber is treated with the veratrine alkaloid veratridine, there is a persistent component of sodium conductance that appears rapidly (within milliseconds) on depolarization and that decays very slowly (time constant 0.1—1 s) on repolarization (see ULBRICHT, 1974). Electrophysiologically, this holding open of the sodium channels seems to be accomplished by an action of the drug at some site other than that at which tetrodotoxin acts (HILLE, 1968a; ULBRICHT, 1974). Thus, the kinetics of onset and offset of tetrodotoxin action are independent of whether or not the gates have been poisoned with the veratrine alkaloid. Batrachotoxin, a steroid alkaloid obtained from the skin secretions of a Columbian frog, acts similarly to veratridine. By keeping the sodium channels open, batrachotoxin may thus lead to sustained membrane depolarization (see ALBUQUERQUE, 1972) and an increased flux of sodium ions across the membrane (CATTERALL and NIRENBERG, 1973; HENDERSON and STRICHARTZ, 1974). Channels opened by either batrachotoxin or veratridine can be closed by applying tetrodotoxin, which can thus, for example, repolarize a preparation that has run down and become depolarized in the presence of veratridine or batrachotoxin (without, however, restoring electrical excitability).

However, chemical studies indicate that neither batrachotoxin or veratridine interfere with the binding of labeled saxitoxin or tetrodotoxin (COLQUHOUN et al., 1972; HENDERSON et al., 1973). This means, as the electrophysiological experiments on the kinetics of veratridine action had already suggested, that veratrine and batrachotoxin act at a site different from that at which tetrodotoxin works.

B. Local Anesthetics

Electrophysiological evidence suggests that local anesthetic agents do not act at the same location as tetrodotoxin. Thus, the major sites of action of local anesthetics are either within the lipid membrane (where they seemingly cause a membrane expansion) or at some anionic site that is accessible only from the inner surface of the membrane (STRICHARTZ, 1973; see RITCHIE, 1975b). Tetrodotoxin, on the other hand, is only effective when applied at the external surface (NARAHASHI et al., 1966). Again, in agreement with the electrophysiologic findings, local anesthetics do not interfere with the binding of saxitoxin or tetrodotoxin (COLQUHOUN et al., 1972; HENDERSON et al., 1973; BENZER and RAFTERY, 1972).

Support for the idea that local anesthetics act at some quite different site than do the toxins, saxitoxin and tetrodotoxin, comes from the finding of STAIMAN and SEEMAN (1975) that the nerve-blocking potency of tetrodotoxin is increased tenfold by lidocaine. This synergism is characteristic of there being two different sites of action.

C. Hydrogen Ions

Other substances might, however, from electrophysiological experiments, be expected to affect toxin binding. For example, voltage-clamp experiments on single nodes of Ranvier of frog myelinated nerve fibers have shown that hydrogen ions block the sodium currents by a potential-dependent type of binding to a site in the sodium channel that has a pKa of 5.4 at zero membrane potential (WOODHULL, 1973). The value becomes lower at positive membrane potentials, being about 5.6 at −40 mV (which is thought to be the value for the resting potential in mammalian nonmyelinated fibers; KEYNES and RITCHIE, 1965). The possibility that hydrogen ions bind to the same site in the sodium channel as does tetrodotoxin was already implied by HILLE (1971), who proposed that the sodium channel included an acidic oxygen group at the proton binding site that could function as the receptor for binding tetrodotoxin in a blocking position. In a subsequent analysis of the influence of pH on tetrodotoxin binding to myelinated nerve fibers, ULBRICHT and WAGNER (1975a, 1975b) felt that although "no unequivocal explanation of the pH effect on the rate of toxin action can be offered", their results nevertheless suggested that cationic tetrodotoxin molecules and hydrogen ions compete for the same binding site (see also ULBRICHT and WAGNER, 1975a, b). One would expect, therefore, that pH should markedly

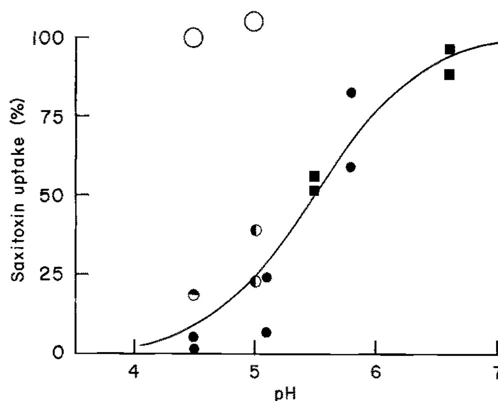

Fig. 8. The dependence on external pH of the saturable uptake of saxitoxin by the desheathed rabbit vagus nerve. The uptakes at the different values of pH are expressed relative to the uptakes by paired nerves from solutions of the same saxitoxin concentration but at pH 7.0. Uptake was measured after 6 h exposure time in solutions containing STX at 6 nM (●), 7 nM (■); 8 nM (○); and 200 nM (○) concentrations. The open circles (○) represent the uptakes at pH 7.0 by nerves that had previously been exposed for 3 h in saxitoxin-free solutions at the pH indicated. The continuous line is a titration curve for a single protonation site with pK = 5.5

influence the uptake of labeled tetrodotoxin. And, indeed, this has been shown for intact nerve fibers (COLQUHOUN et al., 1972; HENDERSON et al., 1973, 1974), homogenized nerves and membrane fractions prepared from such homogenates (REED and RAFTERY, 1976; BENZER and RAFTERY, 1972), and solubilized sodium channels (HENDERSON and WANG, 1972). Thus, in desheathed rabbit vagus nerve (Fig. 8), the uptake of labeled saxitoxin is found to be markedly dependent on external pH. Binding is much reduced in acidic solutions, being virtually abolished at about pH 4.5. An analysis of such curves (HENDERSON et al., 1973) indicates that protons compete with the toxins for binding to a site with pKa 5.9. The binding experiments thus agree well with the electrophysiological results.

D. Metal Cations

The electrophysiological experiments, particularly those of HILLE (see HILLE, 1972, 1975b), led to the conclusion that there is an essential, negatively charged group within the sodium channel of excitable membranes. This group seems to be important in lowering the free energy required to remove the water shell from the metal cations as they pass through the narrow selectivity filter of the channel. It also seems to be part of the receptor for tetrodotoxin and saxitoxin. HILLE (1971, 1972) found that the ionic currents carried by certain ions that can pass through the sodium channel, including lithium and thallium, are smaller than would be predicted from the Goldman-Hodgkin-Katz flux equation, given their permeability relative to sodium ions calculated from their measured reversal potential. These effects are markedly voltage-dependent, and HILLE concluded that the results are well fitted by supposing that the different cations, including hydrogen ions, interact with a single major binding site about 25% of the way across the membrane potential drop. The apparent dissociation constant for thallous (Tl^+) ions at the resting potential is 22 mM (see HILLE, 1975a). The corresponding value for calcium (WOODHULL, 1973) is similar in size. Again, in agreement with these electrophysiological results, calcium and thallous ions were found to compete with the labeled toxins for their binding sites with equilibrium dissociation constants for binding of the same order as those suggested electrophysiologically, namely 20–30 mM (HENDERSON et al., 1973).

Although the values of K_i obtained were somewhat scattered, they did not seem to depend in any systematic way either on the concentration of labeled toxin or on that of the inhibitory cation. There is thus excellent agreement between the concentrations of calcium, thallous, and hydrogen ions required to block sodium currents in frog nodes and the concentrations required to produce corresponding decreases in the binding of the toxin. All the evidence is consistent with the suggestion that the same site is involved in each case, namely, a binding site that may be the principal coordination site for cations as they pass through the sodium channel.

A variety of other cations have also been studied in the binding experiments. The affinity of the channel for trivalent cations is high, the values of K_i being 1 mM or less. The affinity for divalent cations is somewhat less; all divalent

cations studied, with the exception of beryllium, have K_i values of about 20 mM. Monovalent cations, with the exception of thallium, seem to have a very poor affinity for the site, the K_i values being 100–1000 mM (intact rabbit vagus). On general grounds, such a poor affinity makes sense because an ion with a high affinity would be bound too tightly and would not be so readily able to carry the large current necessary to generate the action potential. This value agrees quite well with the value of 380 mM obtained by HILLE (1975a) for the sodium channels of frog node of Ranvier.

It is interesting that REED and RAFTERY (1976), studying membranes isolated from eel electroplaques, found a much higher affinity for sodium and other monovalent cations; the value of K_i for sodium was 70 mM. Whether or not this represents a true difference in the electroplaque preparation remains unclear. For lithium ion, the value of K_i was 160 mM on the node of Ranvier (HILLE, 1975), 80–340 mM for rabbit vagus nerve (HENDERSON et al., 1974), and 60 mM on the membrane preparation (REED and RAFTERY, 1976).

IX. Effect of Surface Charge

The effect of cations on the action of tetrodotoxin and saxitoxin on nerve membranes has been discussed above as if it resulted from a direct specific competition between the cations and the toxins at the same binding site. However, it could also result from a general reduction by the added cations of the local negative surface potential and hence, of the local concentration of toxin. For the concentration of toxin, $[T]_r$, in the immediate vicinity of a receptor that is embedded in a membrane whose surface potential because of attached fixed charges is ψ, differs from the concentration of toxin in the bulk of the solution, $[T]_b$, according to the Bolzman relation:

$$[T]_r = [T]_b e^{-\psi ZF/RT} \tag{13}$$

where Z is the valency, F Faraday's constant, R the gas constant, and T the absolute temperature. The added cations, by making the surface potential less negative, would reduce the concentration of toxin in the immediate vicinity of the receptor and so decrease the toxin's apparent affinity for the site and increase the value of its apparent equilibrium dissociation constant. This possibility is amenable to experiment because at physiological values of pH, tetrodotoxin is a monovalent cation whereas saxitoxin is a divalent cation. Thus, if simple competition were responsible for the interactions between the metal cations and the toxins, both toxins would be equally affected. However, if the effect is mediated by a change in surface charge, saxitoxin, being divalent, would be more affected than tetrodotoxin.

The importance of surface charge was already suggested when the effects of altering external calcium on the binding of saxitoxin and tetrodotoxin were investigated (Fig. 9). As Figure 9 (which is based on data from HENDERSON et al., 1974) shows, increasing the calcium concentration decreases the affinity

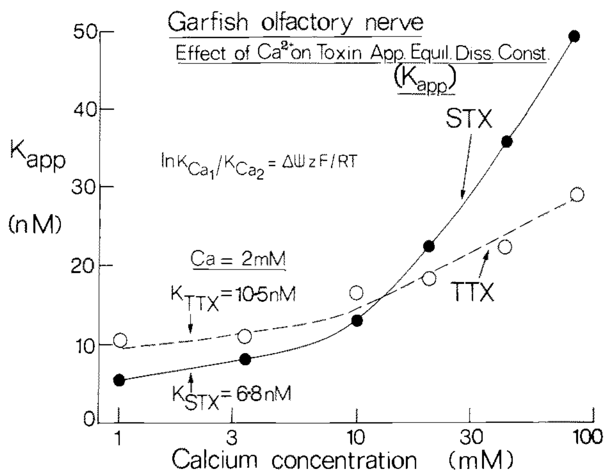

Fig. 9. The effect of external calcium concentration on the apparent equilibrium dissociation constants (K_{app}) for saxitoxin (●) and tetrodotoxin (○) for the garfish olfactory nerve. The solid line is a best fit by eye to the experimental points for saxitoxin. The interrupted line is a theoretical line based on this; for full explanation, see text. The values for K_{app} (i.e., K_t) at the calcium concentration in normal bathing medium (2 mM) are indicated by K_{TTX} and K_{STX}

(i.e., increases the equilibrium dissociation constant) for both saxitoxin and tetrodotoxin. Saxitoxin, however, is much more affected than tetrodotoxin as would be predicted on the surface charge hypothesis. Indeed, if one assumes that the effect of the cations on the binding of the toxins is *entirely* mediated by a change in surface charge, one can calculate from the saxitoxin results how ψ varies with external calcium and then use this relation to determine how the value for K_t for tetrodotoxin should depend on these changes in external calcium concentration, allowance being made for the different valencies of the two cations. The broken line in Figure 9, which is calculated in this way from the saxitoxin results, clearly agrees well with the experimental observations for tetrodotoxin.

The possibility has been examined in more detail by HILLE et al. (1975) in voltage-clamped, single frog myelinated fibers. Current-voltage curves were obtained from nodes of Ranvier in control bathing solution containing 2 mM calcium and in a similar solution containing 20 mM calcium. The concentrations of both tetrodotoxin and saxitoxin to halve the sodium currents in both solutions were then determined. As expected, the dissociation constants for both toxins, which were both about 1.5 nM in normal Ringer solution, were higher in the high calcium solution. Simple competition with calcium should have raised the apparent equilibrium constant, K_t for both toxins by the same factor. Instead, K_t for tetrodotoxin was increased by a factor 1.34, whereas that for saxitoxin was increased by a much larger factor, 1.87. These require quite divergent values for the equilibrium dissociation constant, K_i, for calcium, which would have to be 51 mM based on the tetrodotoxin results and 19 mM based on the saxitoxin results. However, if the results were mediated by a change in surface potential alone, the fact that the divalent saxitoxin was much more strongly affected than the monovalent tetrodotoxin is readily accounted for; and quantita-

tively, the results for both toxins could be explained by a change in surface potential of 7.2–7.6 mV.

Whereas the experiment above does not exclude the possibility of simple competition between the cations and the toxins, it does establish the importance of changes in surface potential. It is interesting that this calculated apparent potential change at the toxin receptor is only 30 and 50% of the 15 and 20 mV changes seen respectively by the *h* and *m* gates of the same fibers with the same change in calcium concentration (HILLE, 1968b).

X. Chemical Modification of the Sodium Channel

A. Enzymic Attack

The saxitoxin and tetrodotoxin binding site in the sodium channel is relatively stable while still in the membrane. However, it becomes much less stable when solubilized. Thus, although the solubilized material can be kept at 0–4° C for a period of several days with little or no drop in its ability to bind toxin, even after 1 h at room temperature (20° C), the binding activity completely disappears (HENDERSON and WANG, 1972; BENZER and RAFTERY, 1973).

BENZER and RAFTERY (1972), REED and RAFTERY (1976), and VILLEGAS et al. (1973) have examined the effects of various proteolytic enzymes on both homogenized and solubilized preparations of membranes of nerve and eel electroplaque. In homogenized preparations, the only enzymes found capable of inhibiting toxin binding were phospholipase A and two proteases, chymotrypsin and pronase. Prior treatment of the membranes with phospholipase A for 1 h enhanced the effects of the latter two proteases, indicating that a part of the tetrodotoxin binding component may be embedded in a phospholipid environment that may partly protect it against attack by proteolytic enzymes. After phospholipase A treatment, trypsin becomes effective in reducing binding in homogenized preparations. In the solubilized preparation, even in the absence of phospholipase A, however, trypsin, chymotrypsin, and pronase are now all effective in markedly reducing binding. It seems that in the solubilization procedure a phospholipid component protecting the membrane is lost or destroyed, but another phospholipid whose presence is necessary for the integrity of the channel is retained by the membrane in sufficient amount to maintain its ability to bind toxin. In both preparations, ribonuclease, deoxyribonuclease, hyaluronidase, and neuraminidase treatment do not decrease binding.

B. Modification by Reagents Specifically Blocking Certain Groups

In the experiments described above, some clue to the structure of the sodium channel and its surrounding membranes was sought by examining how certain broad classes of enzyme affect its ability to bind saxitoxin and tetrodotoxin.

Experiments have also been done in which various organic functional reagents interacting with amino acid residues in peptides have been studied.

Following HILLE's (1968b) and WOODHULL's (1973) conclusion that an ionized acidic group with a pKa of 5.2–5.4 (at zero membrane potential) seems to form part of the sodium channel (and thus of the receptor for tetrodotoxin), SHRAGER and PROFERA (1973) examined the effect on binding of [^{3}H] tetrodotoxin to the sodium channel of reagents that are capable of blocking carboxyl or phosphate groups, the likely candidates for this ionized acidic component. In this method, carboxyl (or phosphate) groups react reversibly with a water-soluble carbodiimide and are then attacked by a nucleophile to form a stable derivative. The final result is a covalent linkage of the carboxyl group to the nucleophile. Using tetrodotoxin binding by crab claw nerves as the indicator, they found that the nucleophile alone had little or no effect on tetrodotoxin binding. However, when the carbodiimide was also present, binding was reduced to about 15% of its control value. Although these and other results do not rule out the possibility that a phosphate group may be involved, SHRAGER and PROFERA (1973) felt that the observed blocking action of the carbodiimide and nucleophile is likely to be due to a reaction with a carboxyl rather than a phosphate group. These chemical results are thus consistent with the idea of HILLE (1968b) that a carboxyl group forms an integral part of the tetrodotoxin receptor, which in turn forms part of the sodium channel.

In related studies on voltage-clamped single myelinated nerve fibers, KEANA and STÄMPFLI (1974) introduced a note of caution into the interpretation of the effects of carbodiimide-nucleophile treatment on tetrodotoxin binding to nerve. For KEANA and STÄMPFLI (1974) showed that the effect of a very closely related carbodiimide is primarily on the potassium channels in low concentrations; prolonged application (and possibly the tenfold higher concentrations used on crustacean nerve by SHRAGER and PROFERA (1973) and subsequently by BAKER and RUBINSON (1975)) leads to a nonspecific "breakdown" of the fiber, which is manifest in a dramatic increase in the leakage current. Perhaps, suggest KEANA and STÄMPFLI (1974), the effects of the carbodiimide-nucleophile treatment on tetrodotoxin binding on crustacean nerve have little to do with effects on intact sodium channels; rather, they reflect a nonspecific breakdown of the crab nerve membrane. REED and RAFTERY (1976) made a similar suggestion on the basis of experiments on the binding of [^{3}H]tetrodotoxin to a preaparation of electroplaque membrane fragments. They noted that treatment with a water-soluble carbodiimide produces such extensive modification of the surface carboxyl groups that the membrane aggregates, reflecting perhaps gross changes in the charge distribution on the membrane surface. The fact that this could not be prevented by excess of tetrodotoxin was taken to indicate a lack of specificity of the carbodiimide action. However, there is some disagreement on the validity of this observation, as the discussion of the results of BAKER and RUBINSON (1975) in the paragraph below will show.

SHRAGER and PROFERA (1973) had shown that the bulk of the sodium channels treated with carbodiimide and nucleophile are no longer able to bind tetrodotoxin. The sodium channels, therefore, should be insensitive to tetrodotoxin; but since the nerves in their experiments were inexcitable, they were unable to test this critical

point. BAKER and RUBINSON (1975) carried out experiments similar to those employed by SHRAGER and PROFERA (1973), but with the difference that the conditions were such that the nerves continued to conduct impulses, and they were able to demonstrate that such nerves do indeed have a greatly reduced sensitivity to both tetrodotoxin and saxitoxin. This finding suggests that either the sodium channel or its immediate environment is modified by the chemical treatment such that the binding of both saxitoxin and tetrodotoxin is markedly impaired without affecting the access of ions to the voltage-sensitive sodium channel. BAKER and RUBINSON (1975) excluded the possiblity that the reaction with the carbodiimide was with thiols, phenols, or amines. The two remaining possibilities, that the carbodiimide reaction involves membrane-bound phosphate or membrane bound carboxylate, are more difficult to distinguish from one another experimentally. They were able, however, to demonstrate a certain specificity of action of the carbodiimide treatment. As a counterpart to the demonstration by SHRAGER and PROFERA (1973) that the carbodiimide-nucleophile treatment reduces tetrodotoxin binding, BAKER and RUBINSON (1975) showed that tetrodotoxin is indeed able to protect against the chemical modification produced by the reagents. This seems to remove one of the main arguments brought forward to support the suggestion (KEANA and STÄMPFLI, 1974; REED and RAFTERY, 1976) that the results with carbodiimide treatment reflect a massive, nonspecific destruction of the membrane.

Although their own attempts were unsuccessful, BAKER and RUBINSON (1974) pointed out that this protection by the toxins might eventually be very useful in attaching a covalent label to the sodium channels. Since the sodium channel would thus be affinity-labeled, this would allow a partially purified solubilized preparation of saxitoxin or tetrodotoxin binding sites to be further purified.

D'ARRIGO (1976) has studied the cationic binding site in the sodium channel, to which both toxins and sodium probably bind, by studying blocking effects of the uranyl ion (which specifically binds to phosphate groups) and methylene blue (which binds to carboxyl groups). This work suggests that the acidic anion moiety is likely to be a carboxyl rather than a phosphate group.

XI. Different Kinds of Sodium Channel in Excitable Membrane

A. How Many Kinds of Channel Are There?

All discussion so far has been based on the supposition that the channel reacts monomolecularly with the toxin molecule in the process of becoming blocked. As pointed out earlier, the evidence for this, though fairly reasonable, is not completely conclusive. The chemical experiments, particularly those of KEANA and STÄMPFLI (1974), question to some extent the validity of this general scheme; they raise the possiblity that there may be more than one kind of sodium channel. In their experiments with carbodiimide, they suggested that there might be at least two populations of structurally different potassium channels that differ in their reactivity towards the carbodiimide. If, then, it is possible for there to be

several species of potassium channel, might there not also be several species of sodium channel? This suggestion has recently found electrophysiological support in the experiments of SEVCIK (1976). In experiments on voltage-clamped squid giant axons in the presence of a variety of concentrations of tetrodotoxin, expecially low concentrations, SEVCIK (1976) found evidence for two types of sodium channel. The bulk of the channels are associated with receptors having the usual equilibrium dissociation constant for tetrodotoxin of 4.9 nM. However, about 20% of the channels are associated with receptors with an extremely high affinity for tetrodotoxin, whose apparent equilibrium dissociation constant for tetrodotoxin is about 0.1 nM. These observations may account for the apparent irreversibility of tetrodotoxin action reported by CUERVO and ADELMAN (1970). Even though the reaction of tetrodotoxin with both types of receptor studied by SEVCIK (1976) is fully reversible, in the case of the very high affinity channel a rather long time would be required to wash out sufficient toxin from the preparation to reduce the local concentration to below the extremely low value of K_t. If the low value of K_t reflects a reduction in the backward rate constant, k_2, the time constant of recovery, k_{off}, (0.1 min^{-1} for the normal channels) mights be as low as 1/50 th for the high affinity channels (see Sect. XII.D. for discussion of k_{on} and k_{off}). Thus, hours of washout rather than minutes are required.

It should be pointed out, however, that although one can thus operationally distinguish two components of tetrodotoxin sensitivity (SEVCIK, 1976), this does not mean necessarily that from the molecular point of view there are two kinds of channel. For example, identical channels will have different affinities for toxin in different environments. The variation in K_t noted by SEVCIK (1976) might reflect just such a difference — in local ionic strength or in local surface potential.

B. Saxitoxin- and Tetrodotoxin-Resistant Sodium Channels

Action potentials not based on sodium ion fluxes are insensitive to saxitoxin and tetrodotoxin. Thus, the toxins are without effect on a variety of calcium-based action potentials, for example, of: barnacle muscle (HAGIWARA and NAKAJIMA, 1966), guinea pig taenia coli muscle (KURIYAMA et al., 1966), procaine-treated crayfish muscle (OZEKI et al., 1960), snail neurones (MEVES, 1966), molluscan muscle (TWAROG et al., 1972), procaine-treated amphioxus muscle (HAGIWARA and KIDOKORO, 1971), TEA-treated leech neurones (KLEINHAUS and PRITCHARD, 1975), and frog motoneurones (BARRETT and BARRETT, 1976). All action potentials that are blocked by the toxins are based on sodium currents. The converse, however, is not necessarily true, and several examples are now known of sodium-based action potentials that are tetrodotoxin- or saxitoxin-resistant, as for example: the nerves and muscle of Puffer fish and of the newt *Taricha* (KAO and FUHRMAN, 1967; GRINNEL, 1975), nerves of molluscs (TWAROG and YAMAGUCHI, 1975), denervated mammalian muscle (REDFERN and THESLEFF, 1971b; COLQUHOUN et al., 1974; HARRIS and THESLEFF, 1971; MARSHALL and WARD, 1974), embryonic and newborn rat muscle (HARRIS and MARSHALL, 1973), rat skeletal muscle L6 myotubes (SASTRE and PODLESKI, 1976).

Some understanding of an underlying explanation of these toxin-resistant action

potentials comes from the experiments with carbodiimide-nucleophile treated nerves (BAKER and RUBINSON, 1975). These experiments make it clear that the group in the membrane involved in the sensitivity of the action potential to tetrodotoxin and saxitoxin can, under appropriate circumstances, be dissociated from the ability of the channels to generate action potentials; thus, sensitivity to the toxins can be abolished, yet the channels are still physiologically functional (BAKER and RUBINSON, 1975). These findings, together with the suggestion of HILLE (1976) that the antechamber to the sodium channel (Fig. 2) may be important for the interaction of the toxins with the channel, may provide an explanation for the fact that many sodium channels are, anomalously, tetrodotoxin-resistant or saxitoxin-resistant. For the antechamber, or some such component of the channel, may be modified so as to interfere with binding (and efficacy of the toxins) without in any way interfering with the components of the channel beyond, including the selectivity filter and the anionic site that normally binds the cationic head of the toxin. The entry of sodium ions to such a channel is unimpeded and action potentials can be generated; access by the toxins, however, is denied.

Such an explanation would account for the fact that whereas sodium channels in normal nerve are virtually always sensitive to the toxins, there are some special exceptions: the nerves of the Puffer fish and the nerves of the American newts *Taricha* are insensitive to tetrodotoxin. Interestingly, the nerves of both are sensitive to saxitoxin (KAO and FUHRMAN, 1967). The nerves of molluscs (TWAROG and YAMAGUCHI, 1975), however, are insensitive to both tetrodotoxin and saxitoxin. Furthermore, some sodium channels of denervated mammalian skeletal muscle become tetrodotoxin-resistant (REDFERN and THESLEFF, 1971b; COLQUHOUN et al., 1974; RITCHIE and ROGART, 1977b); they also become saxitoxin-resistant, but to a lesser extent (HARRIS and THESLEFF, 1971; D. COLQUHOUN, personal communication). These latter channels are not formed by modification of existing channels. Rather, they are newly synthesized channels, which do not appear if protein synthesis is inhibited (GRAMPP et al., 1971). Finally, it seems that the cardiac action potential is relatively tetrodotoxin-resistant (BAER et al., 1976), even that part where the early depolarizing current is carried by sodium ions. The chemical experiments described provide some insight into a possible explanation for this tetrodotoxin-resistance of a variety of sodium-dependent action potentials that is well worth future study.

XII. The Significance of K_t

Much effort has been exerted in determining the value of K_t in a variety of different kinds of tissue. These results unanimously agree that the value of the equilibrium dissociation constant is extremely low, being of the order of a few nanomolar. The reason for this effort is largely for purposes of identification. If the drug under study, particularly in the labeled experiments, does not have an extremely high affinity for excitable membranes, or extracts of them, it is unlikely

to be saxitoxin or tetrodotoxin. Several points, however, should be made in this connection.

A. The Constancy of K_t

The first point is that although one expects to obtain values of K_t experimentally that are of the order of a few nanomolar, one would not expect complete agreement in the results of different studies. To begin with, the determination of K_t, especially in intact preparations, is subject to considerable experimental variation; it is not uncommon to find a twofold variation in the value of K_t even in different facets of the same report. The main reason, however, that one would not expect absolute agreement is that even if one were dealing with the *identical* receptor, the fine structure of the membranes of the different species will not necessarily be identical; in addition, the ionic environment will certainly differ markedly in amphibian, mammalian, and crustacean preparations. The resultant variation in the surface potential alone would have marked effects on the observed value of the equilibrium dissociation constant. Only when the experiment is done under a standardized set of conditions would the K_t values obtained with preparations from a variety of different sources be expected to be the same. The highly purified, solubilized preparation might in the future be of value in this respect. Under sufficiently standard conditions, differences in the K_t values obtained in different preparations obtained from the different sources might reveal the existence of even minor differences in the molecular structure of the sodium channel from one type of preparation to another.

B. K_t and Purity

The mere finding of a low value for K_t does not in itself say anything about the purity of a labeled preparation. For the determination of K_t is essentially a determination experimentally of the concentration at which the uptake that is being studied reaches the halfway mark. This concentration can be measured by *any* marker in the solution. It is not necessary to have the label specifically attached to the toxin; a label in some other component, or even, under the appropriate conditions, just a color added to the stock solution, would do just as well.

C. Electrophysiological Determination of K_t From Measurements of Maximum Rate of Rise of Action Potential

The final point, perhaps somewhat trivial, is that the electrophysiological measurements of K_t made for comparison with a chemically determined value must be based only on measurements of $\bar{g}_{Na}$ (e.g., CUERVO and ADELMAN, 1970; HILLE 1968a). Parameters dependent on $\bar{g}_{Na}$, but not linearly related to it, such as the maximum rate of rise of the action potential, $\dot{V}_A$, may give misleading results. The reason is that by the time the maximum velocity of depolarization in a spike

occurs, sufficient time may have elapsed — especially when the rate of rise is slowed by saxitoxin or tetrodotoxin — for the sodium conductance to be expected to have fallen considerably from its maximum value $\bar{g}_{Na}$ (being reduced by m^3h in squid axons, for example). This expectation is in fact confirmed experimentally. SCHWARTZ et al. (1973), for example, showed that whereas 10.9 nM tetrodotoxin is necessary to reduce V_A to half its original value in single voltage-clamped frog nodes of Ranvier, the true value of K_t based on voltage-clamp experiments is 3.6 nM. A similar discrepancy (COLQUHOUN et al., 1974) exists between the concentration of tetrodotoxin to halve the maximum rate of rise of the action potential in rat diaphragm muscle (40 nM) and the value of K_t determined from experiments on the uptake of labeled toxin (4.7–7.8 nM).

Ignoring these reservations on the significance of determinations of K_t may well lead to conclusions that are erroneous, or at best misleading.

D. Determination of K_t From Kinetic Experiments

When an intact muscle or nerve is exposed to tetrodotoxin, the blocking action takes considerable time to develop. For example, SCHWARTZ et al. (1973), surveying their own work and that of others, noted that the onset of action on exposure of a nerve to 15 nM tetrodotoxin (at 20° C) has a time constant in the rabbit vagus of 5.4 min, 6 min in lobster axons at 7°, and 2.4 min in the squid. In the single nodes of Ranvier from frog fibers, however, the corresponding time constant is only 17.5 s.

The reason for the slow onset times in most preparations is due to the slow passive diffusion of toxin to its final receptor site. The speed of diffusion is very much reduced because the rapid binding of the toxin to the sites markedly retards the rise in toxin concentration in the compartment in which the receptors are situated and hence slows the rate of receptor occupancy. In this limited biophase model, washing out of the toxin may be also considerably slowed compared with simple diffusion of a nonbinding molecule from the compartment. COLQUHOUN et al. (1972), analyzing the kinetics of onset and offset in such a limited biophase system, showed that the diffusion in this system behaves as though the diffusion coefficient were not constant but varied with concentration. Numerical integration of the diffusion equations showed that over a large range of conditions, both the rate constant for offset and that for onset of action (k_{off} and k_{on}) ought to be smaller than the forward and backward rate constants for the true reaction between the receptor and the toxin (k_2 and k_1) but by the same factor which is approximately equal to M/K_tV where V is the volume of the compartment containing the receptors. The interesting result of this analysis is that the ratio k_{off}/k_{on} gives the same value as k_2/k_1, namely the equilibrium dissociation constant K_t. This leads to the surprising conclusion (COLQUHOUN and RITCHIE, 1972a) that the equilibrium dissociation constants calculated from kinetic measurements of the onset and offset of action, on the wrong assumption that these rates are controlled solely by the drug receptor interaction, are roughly correct. In the rabbit vagus, COLQUHOUN and RITCHIE (1972b), on the basis of their kinetic measurements, obtained a value for K_t of 3.2 nM, which compares well with their value of

3–5 nM found in the electrophysiological experiments on the same nerve (COLQUHOUN and RITCHIE, 1972a). Similarly, in experiments on the squid giant axon, CUERVO and ADELMAN (1970) found values for k_{off} of 0.19×10^{-2} s^{-1} and values for k_{on} of 0.0034×10^{-8} M^{-1} s^{-1}. The magnitude of the dissociation constant derived from these values is 5.7 nM, which again agrees well with the value 3.3 nM, directly determined from equilibrium measurements in the same experiments. The values for k_{on} and k_{off} obtained in single nodes by SCHWARTZ et al. (1973) may come quite close to the true values k_2 and k_1. Certainly, in the preparation where a diffusion barrier is least likely to intervene, namely the solubilized sodium channel preparation, the value obtained for k_2 (HENDERSON and WANG, 1972), 1.58×10^{-2} s^{-1}, is virtually identical to the value 1.42×10^{-2} s^{-1} obtained in the myelinated experiments. However, even in the solubilized preparation, it is not clear that diffusion barriers are completely absent. The fact (see Sect. VI.C.) that the size of the solubilized sodium channel (HENDERSON and WANG, 1972) seems to be considerably larger than that in vivo (LEVINSON and ELLORY, 1973) suggests that the solubilized channel is associated with, and perhaps embedded in, a micelle of detergent, which could well present a barrier to diffusion.

The analysis of COLQUHOUN and RITCHIE (1972a) and of COLQUHOUN et al. (1972) make it clear that under the conditions normally met in biological tissues, diffusion of a drug acting in such small concentration, as do saxitoxin and tetrodotoxin, into a compartment to which access is limited by diffusion is going to be slowed by several orders of magnitude. This is why in the experiments of Figure 4, for example, a long exposure time (8 h) was used to ensure equilibration, especially at the lower concentrations.

XIII. Future Developments

Several areas of future work seem to be indicated at the present time.

Firstly, as pointed out in Section VI.C. chemical purification of the sodium channel in solubilized preparations is still imperfect. A pure crystalline preparation might allow the possibility of determining the structure of the channel by X-ray crystallography. It would certainly allow the channel to be studied by a variety of well-established biophysical and biochemical techniques.

Secondly, further steps toward improving the marker used in identifying the channel seem necessary. At the present time such a marker is likely to continue to be either the currently available tritiated saxitoxin or tetrodotoxin (or some derivative of these compounds). One problem at the moment is in the specificity of the labeling. The recent exchange-labeling method, transferring tritium from tritiated water to saxitoxin, is a major step. Even here, however, considerable difficulty remains. The fact that both saxitoxin and tetrodotoxin can now be made synthetically raises the possibility that a stable label can be put in a specific part of the tetrodotoxin molecule. Failing this, labels attached to the recent derivatives of tetrodotoxin produced by TSIEN et al. (1975) or of saxitoxin produced by GHAZAROSSIAN et al. (1976) show much promise.

Thirdly, a more extensive study of the kind of interaction between the toxin and the receptor could be undertaken. The electrophysiological studies in non-

myelinated fibers, for example (COLQUHOUN and RITCHIE, 1972a), could not exclude the possibility that two toxin molecules were reacting with each receptor, and the binding studies of the same tissue showed too much experimental variation to be sure on this point. The major piece of evidence in favor of a simple monomolecular reaction remains that illustrated in Figure 4 for myelinated nerve and a similar experiment for squid by CUERVO and ADELMAN (1970).

Finally, further binding studies associated with the development of stable and active saxitoxin and tetrodotoxin derivatives may yield a clinical harvest quite apart from a better understanding of basic mechanisms of the physiology of the sodium channel. For such agents might provide a basis for newer and better local anesthetic agents. Both types of molecule, because of their specificity, could, if associated with an alklating group on them, provide the basis for long-lasting anesthetic agents (see RITCHIE, 1975b).

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The Physiology, Pharmacology, and Biochemistry of the Eccrine Sweat Gland

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I. Introduction

The importance of eccrine sweat secretion for control of body temperature in man has stimulated many investigators to study various aspects of this interesting but virtually unexplored exocrine gland. Man has $3–4 \times 10^6$ eccrine sweat glands distributed over nearly all the body surface (KUNO, 1956), and each gland weighs about 30–40 μg net weight (SATO, K., unpublished data). Thus, it takes only simple arithemtic to recognize that the eccrine sweat glands total roughly one kidney, ie., 100 g. Man can perspire as much as several liters per hour and 10 liters per day (KUNO, 1956), which is far greater than the secretory rates of other exocrine glands, such as the salivery and lacrymal glands and pancreas. The secretory activity of the human eccrine sweat gland consists of two major functions: (1) secretion of an ultrafiltrate of plasma-like precursor fluid by the secretory coil in response to locally released acetylcholine, and (2) reabsorption of sodium in excess of water by the duct, thereby producing a hypotonic skin surface sweat. Under extreme conditions where the amount of perspiration reaches several liters a day, the ductal reabsorptive function assumes a vital role in maintaining homeostasis. In addition to the secretion of water and electrolytes, the sweat gland serves as an excretory organ for heavy metals, organic compounds, and macromolecules.

As compared with the recent progress being made in the study of other transporting epithelia including the exocrine glands, eccrine sweat gland research lags far behind, and it is an undeniable, albeit deplorable, reality that eccrine sweat gland function is a relatively unexplored and neglected spot in the field of medical knowledge. This is especially true in regard to the biochemical and membrane biophysical aspects of this gland, which are, ironically enough, the

very areas that are the subjects of this review. I have thus found myself with no choice but to rely heavily on my own unpublished recent data to fill the gaps of knowledge and to open further discussions, in the expectation that such an unorthodox approach will eventually help to stimulate and encourage future research on this unexplored exocrine gland by other investigators. Recent sharp decline in the number of publications on physiology and biochemistry of the sweat gland appears to be due mainly to the lack of methologic progress at the time when such sophisticated methods as micropuncture, vascularperfusion both in vitro and in vivo, microperfusion of the ductal system, electrophysiologic approaches, etc., have been routinely empolyed by many physiologists in the study of other exocrine glands. In biochemical fields, there is no necessity to mention that both pancreas and salivary glands have been subjected to intensive studies. It should be mentioned, however, that there have been only a few sporadic attempts to introduce such methodologic innovations into the field of eccrine sweat gland physiology during the past decade; namely, the micropuncture method of SCHULZ et al. (1965), the microinduction of sweat secretion from an isolated eccrine sweat gland in vitro (SATO, 1973b), and in vitro microperfusion of the sweat duct by MANGOS (1973a). In the author's laboratory, an extensive effort has been made during the past 2 years to improve the original in vitro method (SATO, 1973b) and to apply it to answer a variety of questions surrounding this exocrine skin gland hitherto unanswered with classic approaches. Although most of these data are still unpublished. I have chosen to draw upon them wherever appropriate to make the discussion more stimulating and provocative.

II. Classification of Skin Glands and the Problem of Distinction Between Apocrine and Eccrine

A. Classification and History

In 1887, Ranvier divided the skin glands into holocrine and merocrine glands according to the differences in mode of secretion. According to his concept, holocrine secretion represents the disintegration or casting off of the secretory cells, as in the sebaceous gland, whereas merocrine secretion is accomplished without accompanying damage to the secretory cells. In 1917, SCHIEFERDICKER attempted to subclassify the merocrine glands into apocrine and eccrine glands but made a point that apocrine secretion has features of both merocrine and holocrine secretion. This holocrine-like component of apocrine secretion is usually called decapitation or necrobiotic secretion and was once regarded as a major point of distinction between aspocrine and eccrine.

The distinction between apocrine and eccrine is clear-cut when their morphology, distribution, and embryologic development are compared. In man, apocrine glands occur mainly in the axilla, the mons pubis, the external auditory meatus, the areola, and the circumanal area. Each apocrine gland opens into a hair follicle, and its secretory function does not begin until puberty. It is not clear whether the so-called necrobiotic secretion does indeed occur with apocrine secretion (KUNO,

1956; MONTAGNA, 1959; JENKINSON, 1968). As compared with the eccrine sweat gland, very few studies have been made of the apocrine gland, and its mechanism of secretion remains unknown. Although the role of necrobiotic secretion has been repeatedly denied in apocrine secretion, a recent electronmicroscopic study by SORENSEN and PRASAD (1973) clearly shows that the tiny apical cytoplasmic protrusions are about to pinch off into the lumen in the horse sweat gland, an apocrine-type skin gland. It may be reasonable to assume, therefore, that such a pinching-off secretion (reversal of pinocytosis) without accompanying necrobiosis and/or cell death may occur in the apocrine sweat gland and perhaps also in the eccrine sweat gland, although it probably constitutes only a minor role in the entire secretion. If true, it would conveniently explain how macromolecular substances are secreted into both apocrine and eccrine sweat.

B. Some Aspects of Apocrine Secretion in Animals

Although apocrine sweat glands have been the subject of numerous anatomical and ultrastructural studies, few physiologic studies have been done on this gland. This may be because apocrine sweat is easily contaminated by sebaceous and eccrine secretions. In man, not even the electrolyte concentrations of apocrine sweat are known. JIRKA and KOTAS (1959) analyzed protein and electrolyte concentrations in the skin surface secretion of the horse sweat gland, which is presumably a variant of apocrine sweat gland (KUNO, 1956). Since the sweat had been exposed to air before collection by skin scraping, the hypertonic electrolyte composition observed (in mM: Na. 382–432; K, 48–141; Cl 432) cannot be accepted at face value. However, Na/K ratio of 3–7 calculated from their data may have been relatively unaffected by evaporative loss. JENKINSON and MABON (1973) and JOHNSON (1970) studied the ionic composition of skin secretions in cattle. In both studies, Na and K concentrations were less than 10 mM. Like human apocrine glands, the hair-associated tubular glands in these animals have a relatively short excretory duct. However, since no data is available on the ductal functions in these tubular apocrine-like glands, the electrolyte composition of the primary secretory fluid cannot be deduced. Accurate knowledge of electrolyte composition in the primary fluid and in the secretory cells may offer a direct answer as to whether necrobiotic secretion is indeed the mechanism of apocrine secretion.

III. Anatomy and Innervation of the Eccrine Sweat Gland in Man

A. Gross Anatomy and Embryology

The total number of eccrine sweat glands varies according to different reports. Estimates usually fall between 1.6 and 4×10^6 glands (KUNO, 1956; SZABO, 1962). In the adult, sweat glands are most numerous on the sole of the foot (620/cm^2) and least abundant on the back (64/cm^2) (SATO and DOBSON, 1970a). The size of the gland varies regionally and individually, but it ranges between 30 and 40 μg net

weight (SATO, K., unpublished data). The gland first appears in the $3^1/_2$-month-old fetus on the volar surface of the hands and feet (ELLIS, 1967). Early in the 5th fetal month, anlagen of the eccrine sweat gland appear in the axillary skin and a few weeks later also in other areas of the body. Whereas the anlage of the eccrine sweat gland develops from the epidermal ridge as a cord of epithelial cells growing downward, that of the apocrine gland derives from the side of the hair follicle as a solid epithelial bud in the 5th fetal month. The anlage of the eccrine sweat gland is double-layered, and the lumen is formed by fusion of the intracytoplasmic vacuoles of the luminal cells between fetal months $3^1/_2$ and 8 (HASHIMOTO et al., 1965). During the 8th fetal month, the lumen broadens and the secretory cells simulate those of the adult glands. The origin of the myoepithelial cell is not yet clear, and this structure is not recognizable at least until the 9th embryonal month (ELLIS, 1967).

B. The Fine Structure of the Eccrine Sweat Gland

The eccrine sweat gland consists of two segments, a secretory coil and a duct. Since the structure and function of the eccrine sweat gland differ considerably from one species to another, the major morphologic and functional characteristics of the eccrine gland for each species are summarized in Table 1. Of those listed in the Table, monkey palm sweat glands most closely resemble those of man both structurally and functionally (TERZAKIS, 1964; IKAI et al., 1970; SATO, 1973c). Thus, the monkey sweat glands can be used in many cases as a model of the human eccrine gland. The secretory coil of human, monkey, cat, and opossum eccrine sweat glands is composed of three distinct cell types: secretory (clear), dark (mucoid), and myoepithelial cells. The secretory and dark cells occur in approximately equal numbers (Fig. 1). Detailed information is not available on the developmental interrelationships between these three cell types, although TERZAKIS (1964) has postulated that the dark cell may originate from the clear cell based on the observation that a so-called "transitional cell" was observed, although very infrequently, which he claimed contained features of both a clear cell and a dark cell. The dark cells border nearly all the apical (luminal) surface of the secretory tubule and occur either as a cuboid cell which rests on the clear cell or as an inverted pyramidal cell whose wedge-shaped cytoplasmic process extends toward the basement membrane between two clear cells, but they rarely reach the myoepithelial cells or the basement membrane. The mechanism by which the dark cell granules are released from the cell is not known. Although it is widely held that the mucosubstance in sweat is derived from the dark cell granules, direct biochemical proof is lacking. ELLIS (1967) suggested that exocytosis may be the mechnism for the release of the content of granules. Although no convincing morphologic evidence for such a mechanism of degranulation has been presented in human and monkey sweat glands, the electronmicroscopic studies of opossum sweat glands (FORTNEY, 1973) and of mammalian submaxillary glands (DOREY and BHOOLA 1972) clearly show the occurrence of exocytosis in the degranulating process of dark cell granules and zymogen granules respectively.

Table 1. Eccrine sweat glands: morphology, composition of secretion and pharmacologic reactivity

Species	Structure, Secretory coil				Sweat electrolyte composition (mM)						Ductal Na reabsorption	Reactivity		References
					Skin surface sweat			Precursor sweat						
	CC	DC	ME	ICC	Na	K	Cl	Na	K	Cl		CH	AD	
Human	+	+	+	+	H(F)	4–24	H(F)	147	?	122	+	+	+	ELLIS (1967) SATO et al. (1970, 1973) SCHULZ et al. (1965)
Monkey palm [a]	+	+	+	+	H(F)	5–19	H(F)	140[b]	5.5[b]	?	+	+	+	TERZAKIS (1964) IKAI et al. (1970) SATO (1973b)
Cat paw	+	+	+	+	135–160	25–45	80–120	?	?	?	(−)	+	+	MUNGER and BRUSILOW (1961) FOSTER (1966) LLOYD (1959b)
Dog paw	+	?	?	?	10–200 (F)	2–25	30–150 (F)	?	?	?	+	+	?	IKAI et al. (1969) TAKAHASHI (1964)
Opossum paw	+	+	+	+	?	?	?	?	?	?	?	+	?	FORTNEY (1973)
Rat paw	+	−	+	−	4–25	150–200	85	25[b]	160[b]	?	−	+	−	QUTRALE and LADEN (1968) SATO (1974, 1975)
Mouse paw	+	?	?	?	60	160	?	60[b]	>100[b]	?	?	+	+	HAYASHI (1969) SATO, K., unpublished data

CC: clear cell
DC: dark cell
ME: myoepithelial cell
ICC: intercellular canaliculi
+: presence
−: absence

(−): absence not directly proven
(H): hypotonic with respect to plasma
(F): flow dependency
CH: cholinergic stimulation
AD: adrenergic stimulation
?: not yet studied

[a] The eccrine sweat glands of some primates seem to lack dark cells.
[b] Values obtained from in vitro system.

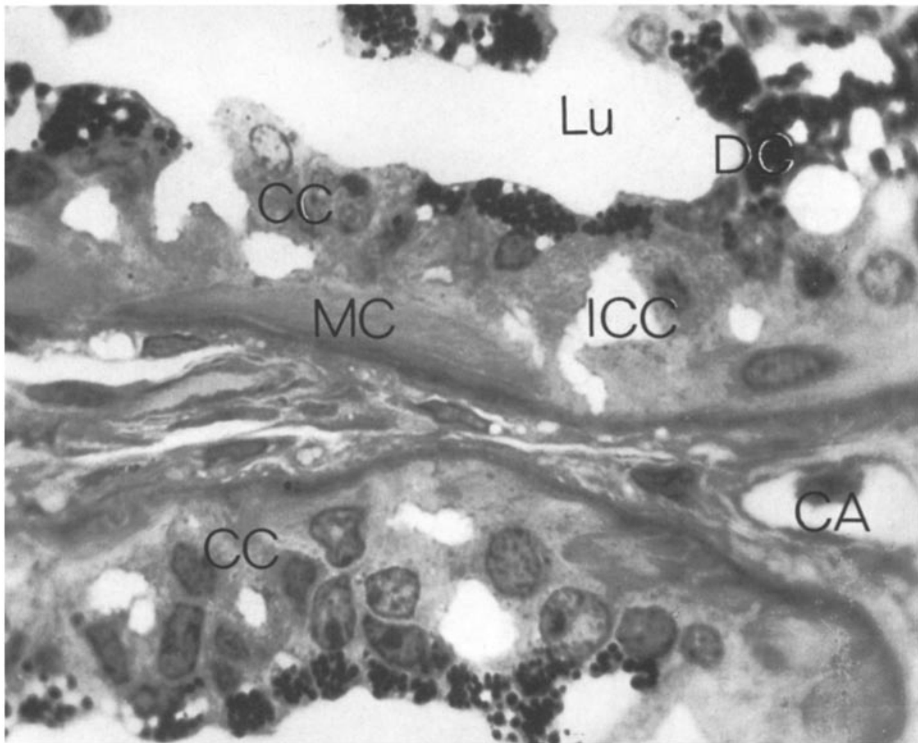


Fig. 1. Light microscopic picture of the secretory coil of an acetylcholine-stimulated monkey palm eccrine sweat gland. A section (1 μm) was cut from an epon-embedded specimen and stained with methylene blue. Lu: lumen, ICC: intercellular canaliculi, MC: myoepithelial cell, CC: clear cell, DC: dark cell, CA: peritubular blood capillary

The clear cells rest either directly on the basement membrane or on the myoepithelial cells. Where two or more clear cells abut, intercellular canaliculi are formed. The canaliculi start immediately above the basement membrane or the myoepithelial cells and open directly into the lumen of the gland. The luminal membrane of the clear cell is rarely exposed directly to the lumen because the luminal surface is nearly totally lined by dark cells. It follows, therefore, that the only membrane of the clear cell directly exposed to the lumen is that part of the membrane facing the intercellular canaliculus (Figs. 1 and 2), which is a pouch extending from the luminal spece. The three-dimensional structure of the intercellular canaliculus is rather difficult to comprehend from the electronmicroscopic pictures. Figures 2 and 3 are the schematic illustration of the canaliculus, which is composed of cell membranes of the two opposing clear cells sealed by a continuous line of terminal bar (or so-called tight junction). Not infrequently, the lateral cell membrane of the dark cells forms part of the orifice of a canaliculus. The membrane of the canaliculus is studded with microvilli, but the number of villi is markedly decreased when the intercellular canaliculus dilates during sweat secretion (SATO et al., 1973). The structural complexity of the secretory epithelium

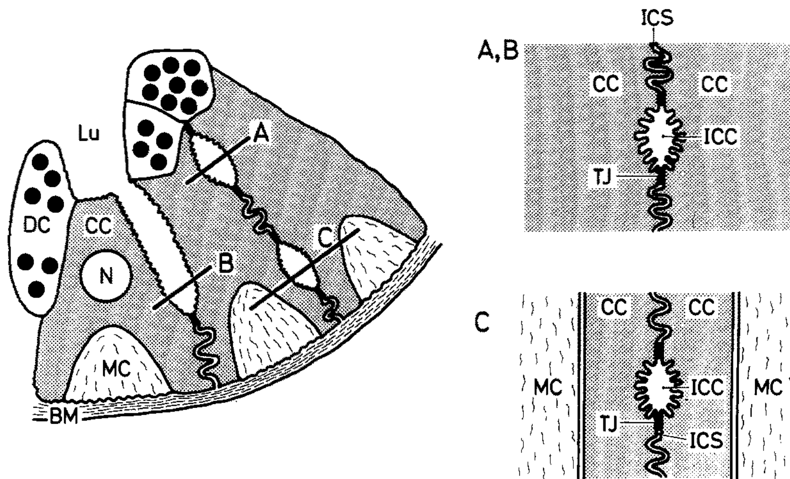


Fig. 2. Diagrammatic representation of the secretory coil and the intercellular canaliculi. A, B, and C represent the height of horizontal sections on the left, and on the right the schematic picture of each section is shown. Other notations are the same as in Fig. 1

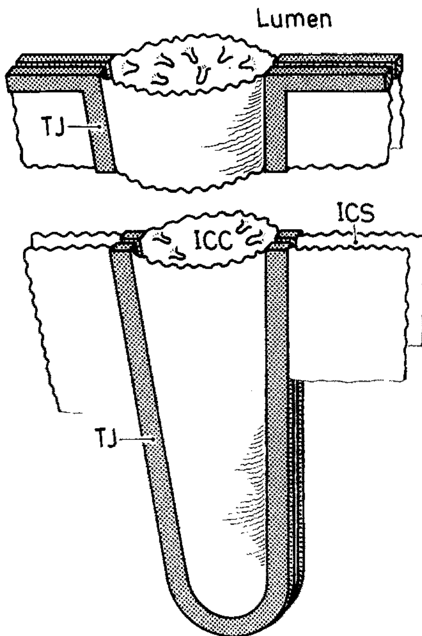


Fig. 3. Diagrammatic representation of the intercellular canaliculi (ICC) and the intercellular space (ICS). Two opposing secretory cells, between which ICC is formed, are not shown. TJ: tight junction (terminal bar)

is such that it was once misinterpreted (SLEGERS and VAN'T HOT-GROOTENBOER, 1971) as being a so-called "forward fluid transporting epithelium" like the kidney proximal tubule or the gall bladder epithelium (DIAMOND and BOSSERT, 1967). However, as shown in Figure 3, the relationship of the tight junction to the lumen is in keeping with the characteristics of the so-called "backward transporting system."

The clear cell contains abundant mitochondria, forms intercellular canaliculi with the neighboring clear cells, and is endowed with an intricate infolding of plasma membrane at its basal side (basal labyrinth) (ELLIS, 1967). Because of its resemblance to other fluid and electrolyte transporting cells, it is generally believed that the clear cell is responsible for secretion of water and electrolytes by the secretory coil. The clear cells also contain varying amounts of glycogen. Histochemical evidence for glycogen depletion in the clear cells during sweating was first demonstrated by YUYAMA (1935). In acclimatized males, however, there is no histochemical evidence for glycogen depletion after sweating (DOBSON, 1962). It is not known to what extent cellular glycogen is utilized as a fuel depot. In an isolated simian sweat gland, neither lactate production (SATO and DOBSON 1973) nor continued sweat secretion (SATO, K., unpublished data) occurs in the absence of glucose or other substrates in the incubation medium. Recently, SPICER et al. (1972) observed in the clear cell of the human sweat gland electron-dense bodies without a limiting membrane. These bodies are smaller than 1 μm in diameter and differ from the secretory granules of dark cells by the absence of a limiting membrane. They also differ from the small acid phosphatase-positive lysosomal bodies present in both dark and clear cells (SPICER et al., 1972). Since these bodies are always found in close proximity of the zona occludens (or tight junction) in the region of the intercellular canaliculi, SPICER et al. (1972) referred to these bodies as junctional complex associated bodies (JCA). These investigators speculated that JCA contribute the material(s) to the lateral part of the zonula for either its biosynthesis or replenishment.

Spindle-shaped myoepithelial cells lie on the basement membrane and abut the clear cells, although it is not rare for the elongated cytoplasmic processes of dark cells to come into contact with them. The myoepithelial cell is filled with masses of myofilaments and arranged more or less parallel to the course of the secretory tubules. It is rather infrequent that foot processes of two opposing myoepithelial cells are bound to each other, and for this reason, the myoepithelial cells are generally regarded as discontinuous cells. The function of the myoepithelial cell is not clear, but several hypotheses have been postulated. These include:

1. Myoepithelial contraction for expulsing the preformed sweat onto the skin surface.
2. A supportive structure for the tubule.
3. Myoepithelial contraction opens the intercellular spece in the basal labyrinth, controlling the flow of fluid and metabolites to the secretory epithelium (ELLIS, 1967).

The first hypothesis appears unlikely because during the resting state, the lumen of the secretory coil is collapsed, and thus little fluid is present to be expulsed on stimulation (SATO, K., unpublished data). Furthermore, the contraction of the secretory coil is never a repetitive and pulsatile one but continues persistently during sweat secretion in vitro in response to a given constant dose of acetylcholine. Pharmacologic responsiveness of the myoepithelial cells will be elaborated under the pharmacology section of this review.

The duct consists of an outer ring of peripheral or basal cells and an inner ring of luminal or cuticular cells. It seems that the proximal (coiled) duct is functionally more active than the distal straight portion because Na-K-ATPase

activity (SATO et al., 1971) and the number of mitochondria (ELLIS, 1967) is higher in the proximal portion. The luminal cytoplasm of the ductal cells forms a cuticular border consisting of a dense layer of tonofilaments. In a strict sense, it is not a true cuticle since the surface of the luminal cell membrane is not covered with cuticular substance, but the unit membrane of its luminal microvilli is directly exposed to the luminal space (ELLIS, 1967). The basal cells rest on the basement membrane which is continued from the secretory coil. These cells contain large nuclei, and the narrow cytoplasmic space i loaded with mitochondria, suggesting an active role in ductal Na reabsorption.

C. Innervation

The control center of sweating is situated in the hypothalamus, which acts as a thermostat for regulating the body temperature. Opinion is divided as to which afferent stimuli act as a major sudorific drive for the hypothalamic sweat center: core temperature, skin temperature, neuromuscular drive, subdermal temperature, or muscle temperature (SMILES and ROBINSON, 1971). Hypothalamic efferent sweat impulse descends through the brain stem and the spinal tract, crosses at various heights, and ends in the lateral horn where new neurons start. The nerve surrounding the sweat glands is composed of nonmyelinated class C fibers of the symphathetic post ganglionic fiber, but physiologicly and pharmacologicly, the gland behaves as if it is parasympathetic or cholinergic (DALE and FELDBERG, 1934). Recently, however, UNO and MONTAGNA (1975) successfully demonstrated the presence of a loose network of catecholamine-containing nerves around the sweat glands of monkey paws using a fluorescent histochemical method. Since the monkey palm sweat gland shows the sudorific responses to pharmacologic drugs, as does the human eccrine sweat gland in the general skin surface in vivo and in vitro (SATO 1973; SATO et al., 1973; SATO, K., unpublished data), the dual cholinergic and adrenergic innervation as demonstrated in the monkey palm sweat gland may not be confined only to this species of animal.

D. Emotional Sweating

Sweating induced by emotional stress can occur over the whole skin surface, but usually it is confined to palms, soles, axillae, and forehead. Despite the differences in its response to stimuli, there is little evidence that palmar sweat glands function differently from other sweat glands (COLLINS, 1962).

E. Denervation Hypersensitivity

It seems that disagreement among investigators regarding the presence or absence of postdenervation hypersensitivity of the sweat gland depends mainly on the animal species studied. In the human eccrine sweat gland, COON and ROTHMAN (1941) and KAHN and ROTHMAN (1942) failed to observe sweat response to

intradermal injection of nictone or acetylcholine after denervation. For this reason, it has been widely regarded that the human sweat gland is the only eception to Canon's law (the development of supersensitivity of any organ to the transmitter substances after postganglionic denervation). A more recent study by SILVER et al. (1963) on the human sweat gland also supported the above contention. However, in cat and monkey sweat glands, evidence suggests that the pharmacologic responsiveness of the denervated sweat glands conforms to Canon's law. NAKAMURA and HATANAKA (1958) showed that eccrine sweat glands in the foot pads of the cat were hypersensitive to adrenaline, nicotine, and Mecholyl (these authors measured sensitivity using the threshold concentration of the drug which elicited a minimal sweat response) for several weeks after denervation, and the effect of these drugs lasted longer than in normal foot pads. SAKURAI and MONTAGNA (1965) observed in the completely denervated hands of *Lemur mongoz* that, although the onset of response of the denervated sweat gland to Mecholyl was delayed, the duration of the sweat response was longer than in normal hands. The response to Mecholyl was present even at 13 weeks after denervation despite the demonstration that histochemically demonstrable acetylcholinesterase disappeared 4–7 days after the nerve section. Most recently, REAS and TRENDELENBURG (1967) carefully determined the threshold concentrations of acetylcholine and pilocarpine infused intra-aortically on denervated cat paw sweat glands. After denervation, they observed that changes in sensitivity to the drugs occurred in two phases. During the first 2 postoperative days, subsensitivity was noted when the glands still responded to electrical stimulation of the peripheral nerve stump. The acetylcholine content of the hairless foot pads declined to about 50% of normal, and choline acetylase activity was nearly normal. From the 4th postoperative day onward, pronounced supersensitivity was observed, by which time peripheral transmission had failed, the acetylcholine content fell below 20% of normal, and choline acetylase activity was clearly reduced. Daily pretreatment with pilocarpine prevented the development of denervation supersensitivity whereas daily pretreatment of normal cat with a ganglion blocking agent (chlorisondamine) for 4 days caused supersensitivity. These investigators thus concluded that the sensitivity of the sweat gland is inversely related to activity of the gland, inactivity of the gland causing supersensitivity and vice versa.

IV. Pharmacologic Responsiveness of the Eccrine Sweat Gland — in vivo Studies

A. The Effect of Catecholamines on Cholinergic Sweating

The responsiveness of the eccrine sweat glands to intradermal injection of acetylcholine has been well established (RANDAL and KIMURA, 1955). Until UNO and MONTAGNA (1975) demonstrated catecholamine-containing nerves as well as the terminal structure of the adrenergic nerve around the eccrine sweat gland, the cholinergic fiber had been regarded as the only nerve supplying the sweat gland (CHALMERS and KEELE, 1951; FOSTER et al., 1970; FOSTER and WEINER, 1970).

Thus, it appears that the observations by UNO and MONTAGNA (1975) may lead to revival of the old "dual innervation theory" (HAIMOVICI, 1950), and the problems concerning the functional significance of adrenergic component of innervation must be reevaluated in the future. Human eccrine sweat glands also respond to intradermal or intra-arterial adrenaline (CHALMERS and KEELE, 1951; WADA, 1950; FOSTER et al., 1970; FOSTER and WEINER, 1970). Quantitatively, adrenergic sweat is only 10% that of cholinergic sweat (SATO, 1973a). The possible interaction between acetylcholine and adrenaline has been a matter of controversy. The observations reported by various investigators on the effect of adrenaline on cholinergic sweating are often in conflict with one another. KUNO (1965) has claimed that the action of adrenaline is to potentiate the sudorific action of acetylcholine and has postulated that circulating adrenaline will potentiate the nervous response. KUNO (1965) based his postulate on the observation that iontophoresis of a mixture of acetylcholine and epinephrine yielded a much higher sweat rate than did the iontophoresis of acetylcholine alone. Using a different experimental approach, TERADA (1966), however, was unable to confirm KUNO's (1965) observation. TERADA (1966) observed that systemic administration of a small dose of adrenaline either had no effect on or actually inhibited thermally-induced sweat secretion. However, he found that sweat secretion induced by physical exercise is intensified by systemic administration of adrenaline.

FOSTER et al. (1970) and FOSTER and WEINER (1970) also failed to reproduce KUNO's (1965) data using intradermal injection instead of iontophoresis of a mixture of acetylcholine and epinephrine. Because of these disagreements, DOBSON and SATO (1972) repeated KUNO's (1965) iontophoresis experiment and were able to observe an enhancement of sweat rate by addition of adrenaline to acetylcholine only on the palm but not on the forearm. On the forerarm, the sweat rate was lowered whenever vasoconstriction was present, but on the palm, it was maximally stimulated when there was a mild vasocontriction. It was later noticed by SATO (SATO, K., unpublished data) that the sweat rates usually induced by iontophoresis of acetalcholine are in the submaximal range because intradermal injection of the same concentration of acetylcholine induces more than two times higher sweat rate when tested on the symmetric test site. Since in the submaximal sweat rate range the secretory rate may be a function of periglandular acetylcholine concentration (SATO, 1973b), it is highly possible that KUNO's (1965) observation as well as our data on the palm may simply reflect an increase in periglandular acetylcholine concentration due to a mild vasoconstriction of the dermal blood vessels (and thus decreased disappearance of acetylcholine), but the vasoconstriction was not strong enough to interfere with oxygen and nutrients supply to the gland. Nevertheless, the above explanation of the possible artifacts does not refute TERADA's (1966) observation that a systemically administered small dose of adrenaline enhanced sweating during exercise. In fact, evidence has been obtained indicating that circulating adrenaline plays an important role in the control of sweating. ROBERTSHAW et al. (1973) reported that stump tail macaques (anthropoid primates) could sweat at a rate 50% higher during exercise in heat than during heat exposure alone. When the adrenal glands of these primates were denervated, a procedure that drastically reduces the release of adrenaline during

exercise, there was no enhancement of sweat rate due to exercise. Infusion of epinephrine to these denervated macaques restored the higher sweat rate seen in normal animals during exercises.

The specificity of adrenergic sweating has been repeatedly documented (WADA, 1950; HAIMOVICI, 1950; CHALMERS and KEELE, 1951) in that priscol or dibenamine, but not atropine, blocked adrenergic sweating. It appears, therefore, that specific adrenergic receptors are present in the sweat glands. It should be note, however, that the presence of an adrenergic component in the nerves surrounding the sweat gland should not necessarily mean that adrenaline is directly involved as a sudorific agent. The only instance where the direct role of epinephrine as a sudorific agent has been assumed is in the sweat response of patients with pheochromocytomas. FOSTER et al. (1970) and PROUT and WARDELL (1969) do not favor an adrenergic mechanism of sweating during attacks of pheochromocytoma, based on their observations that hyoscine abolishes the sweat response in these patients (PROUT and WARDELL, 1969) and that the dose of intra-arterial adrenaline required to stimulate sweat secretion is rather high (FOSTER et al., 1970). Whatever the role of adrenaline, the undisputed fact is that thermal sweating as well as nervously excited sweating (including that induced by electrical nerve stimulation) are completely inhibited by atropine. Such an apparently clear-cut fact naturally leads to a simple but attractive conclusion that there are only cholinergic sudomotor fibers and thus the cholinergic mechanism in the eccrine sweat gland (CHALMERS and KEELE, 1951; FOSTER et al., 1970; FOSTER and WEINER, 1970). Nevertheless, lingering questions remain concerning the role of an adrenergic component of the sweat gland. Why is catecholamine present in the periglandular nerve? Why does the sweat gland respond to adrenaline if an adrenergic component is of no use at all? Why does adrenaline stimulate exercise-related cholinergic sweating? Why does adrenaline exert such a metabolic effect as stimulation fo the pentose cycle in vitro (SATO, 1973a)?

B. The Effects of α and β-Adrenergic Agonists and Their Respective Blockers

Adrenaline (epinephrine) as well as noradrenaline (norepinephrine) have both α and β-stimulating effects. It is, therefore, of interest to decide whether α or β-adrenergic stimulation plays the major role and whether adrenergic and cholinergic stimulations share the same receptor site at the cell membrane. As early as 1950, HAIMOVICI attempted to differentiate the effect of each type of adrenergic stimulation on human eccrine sweat glands using an α-blocker (dibenamine) and a β-stimulator (isoproterenol). Although he observed a sudorific response in 30% of human subjects after intradermal injection of isoproterenol, the observation that adrenaline-induced sweating is completely blocked by dibenamine led him to conclude that the α-effect of adrenaline is responsible for the sudorific response to adrenergic drugs.

Since HAIMOVICI's (1950) study, there have been several other publications that

have dealt with this subject. Most of these investigators used intradermal injections of agonists and antagonists, as did HAIMOVICI (1950), although more refined methods were used to measure the secretory rate. The sudorific effect of isoproterenol has been later confirmed by SONNENSCHEIN et al. (1951) and recently by WARNDORFF (1972). Its effect is blocked by a β-blocker (propranolol) but not an α-blocker (phentolamine) (WARNDORFF and HAMAR 1973). The inhibitory effect of other α-blockers (guanethidine, phentolamine) has also been shown on adrenaline-induced sweating (FOSTER and WEINER, 1970). FOSTER and WEINER (1970) reported that phentolamine, an α-adrenergic antagonist, strongly inhibits cholinergic sweating in man and used this data as evidence that only cholinergic receptors are present in the sweat gland and that phentolamine at high concentrations (0.5–5 mM) blocks the cholinergic receptors. While FOSTER's and WEINER's (1970) postulate may well be worth considering, it is imperative to exclude several alternative interpretations of the observation before reaching such a conclusion. One of the alternatives is the possible action of phentolamine as an inhibitor of energy metabolism, which will be elaborated on in the following section.

In recent years, several investigators claim to have made new observations. Many of these studies were conducted using intradermal injection of the drugs, and periglandular conditions (i.e., periglandular durg concentrations, blood circulation, and thus oxygen and nutrient supply, periglandular electrolyte concentration, skin temperature, endogenous inhibitors, or stimulators, etc) were poorly defined or meticulous control studies were omitted. The confirmation or rebuttal of such observations must, therefore, rely on more refined metholodogy and more precise experimental protocols. For example, HEMELS (1970) claimed that in normal, but not atopic, subjects propranolol increases the intensity of the response of the sweat glands to local injections of acetylcholine. With this observation alone, he postulated that the β-receptor of the sweat gland performs an inhibitory regulatory role for chlinergic sweating. FOSTER et al. (1971), howerver, failed to confirm HEMELS' (1970) observation using a more convincing experimental approach. Because of its potential importance, SATO also studied the effect of propranolol on cholinergic sweating (SATO, K., unpublished data). He used the same doses of propranolol and acetylcholine as HEMELS, but the sweat secretion was induced under oil to avoid evaporative loss, and the sweat droplets were collected by using an oil-filled glass capillary. Interestingly, the area of sweating was consistently larger when proranolol was added to acetylcholine, but the sweat rate per gland near the injected area, where the secretion was the strongest, was the same in the presence or absence of propranolol. Such an observation can be easily explained either by the feeble anticholinergic action of propranolol (as was suggested by FOSTER et al., 1971) or by a possible mild vascular constriction (and thus the reduced diffusive loss of the injected acetylcholine), presumably brought about by selective inhibition of β-component, if any, of endogenous sympathetic tonus (GRANATA et al., 1970). The lack of any stimulating effect of propranolol on an isolated sweat gland stimulated with acetylcholine or the lack of any inhibitory effect of isoproterenol on cholinergic sweating in vitro (see Fig. 17) are in keeping with our unpublished in vivo data and the study of FOSTER et al. (1971). As a conclusion, the β-receptor inhibitory control hypothesis of HEMELS (1970) should be withheld until undisputable evidence is presented.

C. Some Factors Affecting the Quantitative Response of Human Sweat Gland to Intradermal Injection of Acetylcholine

Despite the technical as well as the theoretical uncertainty concerning the local sweat induction by intradermal injection of sudorific drugs, such a method will continue to be employed in clinical or physiologic investigations. FOSTER (1971) made a systematic study of factors that affect the sweat response to intradermally injected acetylcholine. They are: (1) the uniformity of sweating within the test site (which must be achieved by selecting the appropriate size ventilated capsule, i.e., the size of the test area, the volume, and the concentration of drugs to be infused and the method and depth of injection), (2) the construction of dose-response curves, (3) the temperature of the infusate should be the same as that of the skin, 36° C. In addition, the electrolyte composition of the infusate may become important, especially when a large volume of solution is infused. Ca^{++} may be the most important ion because the removal of periglandular Ca^{++} results in complete inhibition of sweat secretion in vitro (SATO, K., unpublished data; see Fig. 23). It is, therefore, essential that these experimental conditions and protocols should be standardized in each laboratory so that reproducibility is established. Even if these factors are satisfactorily controlled, there is little hope that a uniform periglandular drug concentration can be maintained in an in vivo condition. A critical problem may arise if the injected drugs modify blood circulation, as in the case of adrenergic drugs.

V. Pharmacologic Responsiveness of an Isolated Single Eccrine Sweat Gland — in vitro

The theoretical as well as technical problems that surround in vivo sweat gland study have long impeded more precise understanding of the mechanism of sweat formation. It was, therefore, mandatory to develop a new in vitro technique by which the mechanism of secration can be sudied in well-defined experimental conditions. The author (SATO, 1973b) thus attempted to apply the in vitro techniques originally developed for an isolated kidney tubule (BURG et al., 1966) to the siolated single eccrine sweat gland. Eccrine sweat glands are isolated from monkey palm biopsy specimens and are further dissected in order to study the function of each segment (see Figs. 4 and 5). The validity of the in vitro method is evidenced by the demonstration that acetylcholine (or Mecholyl) -induced sweating is completely inhibited by atropine, low medium temperature (4° C), an inhibitor of active transport (ouabain), or metabolic inhibitors such as cyanide or dinitrophenol. Sweat samples collected directly from the segment of the secretory coil contain Na^+ and K^+ isotonic to plasma, whereas those collected from the open end of the proximal (coiled) duct show Na^+ concentrations lower than 145 mM (20–80 mM) and K^+ higher than 5 mM (5–25 mM), indicating ductal reabsorption of Na^+ and possible secretion of K^+ into the sweat (SATO, 1973b) (Fig. 6).

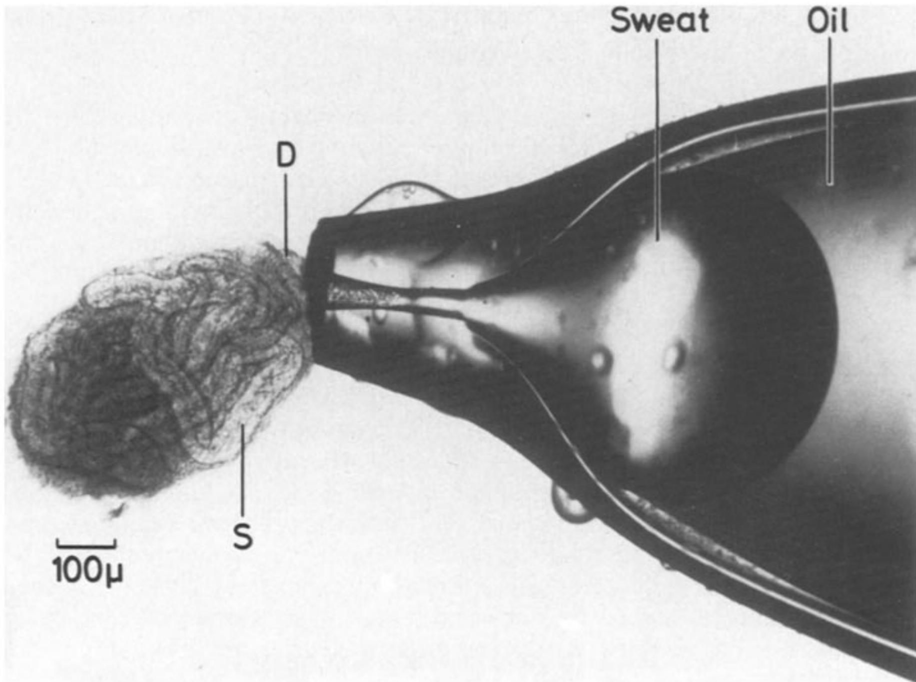


Fig. 4. Photograph of in vitro sweat induction from an isolated eccrine sweat gland (from SATO, 1973b). D: duct, S: secretory coil

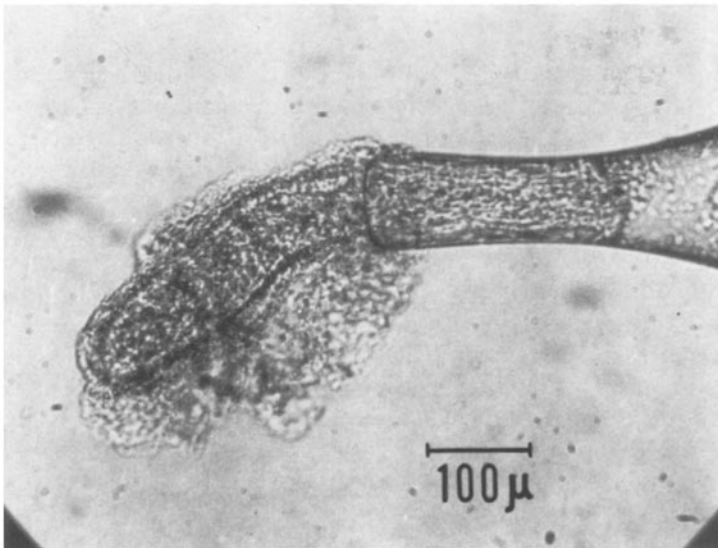


Fig. 5. Photograph of in vitro sweat induction from an isolated segment of the secretory coil

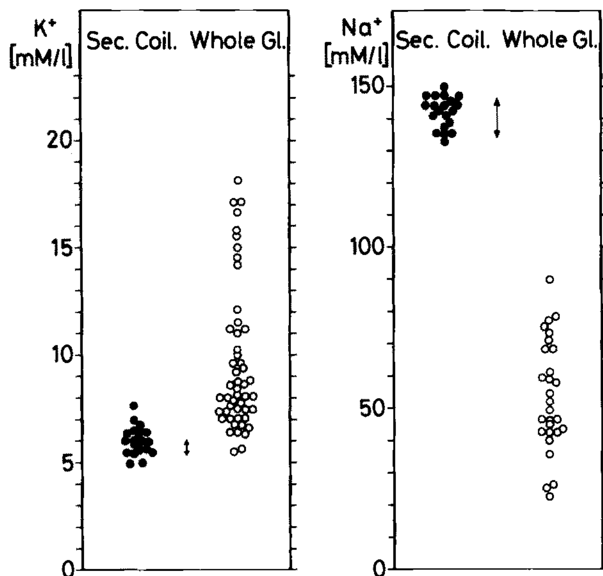


Fig. 6. Comparison between concentrations of Na and K in the secretory coil primary sweat and the sweat collected from the open end of the proximal tubule ("whole gland" in Fig.). The arrows indicate the range of simultaneously measured concentrations of Na and K in the incubation bath (from SATO, 1973b)

As has been described earlier in Section C of this article, the monkey eccrine sweat gland serves as an ideal experimental model for the human eccrine sweat gland. Because of the tissue availability, functional stability in vitro, sameness of function as the human sweat gland, relative uniformity of function and size, etc., we chose to use the simian palm eccrine sweat glands to a greater extent. Since most of the data are relatively new and have not yet been published, emphasis will be placed only on some essential points of interest pertinent to the mechanism of secretion.

A. Response to Cholinergic and Adrenergic Drugs

Most isolated sweat glands maintain relatively constant secretory activity for several hours under constant optimal incubation conditions such as temperature (37° C), medium (Krebs-Ringer bicarbonate), nutrient (glucose 5.5 mM), protein (0.5% bovine albumin), vigorous stirring of the medium with gas lift (5% CO_2+ 95% O_2), the concentration of acetylcholine (0.5–1×10^{-6} M), pH (7.4). In a previous paper (SATO, 1973b), it was tentatively suggested that the siolated sweat gland responds quantitatively equally to both acetylcholine and epinephrine (adrenaline) in an in vitro condition. However, it was later found that the earlier conclusion does not necessarily apply to all the sweat glands thus far studied; some glands do respond equally to both drugs, but the other glands respond rather poorly to epinephrine (Fig. 7). In the experiment shown in Figure 7,

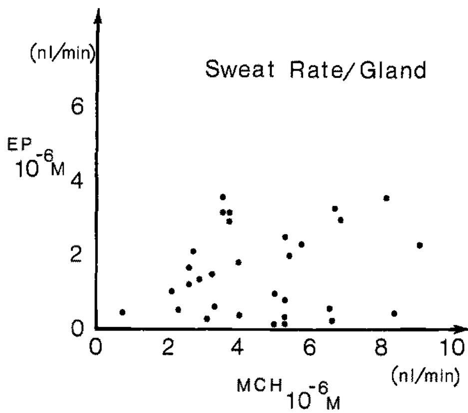


Fig. 7. Comparison of epinephrine (EP)-induced and Mecholyl (MCH)-induced sweat rates in isolated monkey sweat glands in vitro. Each plot represents a single sweat gland. Sweat rate is expressed as nl/min/gl. See text for detail

the sweat gland was first stimulated with epinephrine 10^{-6} M, a concentration range which usually gives a maximal secretory response; then the incubation medium was changed to a new medium containing 10^{-6} M Mecholyl. The secretory rates at the plateau level were plotted for both drugs. As can be seen in Figure 7, the majority of the glands showed a stronger secretory response to Mecholyl than to epinephrine. The following question arose: is the responsiveness to epinephrine a fixed property of a gland or does it change under certain circumstances? For those glands which exhibited a poor response to 10^{-6} M epinephrine, a higher dose of the drug did not particularly enhance the secretory rate. However, as shown in Figure 8, the same sweat gland showed an enhanced secretory response to the same dose of epinephrine after the sweat gland had previously been stimulated in a Mecholyl-containing medium. The enhanced secretory response to the second adrenergic stimulation may not be due to residual effect of Mecholyl or contamination of Mecholyl because the medium was washed several times and the new medium contained 10^{-7} M atropine. Figure 9 summarizes the quantitative response

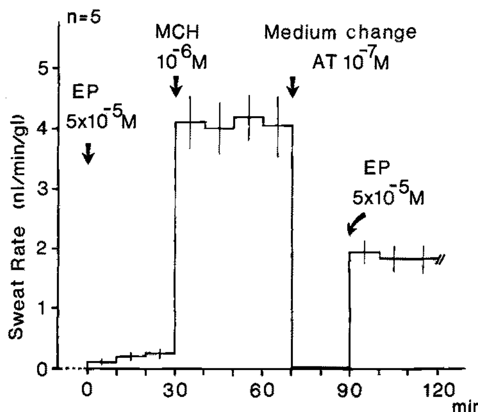


Fig. 8. Responsiveness of an isolated sweat gland to epinephrine before and after Mecholyl stimulation. N: the number of sweat glands studied, AT: atropine. At 70 min, the incubation medium was replaced by 10^{-7} M atropine-containing medium. At 90 min, epinephrine was added to the incubation bath. Throughout this article, the vertical bar indicates ±SEM

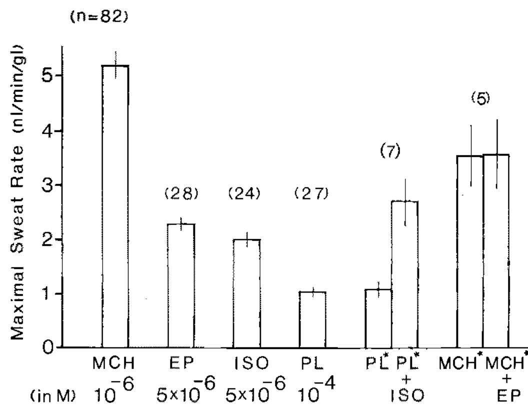


Fig. 9. Comparison of maximal sweat rates induced by various pharmacologic drugs. All drug concentrations are expressed in mol/l (M). ISO: isoproterenol, PL: phenylephrine. The asterisk indicates separate experiments where the sweat glands were stimulated first by a single drug (PL or MCH) and later by a second drug (ISO or EP). The numbers in parentheses are the number of experiments

of the isolated sweat gland to cholinergic and adrenergic drugs. Epinephrine-induced mean secretory rate was approximately half that of Mecholyl stimulation. Interestingly, both an α (PL, phenylephrine) and a β-adrenergic stimulant (ISO, isoproterenol) induced fluid secretion. The effect of isoproterenol was slightly lower than that of epinephrine but was two times higher than that of phenylephrine. There was a partial additive effect when isoproterenol was added to phenylephrine-containing media. However, there was no further stimulation when epineprine was added to Mecholyl-containing media. As already shown in Figure 8, the converse is not true; when Mecholyl was added in the presence of epinephrine in the bath, the sweat rate was enhanced to the level of Mecholyl stimulation alone. Since the concentration of α or β-adrenergic agonist used was already a suprasaturation concentration and yet there was a partial additive effect between them, it may be tentatively suggested that the sweat gland has two separate adrenergic receptors, α and β, and the cholinergic receptor.

A number of questions arise at this point. What is meant by the so-called receptors? Are they the binding sites for different stimulants? If so, what events follow in the membrane or in the cell as a result of the drug-receptor interaction? Why does the stimulation by different drugs result in the same consequence, the secretion of isotonic fluid, but differ in quantity? Are second messenger(s) involved after stimulation? Before considering these questions, however, we must frist examine the specificity of adrenergic α and β-receptors and their respective stimulants by means of respective inhibitors.

B. The Effect of Adrenergic α and β-Antagonists on Adrenergic α and β-Agonists and Cholinergic Drugs

Contrary to our expectation before beginning this series of experiments, we found a cross interaction between adrenergic agonists and antagonists. As shown in

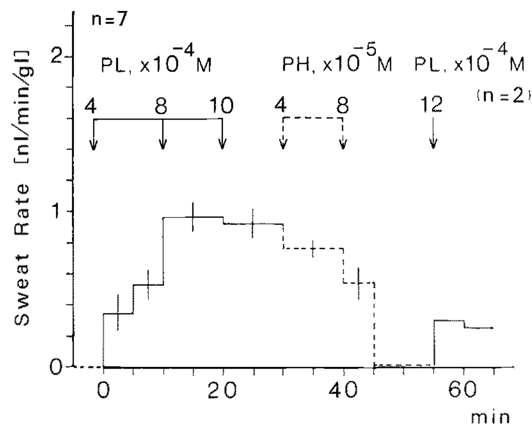


Fig. 10. Effect of phentolamine (PH, α-adrenergic antagonist) on phenylphrine (PL, α-adrenergic agonist)-induced sweat secretion in vitro. The drugs were added to the medium as indicated by the arrows

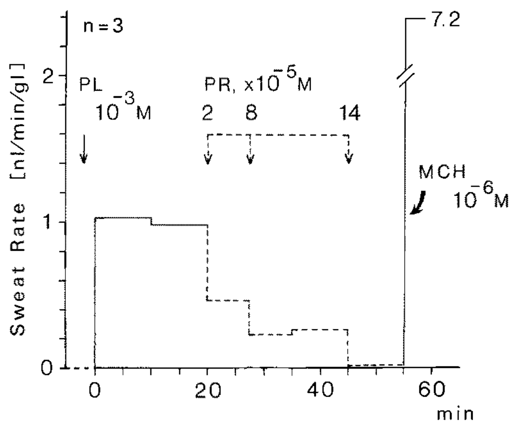


Fig. 11. Effect of propranolol (PR, β-adrenergic antagonist) on phenylephrine (PL, α-agonist)-induced sweat secretion in vitro. The mean of three experiments

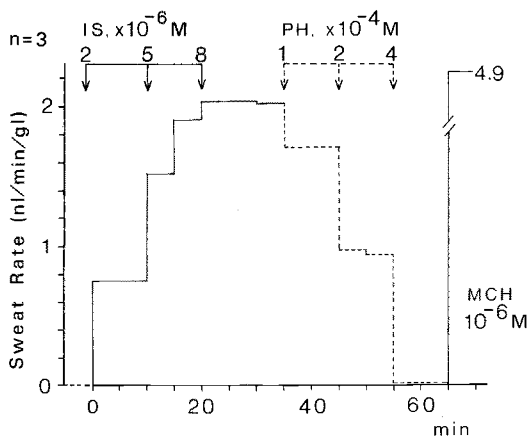


Fig. 12. Effect of phentolamine (PH, α-antagonist) on isoproterenol (IS, β-agonist)-induced sweat secretion in vitro. The mean of three experiments

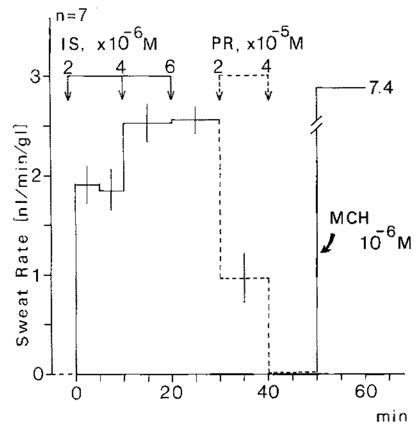


Fig. 13. Effect of propranolol (PR, β-antagonist) on isoproterenol (IS, β-agonist)-induced sweat secretion in vitro

Figures 10–13, we matched the two agonists and the two antagonists. The final concentrations of agonists chosen were those which gave the maximal secretory response. When the maximal secretory response was obtained, the antagonists were added to the (agonists-containing) incubation medium in small steps until a complete inhibition of secretion was achieved. For 10^{-3} M α-agonist (PL, phenylephrine), which gave the maximal secretory response, 8×10^{-5} M α-antagonist (PH, phentolamine) was needed to block sweat secretion, whereas a slightly higher concentration of β-antagonist (PR, propranolo, 1.4×10^{-4} M) was required to inhibit the action of the α-agonist (Figs. 10 and 11). In contrast, the β-agonist (IS, isoproterenol, 6.8×10^{-6} M)-induced fluid secretion was blocked by propranolol (β-antagonist) at a concentration (4×10^{-5} M) one order of magnitude lower than that of the α-antagonist (phentolamine, 4×10^{-4} M). Thus, despite the presence of a strong cross inhibition, it is clear that the effect of β-agonist is more sensitive to the β-antagonist than to the α-antagonist.

The concentration of phentolamine, 4×10^{-4} M, needed to block the effect of isoproterenol is rather high, and it is not certain that phentolamine at this high concentration works in a pharmacologically specific way. This question arose partly because FOSTER and WEINER (1970) reported that infusion of 0.5–5 mM phentolamine into the dermis blocks even in vivo cholinergic sweating in humans. FOSTER's and WEINER's (1970) report prompted us to test the effect of phentolamine on cholinergic sweating using our in vitro system. Varying doses of Mecholyl were matched with different doses of phentolamine. After constant Mecholyl sweating was maintained for 20–30 min., various concentrations of phentolamine were added to the incubation bath at time zero (Fig. 14). Regardless of concentrations of Mecholyl, 2×10^{-4} M phentolamine was not significantly effective, but at 10^{-3} M phentolamine, the sweat rate was drastically suppressed. The subsequent washout of the incubation bath and addition of 10^{-4} M Mecholyl did not reverse the phentolamine-induced inhibition in four of six sweat glands studied and only partially reversed it in the other two glands. These observations lead us to suspect that the effect of phentolamine at high concentrations (e.g., 10^{-3} M) might be nonspecific, presumably through inhibition of membrane transport or energy metabolism. If so, the ductal function should also be affected by phentolamine, and in fact this was found to be the case. As is shown in Figure 15, phentolamine-induced suppression of sweat rate was accompanied by a high sweat sodium concentration when sweat was collected from the open end of the proximal tubule, whereas this was not the case after inhibition of secretion with atropine. Since sodium (Na) concentration of the precursor fluid is always isotonic (Fig. 16), regardless of sweat rate or the presence or absence of inhibitors, the observation shown in Figure 18 indicates the inhibition of ductal Na

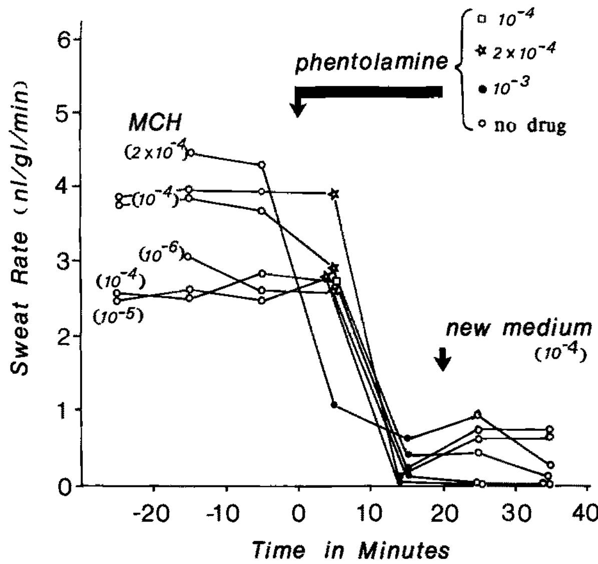


Fig. 14. Effect of phentolamine (α-antagonist) on cholinergic sweat secretion induced by various doses of Mecholyl (MCH) in vitro experiments. The numbers in parentheses are the concentrations of MCH. Each symbol indicates a different concentration of phentolamine. Phentolamine was added to the medium at time zero. Each line represents a single experiment

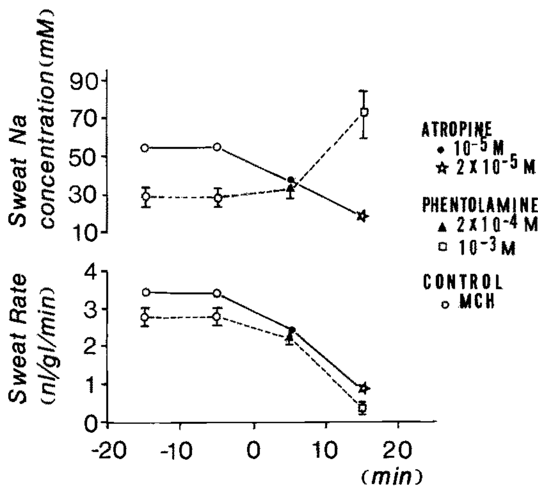


Fig. 15. Effect of atropine and phentolamine on Na concentration and sweat rate of the proximal ductal sweat in vitro experiments. The drugs were added at time zero

reabsorptive function by 10^{-3} M phentolamine. Whether this nonspecific action of phentolamine can explain the inhibition of isoproterenol-induced sweat secretion is not known at present. However, in the latter case the sweat gland still retained responsiveness to Mecholyl after phentolamine addition (Fig. 12), indicating that the so-called "nonspecific inhibiting effect" can not fully account for the blockade of isoproterenol's action by phentolamine.

HEMELS' (1970) postulate that propranolol enhances the effect of acetylcholine was directly tested on the isolated sweat gland in vitro (Fig. 17). For this purpose,

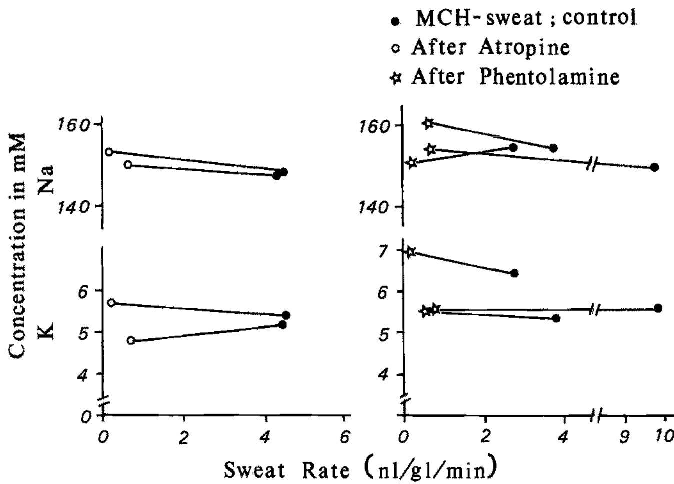


Fig. 16. Effect of phentolamine and atropine on sweat Na concentration of primary sweat. Sweat samples were collected directly from the secretory coil. In vitro experiments

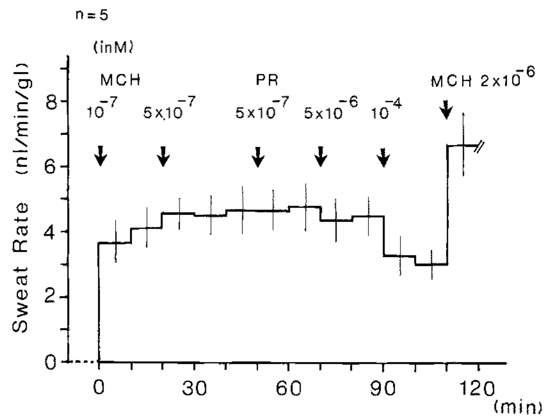


Fig. 17. Effect of propranolol (PR) on Mecholyl-induced sweat secretion in vitro. The submaximal concentration of MCH was first used to examine whether sweat rate was further enhanced by propranolol

a submaximal dose of Mecholyl was chosen (5×10^{-7} M) and varying doses of propranolol were subsequently added to the incubation bath. In Figure 17, it can be seen that propranolol did not enhance the secretory rate further; instead, it tended to suppress, although very slightly, the secretion at its high concentration, 10^{-4} M. The subsequent addition of a higher dose of Mecholyl, 2×10^{-6} M, induced a maximal secretory rate, indicating that the initial dose of Mecholyl was indeed a submaximal concentration. Furthermore, the absence of any inhibitory effect of isoproterenol on Mecholyl-induced sweat secretion (SATO, K., unpublished data) may add to the weight of evidence against the idea of the β-receptor inhibitory control theory of HEMELS (1970).

In summary, the isolated eccrine sweat gland responds to cholinergic as well as both adrenergic α and β-stimulations. The order of the sudorific effect is Mecholyl (or acetylcholine) > epinephrine ($\alpha + \beta$) $\simeq$ isoproterenol (β) > phenyl-

eprine (α). The effects of adrenergic agonists were inhibited by the respective antagonists, although there was a cross interaction (inhibition) at a higher concentration of antagonists. A high concentration of phentolamine (10^{-3} M α-antagonist) is inhibitory to both cholinergic and adrenergic stimulations, which is presumably due to the nonspecific action of this drug. In the in vitro system, propranolol (β-inhibitor) neither enhanced nor inhibited cholinergic sweat secretion.

C. Current Status of Salivary Gland Research Concerning the Effect of α and β-Adrenergic Stimulations and the Role of Cyclic AMP (cAMP)

Salivary glands have been subjected to innumerable physiologic and biochemical studies. The abundant information derived from these studies is helpful in conducting sweat gland research because both exocrine glands share many features. It is appropriate, therefore, to briefly review the current status of the pharmacology of the salivary glands in order to compare them with that of the eccrine sweat gland.

Cyclic adenosine 3,5′-monophosphate (cAMP) is present ubiquitously throughhout the animal kingdom, as well as in a number of microorganisms and plays a crucial role in endocrinologic regulation of cell function (SUTHERLAND, 1970). Evidence indicates that cAMP is the intracellular mediator of the activation of the β-adrenergic receptor in the rat parotid gland (BDOLAH et al., 1964; BDOLAH and SCHRAMM, 1965; SCHRAMM, 1967; BATZRI et al., 1971). Using the tissue slice, BATZRI et al. (1971) and recently MANGOS et al. (1975b) using isolated acinar cells observed that the activation of the β-adrenergic receptor by isoproterenol in the parotid acinar cells resulted in release of amylase, whereas the stimulation of the α-receptor by epinephrine plus propranolol caused a rapid flux of K^+ out of the cells. In contrast, K release is not observed after stimulation with isoproterenol (β). Dibutyryl-cAMP (db-cAMP) also stimulated secretion of amylase at concentrations of 10^{-8} M–10^{-5} M, and its effect was not blocked by the β-adrenergic antagonist, propranolol (MANGOS et al., 1975b). It was thus concluded that the two different adrenergic receptors, α and β, serve two separate functions: the α-effect causes K^+ efflux and fluid secretion as does cholinergic stimulation (MANGOS et al., 1975a; BATZRI and SELINGER, 1973; BATZRI et al., 1973; SELINGER et al., 1973; SCHRAMM and SELINGER, 1975), and β-stimulation, mediated by cAMP, causes amylase secretion. Similar differential functions of α and β-receptors have been postulated in heart muscles where α-stimulation causes increased K^+ fluxes and β-stimulation is correlated with an increased cAMP and the iontropic effect of the muscle (DANIEL et al., 1970).

In the salivary gland, micropuncture studies have shown that both cholinergic and β-adrenergic stimulations yield a plasma-like isotonic precursor fluid (YOUNG and MARTIN, 1971), although the maximal flow rate is much less after β-adrenergic stimulation than after cholinergic stimulation. To my knowledge, no comparative study has been made of the effect of selective stimulation of cholinergic and α-adrenergic receptors in the salivary gland fluid secretion in vivo, although BATZRI et al. (1973) demonstrated that injection of phenylephrine (α) into the rat produced three times as much saliva as that of isoproterenol. Question

arises as to whether the K efflux from the gland slices, which is widely used as a measure of cholinergic or α-adrenergic response in in vitro systems, is quantitatively related to secretion of the primary saliva. To make the matter complex, WOJCIK et al. (1975) and more recently LESLIE et al (1976) have demonstrated a strong amylase secretion by both cholinergic as well as α and β-adrenergic stimulations in rabbit or rat parotid slices. Taken together, it appears that both fluid and amylase secretion can result, although to varying degrees, no matter which receptor is stimulated, thus necessitating the further search for other cellular second messengers which lead to common end results. WOJCIK et al. (1975) observed a marked increase in tissue cGMP levels after cholinergic and α (but not β)-adrenergic stimulations but interpreted this effect of α-stimulation as due to endogenous generation of acetylcholine because it was blocked by atropine. At this writing, the place of cGMP as a candidate for the second messenger remains to be established.

The involvement of cAMP in adrenergic (most presumably β) mechanism of fluid secretion is suggested by the fact that db-cAMP infused arterially can induce a low sustained salivary secretion in the mouse sublingual (but not parotid) gland (VREUGDENHIL and ROUKEMA, 1975). In the latter case, theophylline also stimulated a slow secretion of saliva. As in many other tissues, β-adrenergic stimulation results in an elevated tissue level of cAMP (LESLIE et al., 1976). Cholinergic stimulation is the most potent stimulant of isotonic primary saliva and amylase secretion in absolute amounts. However, cholinergic as well as α-adrenergic stimulations are not associated with the tissue accumulation of cAMP (LESLIE et al., 1976) but are accompanied by Ca^{++} uptake (ROSSIGNAL et al., 1974; KOELZ et al., 1977) by the tissue. Although it is not yet completely clear how to integrate these and other biochemical events after cholinergic and adrenergic stimulations to construct a schematic mechanism of secretion, the concensus of opinion at this writing appears to be that both Ca^{++} and cAMP are second messengers in the salivary gland; cholinergic and α-adrenergic stimulations achieve both fluid and amylase secretion by stimulating the influx of Ca^{++} into the acinar cell, whereas the effect of β-adrenergic stimulation is mediated by cAMP (SCHRAMM and SELINGER, 1975; LESLIE et al., 1976; Koelz et al., 1977). The further roles of influxed Ca^{++} or endogenous cAMP in the overall secretory scheme at the cellular level are totally unknown, but the reader is referred to the hypothetic schemes by SCHRAMM and SELINGER (1975).

The salivary glands are more complex functionally and structurally than the eccrine sweat glands in that the former are predominantly enzyme (amylase)-secreting glands, whereas sweat glands mainly secrete water and electrolytes and structurally are simple tubules. It is in this context that the eccrine sweat gland secretory coil has the potential of being a superior experimental model of exocrine gland function.

D. Some Observations Which Suggest that cAMP is the Second Messenger of Adrenergic Sweating

Although we previously failed to induce eccrine sweat secretion in vivo by intradermal injection of db-cAMP in man (SATO, 1974), this was probably due to the

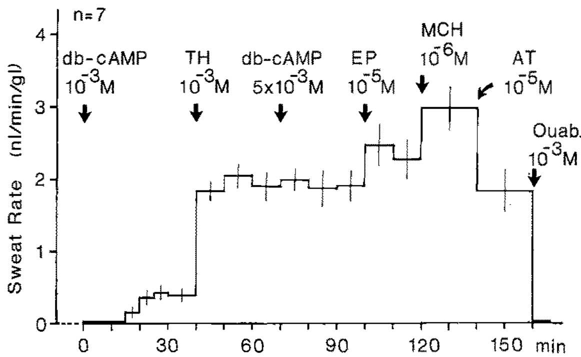


Fig. 18. Stimulation of sweat secretion by dibutyryl cAMP (db-cAMP) and theophylline (TH). Ouab, ouabain in vitro experiments

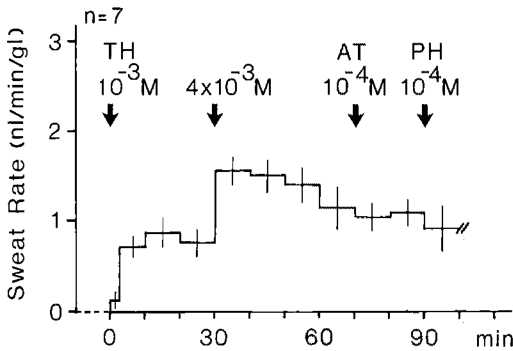
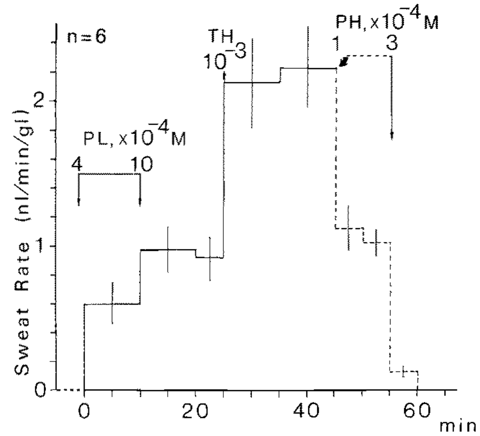


Fig. 19. Stimulation of sweat secretion by theophylline in vitro. AT: atropine, PH: phentolamine

poor permeation of this compound across the secretory cell membrane. As shown in Figure 18, the sweat secretion due to 10^{-3} M db-cAMP (from an isolated sweat gland in vitro) started only after a latent period of 15–20 min. The cAMP-induced sweat rate was low and steady, being about one-tenth that of cholinergic sweating. 10^{-3} M theophylline markedly enhanced the sweat rate, but the addition of epinephrine did not consistently increase the secretory rate. The effect of theophylline, a phosphodiesterase inhibitor, may be partially due to inhibition of the enzymic degradation of exogenous db-cAMP but mainly due to its inhibitory effect on the destruction of endogenous cAMP. As illustrated in Figure 19, theophylline itself is a potent stimulant of eccrine sweat secretion, which is inhibited neither by atropine nor by phentolamine, an α-adrenergic antagonist. Since theophylline is not known to posses a direct pharmacologic action other than its inhibitory effect on phosphodiesterase, the results in Figure 19 can be best interpreted as due to endogenous cAMP. The effect of theophylline was further studied on epinephrine ($\alpha+\beta$) and phenylephrine (α)-induced sweat secretion. Whereas phenylephrine-induced fluid secretion was consistently stimulated by theophylline (Fig. 20), that of epinephrine was not; only three out of 12 glands were stimulated further by theophylline (Fig. 21). However, as can be seen in Figures 20 and 21, the mean secretory rates were similar between epinephrine plus theophylline and phenylephrine plus theophylline, which suggests that there is a limit to the maximal sweat rate inducible by the cAMP-mediated process and that

Fig. 20. Stimulation of phentolamine (PL)-induced sweat secretion by theophylline (TH) in vitro. Note the complete inhibition of sweat secretion at a high phentolamine concentration



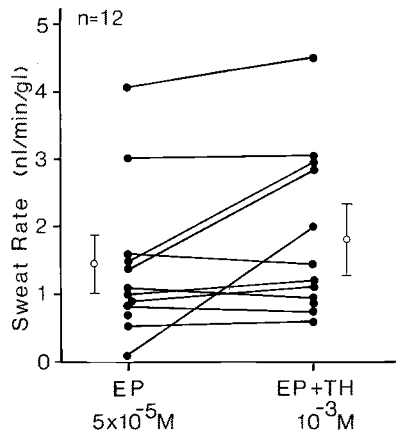


Fig. 21. Effect of theophylline (TH) on epinephrine (EP)-induced sweat secretion in vitro. Experimental protocol is the same as in Fig. 20

theophylline simply raised the phenylephrine sweating to that maximal level. Another observation that suggests a role for cAMP in the stimulation of sweat secretion is the sudorific effect of prostaglandin E_1 (PGE_1). Although investigation is still in its preliminary stage, the maximal secretory rate achieved by PGE_1 is comparable to that of cholinergic stimulation. At 10^{-4} M PGE_1, a sustained high sweat secretion is achieved but a higher concentration, 5×10^{-4} M, appears to irreversely suppress fluid secretion after a period of profuse secretion (Fig. 22). Since PGE_1 is a potent stimulant of adenylcyclase and thus cAMP production in many tissues and its presence is ubiquitous (HITTELMAN and BUTCHER, 1973), we tentatively propose that the effect of PGE_1 on the sweat gland is also mediated by cAMP.[1] If so, it is puzzling to see the large difference in the secretory rate between that of maximal epinephrine sweating and PGE_1-induced sweating.

There appears to be no negative interaction between PGE_1 and adrenergic drugs since PGE_1 fully potentiates the sudorific action of epinephrine in the

[1] Our more recent study has shown a significant correlation between the magnitude of secretory response due to adrenergic drugs and PGE_1 and the tissue level of cAMP.

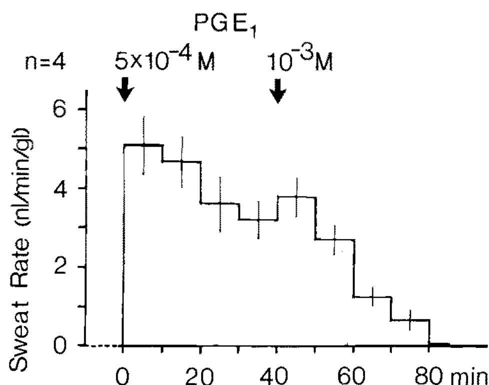


Fig. 22. Effect of a high concentration of prostaglandin E_1 (PGE_1) on sweat secretion in vitro

sweat gland (SATO, K., unpublished data). A negative interaction between PGE_1 and vasopressin has been known to occur, for example, in the toad bladder (FLORES et al., 1974), where each drug activates its own adenylcyclase system, but PGE_1 inhibits the vasopressin-activated adenylcyclase as well as the pharmacologic effect of vasopressin.

Overall, the proposed hormone receptor-second messenger (e.g., cAMP or GMP and/or Ca^{++}) relationship is yet to be proven biochemically or by an isotope flux study. For example, study of the effect of the above pharmacologic agents on the tissue level of cyclic nucleotides will help us determine whether the tentative separation of adrenergic receptors into α and β in the sweat gland is a worthwhile approach.

E. The Pivotal Role of Ca^{++} in the Secretory Processes of the Eccrine Sweat Gland

The critical role of Ca^{++} has been established in many secretory cells which perform a secretory process called exocytosis, i.e., the release of the content of membrane-bound cytoplasmic granules out of the cell by membrane fusion. In these cells, a rise in the concentration of free calcium ions at some critical sites within the cell in response to the physiologic stimuli triggers the exocytotic processes by an unknown mechanism (DOUGLAS, 1968, 1971). The importance of Ca^{++} in exocytosis has also been postulated in the mechanisms of enzyme secretion by the exocrine pancreas (WILLIAMS, 1974; KONDO and SCHULZ, 1976a, 1976b). Except in the insect salivary gland (PRINCE et al., 1973), the role of Ca^{++} has not been firmly delineated in the processes of water and electrolyte secretion in serous exocrine glands. As a preliminary approach, we first tested the effect of Ca^{++} in the incubation bath and then the effect of the calcium ionophore A23187, which acts as a mobile carrier to transfer calcium across cell membranes (REED and Lardy, 1972). Figure 23 shows the effect of Mg^{++} and Ca^{++} removal from the bath on the sweat rate from the isolated sweat gland. When a complete medium (KRB) was replaced by a Mg^{++} free medium, there was no inhibition of the secretory rate. Further addition of Ca-EDTA did not significantly reduce the

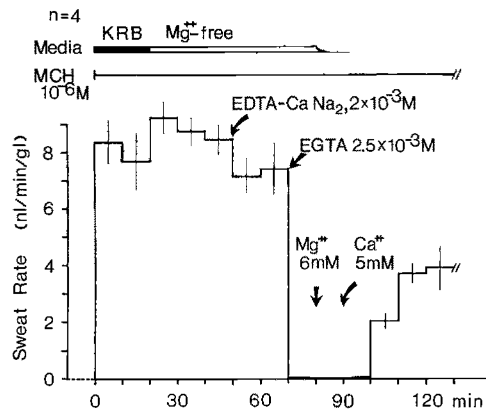


Fig. 23. Effects of extracellular Mg^{++} and Ca^{++} on MCH-induced sweat secretion in vitro. At 20 min, the incubation medium was changed from normal Kreb's Ringer (KRB) to Mg^{++}-free medium. EDTA (disodium-calcium salt) and EGTA were added to the Mg^{++}-free (but Ca^{++}-containing) medium as indicated by the arrows

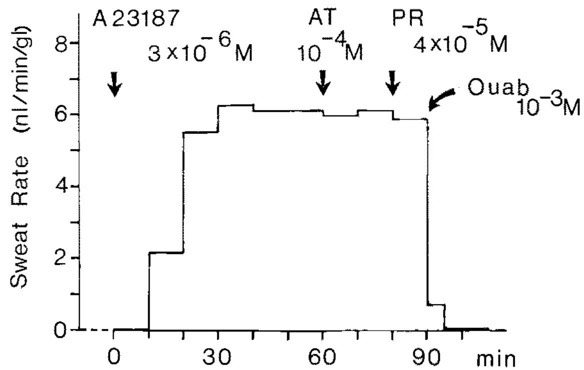


Fig. 24. Stimulation of sweat secretion by a calcium ionophore A23187 and its response to atropine (AT), propranolol (PR), and ouabain in vitro. An illustrative example

secretory activity either. However, when Ca^{++} was chelated with EGTA, the sweat secretion stopped very rapidly and completely. The addition of 6 mM Mg^{++} to the same incubation bath failed to resume secretory response, whereas that of the subsequent addition of 5 mM Ca^{+} did.

The results indicate that extracellular Ca^{++} but not Mg^{++} is essential for maintaining secretion. As shown in Figure 24, the Ca ionophore A23187 at 3×10^{-6} M strongly and persistently stimulated sweat secretion after a latent period of 10 min. The A23187-induced sweat secretion was inhibited by ouabain, an inhibitor of active transport, but not by atropine or propranolol. Thus, it is apparent that Ca^{++} introduced (presumably) into the cell by A23187 stimulates the secretory process at a point beyond the site of action of cholinergic or adrenergic drugs. It must also be emphasized that A23187 is capable of inducing secretion quantitatively as efficiently as acetylcholine, although a high concentration (2×10^{-4} M) of the ionophore seems to damage the cell function after 20–30 min incubation (Fig. 25). Like acetylcholine, A23187 requires Ca^{++} in the external medium. As shown in Figure 26, in the absence of Ca^{++} in the incubation medium, A23187 does not stimulate secretion, but the secretion is resumed on addition of Ca^{++} to the medium. The experiments in Figure 26 can also be interpreted to indicate that, unlike the insect salivary gland (PRINCE et al., 1973),

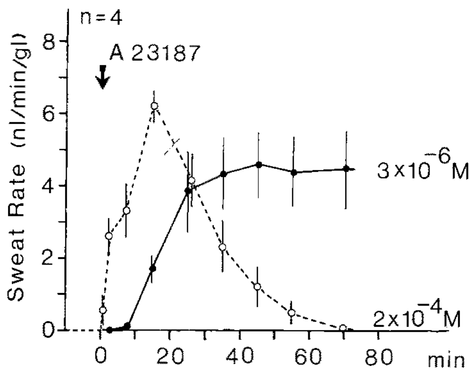


Fig. 25. Effect of 3×10^{-6} M and 2×10^{-6} M A23187 on secretory rate in vitro

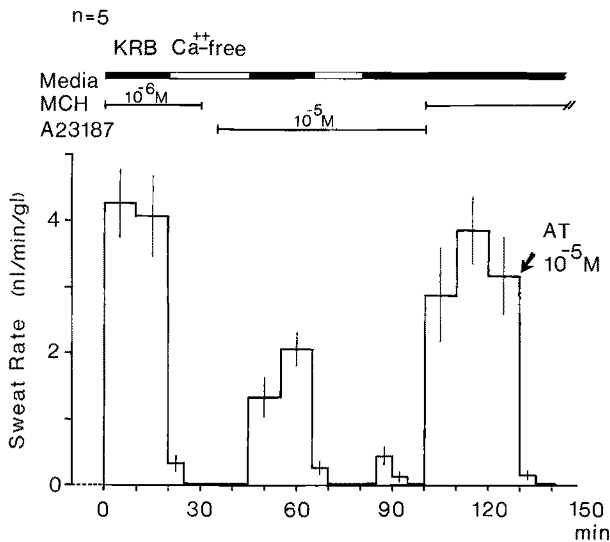


Fig. 26. Effect of Ca^{++}-free medium on MCH- and A23187-induced sweat secretion in vitro. Note that the presence of Ca^{++} in the medium is essential for both MCH- and A23187-induced sweat secretion

the ionophore A23187 cannot mobilize enough cytoplasmic sequestered Ca^{++}, if any, to critical sites in the cell to stimulate secretion and that the sweat gland depends entirely on the extracellular Ca^{++}. Although our present data strongly indicate that the effect of A23187 on eccrine sweat secretion is to facilitate influx into the cell of extracellular Ca^{++}, direct proof must be sought in the future by means of isotope studies as in the insect glands (PRINCE et al., 1973) or in the pancreas (SCHREURS et al., 1976).

The other nonspecific ionophore for divalent cations, X537A, did not show any sudorific effect in vitro over a wide range of concentrations, an observation for which we have no explanation at present. The effect of A23187 on other sudorific agents such as adrenergic drugs, theophylline, cAMP, PGE, etc., simulates that of acetylcholine on these drugs, i.e., the addition of A23187 to a feebly secreting gland results in vigorous secretion as seen after the addition of

acetylcholine (SATO, K., unpublished data). Thus, for practical purposes, it may be fully justified to propose that the influx into the cell of extracellular Ca^{++} is an essential initial step in the events intervening between the pharmacologic (or hormonal) stimulation of the gland and active secretion of ions and water by the secretory cells. The primary, if not the sole, role of acetylcholine may be the introduction of Ca^{++} into the cell, which increases the concentration of Ca^{++} at some critical site(s) in the cytoplasm. Thus far, no evidence exists in the eccrine sweat gland that an exocytotic mechanism is involved in fluid and electrolyte secretion. On the other hand, the secretory cell must not be regarded as composed only of cell membrane, cytoplasmic water, and the machinery for energy metabolism such as mitochondria. There is indirect evidence to suggest the involvement of such cytoplasmic organelles as microtubules in some steps of the secretory process because vinblastine, an antimicrotubular agent, strongly but reversibly inhibits sweat secretion in vitro (SATO, K., unpublished data). It should be noted that the resemblance of the Ca^{++} ionophore to acetylcholine does not necessarily indicate that acetylcholine itself is a Ca^{++} ionophore or vice versa, although the most recent study by KOELZ et al. (1977) suggests that such might be the case. In fact, evidence has been presented in the mouse pancreas which suggests that influx of Ca^{++} by acetylcholine into the acinar cells is triggered secondarily by acetylcholine-mediated influx of Na into the cell (WILLIAMS, 1975). The cellular events that intervene between the influx into the cell of Ca^{++} and the increase in the membrane transport that results in stimulation of electrolyte and water secretion by the secretory cell remain totally unknown in the eccrine sweat glands, as well as in any other exocrine glands. Furthermore, it is not yet known in the exocrine glands how or where other possible candidates for the second messengers such as cAMP or GMP may enter into the scheme. As for cGMP, we have never observed any sudorific effect of db-cGMP at as high as 5 mM on the isolated eccrine sweat gland (SATO, K., unpublished data). In rat parotid slices, BUTCHER (1975) observed that A23187 enhanced tissue level of cGMP (but not cAMP), which are further augmented by theophylline; hence, the negative sudorific effect of db-cGMP in the eccrine sweat gland should not be taken as evidence against possible cGMP action in cholinergic or Ca^{++} ionophore-induced sweat secretion. The interrelationship between cholinergic stimulation and cAMP is still an enigma in the eccrine sweat gland.[2] In the insect salivary gland, the results of extensive studies suggest that 5-hydroxytryptamine (5-HT, the natural stimulant of the insect salivary gland) increases both endogenous cAMP and Ca^{++} influx into the secretory cell (BERRIDGE and PRINCE, 1971; PRINCE and BERRIDGE, 1972). The electrophysiologic study of PRINCE and BERRIDGE (1972) seems to indicate that cAMP stimulates the potassium pump, the putative driving force for this K secreting insect exocrine gland, and that cytoplasmic Ca^{++} simply increases membrane permeability to the chloride ion (Cl^-). The insect salivary gland also respond to Ca ionophore A23187 as it does to the natural stimulant 5-HT. Nevertheless, stimulation of the gland with A23187 lowers the cytoplasmic cAMP level (PRINCE et al., 1973). If so, we are left with the unsolved problem of what stimulates active membrane transport when only Ca^{++} is introduced into the cell as

[2] The most recent unpublished observation by the author has shown that the tissue level of cAMP does not elevate after A23187 or ACH stimulation of isolated sweat glands.

after the A23187 application. Is it cGMP or simply a change in cellular electrolyte concentrations? Despite the current paucity of information about the eccrine sweat gland, we must, at this point in our knowledge, also ask the same question about the cellular events that intervene between Ca^{++} influx and the stimulation of the cation (or anion) pump in the membrane. I will come back to this point once again after presenting our electrophysiologic data.

F. The Function and Pharmacology of the Myoepithelial Cell in the Sweat Gland

There is no direct evidence that myoepithelial contraction is a necessary part of the secretory processes or that myoepithelial contraction must accompany sweat

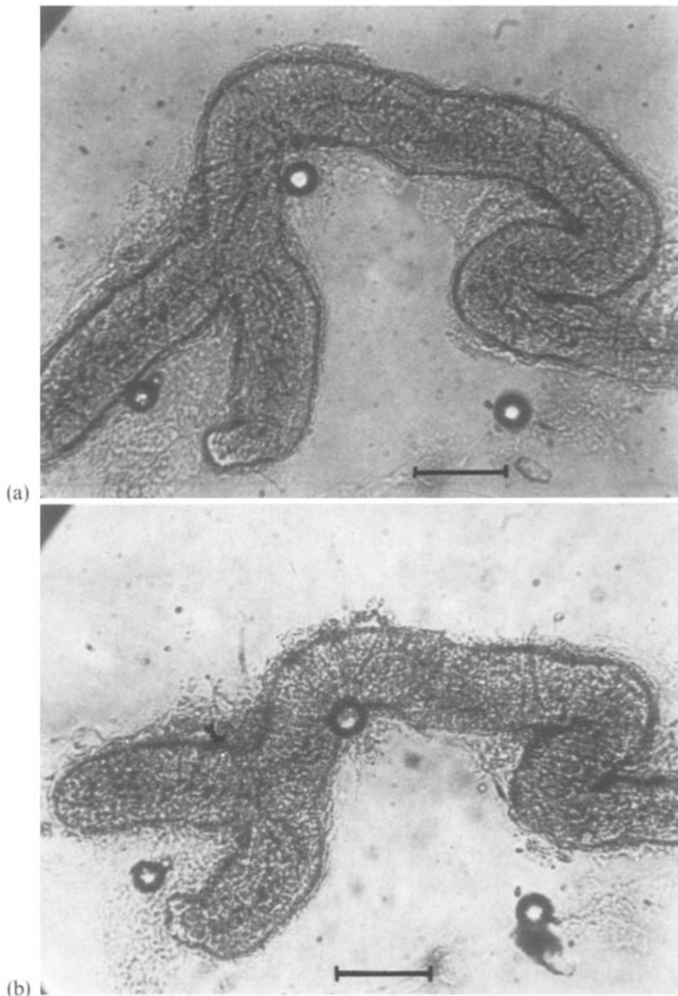


Fig. 27a and b. MCH-induced contraction of a secretory coil segment. This particular secretory coil is bifurcated near its closed end, a rare anomaly. The bar represents 100 μm. (a) unstimulated, (b) after MCH stimulation

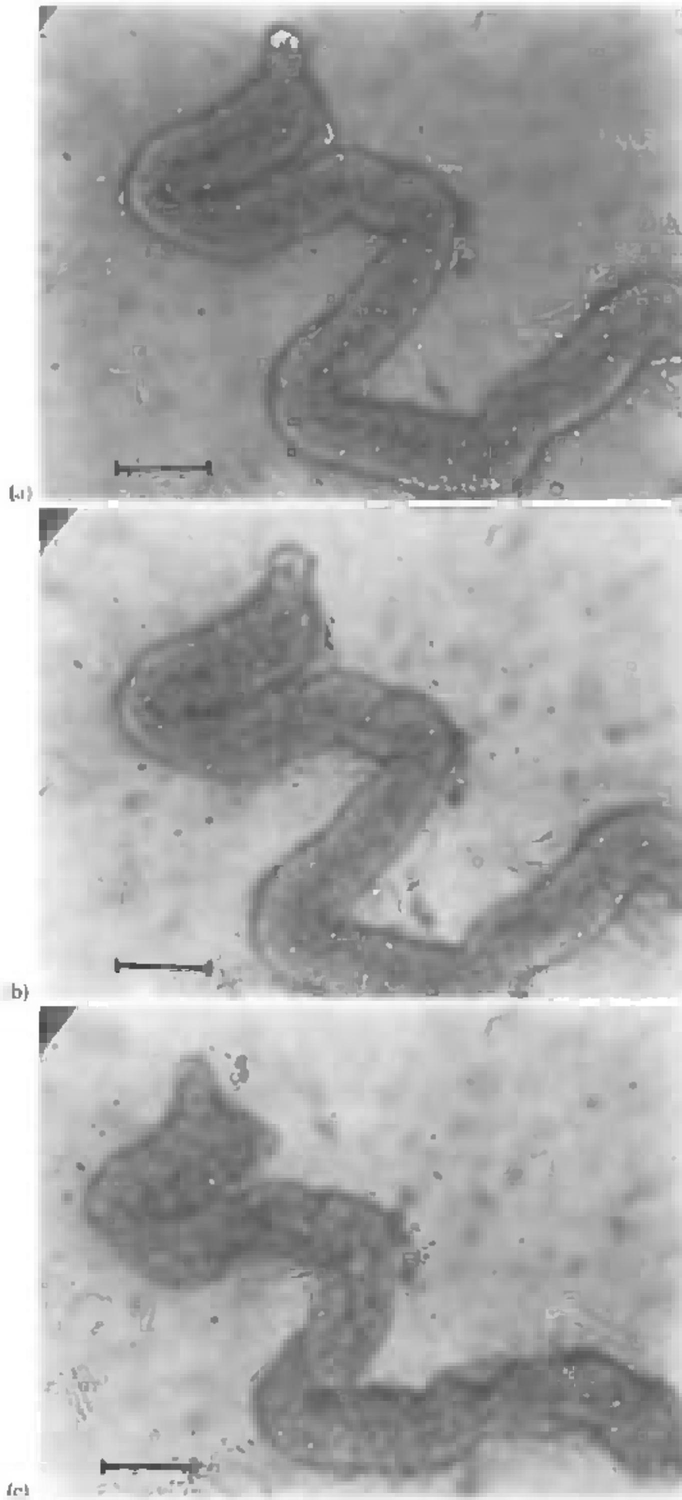


Fig. 28 a–c. Effect of A23187 on tubular (myoepithelial?) contraction. a: unstimulated, b: 5 min after stimulation with 10^{-4} M A23187, c: after the subsequent addition of 10^{-6} M MCH. Note the tubular contraction only in c

secretion. The classic concept of the role of myoepithelial cells is the expulsion of preformed sweat by myoepithelial contraction (ROTHMAN, 1954). In fact, TAKAHARA (cited by KUNO, 1956) observed that the level of sweat in the canulating pipet in vivo was seen rising and falling at 0.5–2 cycles/min at a low sweat rate. However, KUNO (1956) assumed that the periodicity of sweat secretion in vivo is caused by the periodicity of central nerve impulse discharge because the periodicity occurs synchronously with vasomotor tonus waves. Consistent with KUNO's (1956) view, the sweat secretion induced by intradermal injection of pharmacologic agents does not show such a periodicity (FOSTER et al., 1971). Since the periodicity of secretion may also occur due to periodic discharge of acetylcholine and the resultant burst of secretion, we chose to directly observe the tubular contractions in response to pharmacologic agents and analyze them photographically. Figure 27a is a segment of the secretory coil of the isolated monkey eccrine sweat gland. The bifurcation near the closed end of the coil is a rare anomaly, but it was a helpful landmark of the secretory coil. The lumen of the secretory coil was nearly collapsed before stimulation. When acetylcholine was added to the medium, the secretory coil immediately began to contract to approximately two-thirds of the original length (Fig. 27b). A few seconds were required for the contraction to reach its peak, but thereafter the coil remained in the contracted state as long as acetylcholine was present in the bath. The lumen of the secretory coil dilated only slightly during stimulation, but usually it did not exceed 10 μm. The tubular contraction was found to be completely reversible by either washout of the medium or the addition of atropine. Similar photographic analyses were performed for α and β-adrenergic agents, which showed no tubular contraction.

The Ca^{++} ionophore A23187, another strong stimulant of sweat secretion, was used at high concentration, 2×10^{-4} M, to induce a rapid sudorific response. No contraction was observed (Fig. 28a, b), but the subsequent addition of acetylcholine caused forcible contraction of the tubule. It the tubular contraction observed in these figures indeed represents the myopithelial contraction, then it can be concluded that: (1) the myoepithelial cell responds only to cholinergic stimulation, (2) myoepithelial contraction is not a prerequisite for inducing or maintaining sweat secretion by the secretory coil, and (3) the amount of so-called "preformed sweat" is so small due to luminal collapse before stimulation that the initial myoepithelial contraction does not expulse significant amounts of preformed sweat.

VI. Energy Metabolism of the Isolated Eccrine Sweat Gland

Unlike other anatomically complex organs such as the kidney, the isolated single eccrine sweat gland offers an ideal model for studying the biochemical reactions by which the energy derived from metabolism is used in membrane transport. This is especially true when determining whether a certain substrate is taken up and utilized for the production of energy for sweat gland function, i.e., the secretion of water and electrolytes. In addition, it is relatively easy to obtain estimates of the energy

requirements of the individual sweat glands as well as the total glandular energy production. In the kidney, for example, the major energy requirements for renal work are met by the oxidation of such substrates as free fatty acid, lactate, citrate, glutamine, and perhaps also glucose (COHEN and BARAC-NICTO, 1973). It is important, therefore, to clarify whether the eccrine sweat gland also has a similar capacity to utilize a wide variety of plasma substrates and to contribute to the conservation of blood sugar even under such circumstances as hypoglycemia, starvation, and malnutrition.

A. The Role of Stored Glycogen in Energy Production

In a single isolated monkey sweat gland preparation in vitro, removal of glucose from the incubation medium rapidly abolishes sweat secretion, which is restored to the control level by the addition of glucose to the incubation bath (Fig. 29). The rapidity with which the secretion declines after the removal of glucose from the incubation medium varies from gland to gland, but the secretory rates usually drop exponentially until the secretion stops completely 10–15 min after removal of glucose. On addition of glucose to the medium, the sweat rate recovers rapidly, but it also takes about 10 min until it reaches a plateau level. The removal of glucose for the second time results in a much more rapid exponential drop in sweat rate and leads to complete cessation of secretion usually within 5 min. The same time course of response as the second glucose removal was obtained by repetition of the same procedure. Thus, the amount of sweat secreted in glucose-free media (i.e., after the first medium change) is larger than that after the second or third medium change to glucose-free media by 10–20 nl. On the other hand, when an isolated sweat gland leached of glucose at 4° C for 5 min was stimulated in a glucose-free incubation medium, the secretory response was low and short-lived, totaling less than 20 nl (SATO, K., unpublished data).

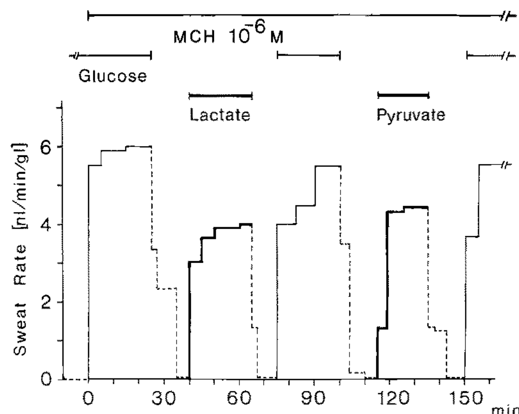


Fig. 29. Substrates utilization (for energy metabolism) of the isolated eccrine sweat glands in vitro. An illustrative example. Concentrations of all the substrates used were 5.5 mM. In order to remove substrate from the medium, the incubation bath was washed several times with a substrate-free medium. Usually 20 to 30 s were required for such a procedure

If we assume that the energy source of the sweat gland placed in a glucose-free medium is mainly cellular glucose and glycogen, the difference in the volume of residual sweat secretion between the first and second washout of the media may represent that portion of energy source supplied by mobilized cellular glycogen. Thus, it follows that whatever is the absolute amount of the cellular glycogen storage, it can only supply the secretory cells with fuel necessary to secrete less than 20 nl of primary sweat. Since the isolated monkey sweat gland can continuously secrete fluid at a rate of 200–400 nl/h, this amount of stored energy source is equivalent to the amount of fuel used up by the gland within several minutes. During the entire period of the experiment in Figure 29, the possible contribution of gluconeogenesis may be minor, if any, because, as demonstrated by SMITH (1970), glycogen synthetase I, the active form of the enzyme, is suppressed during stimulation with Mecholyl. Although the glandular glycogen level has never been quantitatively analyzed in monkey sweat glands, WOLFE et al. (1970) measured it to be 0.98 nmol per gland (as glucose) in human male adults before stimulation and 0.3 nmol after stimulation in vitro. Using histochemical techniques, SMITH and DOBSON (1966) observed that the secretory cells of the palmar sweat glands of the rhesus monkey undergo glycogen depletion after sweating is stimulated in vivo with Mecholyl. They also noted that the decrease in cellular glycogen was accompanied by an increase in phosphorylase, an active form of the enzyme phosphorylase. Glycogen depletion in the isolated human sweat gland in vitro of some 0.6 nmol per gland (WOLFE et al., 1970) is relatively great when compared with the total glucose consumption of 1.45 nmol/h in a monkey sweat gland (SATO and DOBSON, 1973). However, if we consider the larger glandular size, the higher secretory rate, and a higher glycolytic activity than oxidation (i.e., the less efficient utilization of glycogen or glucose for the production of ATP) in the human sweat gland, the amount of glandular glycogen observed in the human sweat gland (WOLFE et al., 1970) is by no means sufficient for supporting prolonged sweat secretion.

In summary, although cellular glycogen is indeed mobilized in the eccrine sweat gland during sweat secretion, its absolute amount is far too small to sustain a continued high rate of sweat secretion. Since it can only supply fuel for a vigorously secreting gland for less than several minutes, the sweat gland must depend exclusively on exogenous substrates as its fuel sources. This is in keeping with the observation that the sweat glands in acclimatized people contain less glycogen than do those in nonacclimatized people (DOBSON, 1962), yet acclimatized people can perspire more readily and profusely than nonacclimatized people on exposure to heat.

B. Substrate Specificity of the Sweat Gland in vitro

As shown in Figures 29–31, in the isolated monkey sweat gland, glucose is the most efficient substrate for the sweat gland function. Although the sweat gland is capable of utilizing both lactate and pyruvate as energy sources, these intermediates of the Embden-Meyerhof pathway appear less efficient than glucose at the same concentration, 5.5 mM. However, since the actual carbon content of glucose is

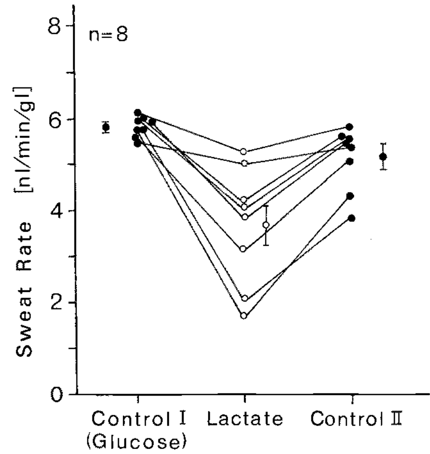


Fig. 30. Comparison of D-glucose and L-lactate (both at 5.5 mM) as substrates for energy metabolism of the sweat gland in vitro. The experimental protocol is the same as in Fig. 29

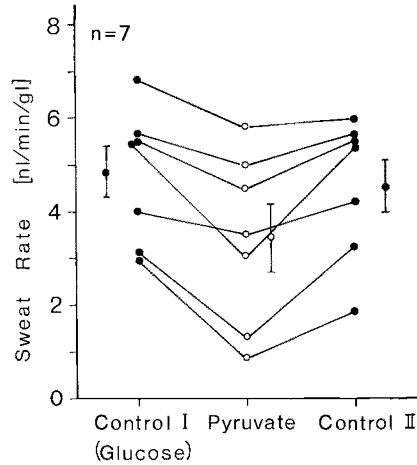


Fig. 31. Comparison of D-glucose and pyruvate (both at 5.5 mM) as substrates for energy metabolism of the sweat gland in vitro

double that of lactate and pyruvate, the data in Figures 30 and 31 may not necessarily support the above statement. Experimental design should insure that the concentration of electrolytes (since both lactate and pyruvate are the sodium salts), osmolarity of the medium, and the amount of carbon are comparable when comparing the two substrates. In practice, however, the plasma level of glucose (5.5 mM) is much higher than that of lactate (1–2 mM) or pyruvate ($\ll$ 1 mM); hence, it may be reasonable to conclude that glucose is a major fuel source for sweat secretion. Among other substrates thus far tested by us using the same experimental protocol, only β-D-fructose could support sweat secretion, but less than 0.5 nl/min/gl. None of the following compounds supported sweat secretion: L-glucose, 3-0-methylglucose, galactose, ATP, phosphoenol-pyruvate, succinate, citrate, malate, α-ketoglutarate, glycine, alanine, acetoacetate, ethanol, β-hydroxybutyrate, propionate (1–2 mM), palmitate (albumin-bound 1–2 mM). It remains to be determined whether the inability of these exogenous compounds

to serve as substrates for energy metabolsim is due to their impermeability through the cell membrane or the unavailability of the enzymic systems necessary for their metabolism.

No study has been done on the mechanism by which glucose, lactate, and pyruvate are transported across the cell membrane. Our preliminary observation (SATO, K., unpublished data) that 5×10^{-4} M phlorizin inhibited sweat secretion by 20–50% when glucose was used but did not inhibit when pyruvate was used as a substrate suggests, but does not prove, the occurrence of carrier-mediated glucose transport as occurs in the kidney proximal tubular and intestinal mucosal brush border or the erythrocyte membrane. Both pyruvate and lactate have low pKa values, and, at pH 7.4, less than 0.1% of the molecules are in nonionic form, the form which is more suitable for transport across the phospholipid membrane by nonionic diffusion. Since the membrane potential of 60 mV, cell interior negative (see Figs. 47 and 48), is also against the passive diffusion of lactic anion, lactate must be transported either by an as yet unknown active mechanism or by the extremely rapid passive diffusion of nonionized lactate followed by anionic trapping inside the cell. In the latter case, however, it is hard to explain the maintainance of the high sweat vs. plasma lactate ratio. SLEGERS (1969) was unable to detect ^{14}C-lactate in the cat paw sweat when ^{14}C-lactate was infused into the systemic circulation; he ascribed this to the inability of lactic anion to passively enter the cell because of its low pKa value. Our data show, however, that lactate does permeate the secretory dell membrane in sufficient quantity. One possible explanation for this disagreement may be that the systemicly injected ^{14}C-lactate in SLEGERS' (1969) in vivo experiment may have actually been taken up by the cell but metabolized and released as $^{14}CO_2$, another that the short ductal segment of the cat paw sweat gland reabsorbed all the ^{14}C-labeled substrates including ^{14}C-lactate or its metabolites. Nevertheless, the question remains unanswered as to why a high lactate concentration in the lumen and, perhaps, also in the cell cytoplasm is maintained if lactate is actually utilized by the cell. It is also possible that in the in vivo cat sweat gland experiment (SLEGERS, 1969) lactate was not taken up significantly by the cell because plasma glucose was also present perivascularly in a much higher concentration, and the secretory cells selectively incorporated plasma glucose over lactate. Hence, the mechanism of substrate transport across the secretory cell membrane is an important field in exocrine gland research which must be explored in the future.

C. Glycolysis and Oxidation of Glucose by the Sweat Gland

Evidence thus far obtained strongly favors the possibility that plasma glucose is the major substrate for energy metabolism by the sweat gland. The degradation of glucose may occur in the sweat gland by four separate pathways: (1) glucose is incompletely oxidized to lactate through the Embden-Meyerhof glycolytic pathway, (2) glucose is oxidized in the reactions of the tricarboxylic acid (TCA) cycle preceded by glycolytic production of pyruvate, (3) glucose is oxidized in the oxidative reactions of the hexosemonophosphate shunt (pentose cycle), and (4) although not yet demonstrated in the sweat gland, glucose may also be oxidized by the

glucuronic acid pathway if the acid mucopolysaccharide present in sweat is actually produced in the sweat gland. In addition, this cycle is also capable of producing pentose phosphate that mixes with pentoses produced by the pentose phosphate shunt (pentose cycle).

The occurrences of active glycolytic metabolism in the sweat gland has been suggested by WEINER and VAN HEYNINGEN (1952), who assumed that the high lactate concentration in sweat was derived not from plasma but from the metabolism of the sweat gland itself. In agreement with this assumption, SCHULZ et al. (1965), WOLFE et al. (1970), SATO and DOBSON (1971, 1973) directly demonstrated lactate production by an isolated eccrine sweat gland in vitro. WOLFE et al. (1970), based on their inability to detect any oxygen consumption by an isolated sweat gland, postulated that anerobic metabolism of glucose to lactate is the only metabolic pathway in the sweat gland for providing energy for the secretory function, but the series of observations obtained in the author's laboratory failed to support this contention. Firstly, interference with the blood circulation in the forearm by applying a tourniquet markedly inhibited local sweating induced by the intradermal injection of pilocarpine. Simultaneously, lactate concentration in sweat rose sharply (SATO et al., 1973), indicating that oxygen supply to the sweat gland is critically important for maintaining sweat secretion in the human. Secondly, an isolated sweat gland in vitro showed a high $^{14}CO_2$ production in both human and monkey, although the monkey glands exhibited a higher oxidative activity than did the human glands (SATO and DOBSON, 1971, 1973). Thirdly, an intimate relationship was observed between oxidative glucose metabolism and membrane transport. For example, drugs such as cyanide, ouabain, or atropine, which inhibit cholinergic sweating in vivo as well as in vitro, also suppress $^{14}CO_2$ production by the sweat gland in vitro, but not necessarily lactate production (which was markedly stimulated by cyanide) (SATO et al., 1969). Furthermore, the specificity of inhibition of both glucose metabolism and sweat secretion by ouabain, a well-known inhibitor of active membrane transport, was proven by use of 2,4-dinitrophenol (SATO and DOBSON, 1973). Since 2,4-dinitrophenol (DNP) uncouples oxidation from phosphorylation, thus producing high cellular ADP levels, the cellular metabolism machinery is intact. Using this characteristic of DNP, the author intended to clarify whether the inhibitory effect of ouabain on both glucose metabolism in vitro and sweat secretion in vivo and in vitro (SATO et al., 1969; SATO and DOBSON, 1973) is the result of inhibition of the ATP-requiring membrane transport (i.e., less ATP utilization causes a low cellular ADP level, which then suppresses energy metabolism) or whether the metabolic machinery itself was damaged by ouabain either directly or secondary to the change in cellular homeostasis. However, the observation that DNP fully reverses ouabain-induced inhibition of glucose metabolism was interpreted by the author as indicating that the cellular metabolic machinery itself was intact, and thus the effect of ouabain represents the tight coupling between ATP-driven membrane pumps and ATP-generating systems (SATO and DOBSON, 1973). Fourthly, DNP at 1 mM completely inhibits sweat secretion in vitro (SATO, K., unpublished data). Although DNP inhibits ATP production at the level of the mitochondrial respiratory chain, it actually stimulates ATP production at the substrate levels (i.e., glycolysis and TCA cycle), indicating that only those ATPs produced by the oxidative phosphorylation appear to support the

secretory activity. Lastly, as shown in Figures 29 and 30, lactate, the ultimate product of glycolysis, can serve as an energy source when glucose is not present.

In summary, the weight of evidence now favors the contention that oxidative phosphorylation of plasma glucose is the main route of energy production, presumably in the form of ATP formation, for fueling the ATP-requiring secretory activity. The role of glycolysis (lactate production) in the overall energy production may be minor, if any, and some other teleologic significance of a high lactate concentration in sweat must be looked for in the future.

D. Pentose Phosphate Cycle in the Sweat Gland

Using isolated monkey eccrine sweat glands, the author attempted to measure pentose cycle activity (SATO, 1973a) according to the method of KATZ and WOOD (1963). Three parameters were measured to calculate the activity of the pentose cycle: glucose utilization and $^{14}CO_2$ production from both G-1-^{14}C and G-6-^{14}C. The amount of glucose metabolized via the pentose cycle, both in absolute terms (nmol/h/gl) and relative to the total glucose utilized (%), was significantly enhanced by both epinephrine and isoproterenol. Propranolol (β-adrenergic antagonist) markedly suppressed the stimulatory effect of epinephrine and isoproterenol. Since dibutyryl cAMP plus theophylline mimicked the effect of isoproterenol, the author suggested that this effect of adrenergic drugs on the pentose cycle is due to its β-effect and is probably mediated by intracellular cAMP. Although cholinergic drugs did not enhance the pentose cycle in relative terms, they did stimulate, although modestly, the absolute amount of glucose metabolized by this cycle. The pentose cycle contributes primarily to the synthesis of lipid, protein, and nucleic acid by providing such precursors as NADPH and ribose.

Although the physiologic significance of the pentose cycle is totally unknown in the eccrine sweat gland, several possible roles should be discussed for the sake of future studies. One of them is the control of glandular growth or hyperplasia. It is a well-established fact that β-adrenergic stimulation induces hypertrophy of the salivary gland (SEIFERT, 1967) and stimulates secretion of a viscous, protein-rich saliva (MANGOS et al., 1969). SEIFERT (1967) observed in parotid and submaxillary glands that isoproterenol stimulated DNA and RNA synthesis, induced hypertrophy and hyperplasia of acinar cells, and increased nuclear division. He also found that these effects of isoproterenol were potentiated by pilocarpine or monoamine oxidase inhibitors and blocked by β-adrenergic inhibitors or by actinomycin. These stimulatory effects were confined to parotid and submaxillary glands which have both cholinergic and adrenergic innervation. In contrast, the sublingual gland, which lacks adrenergic innervation, was not affected by isoproterenol (SEIFERT, 1967). In light of the detection of catecholamine-containing nerves around the eccrine sweat gland (UNO and MONTAGNA, 1975), it may not be far-fetched to speculate that a similar adrenergic control mechanism on synthesis occurs in the dually innervated eccrine sweat gland. Since profuse sweat secretion is often followed by morphologic changes of the secretory cell as observed by the ordinary hematoxylin-eosin stained histology sections (i.e., decrease in height of secretory cells, vacuolization, and pyknosis of nuclei; DOBSON, 1960, 1962), although the

secretory functions of such glands remain unchanged, it may be reasonable to assume that the sweat gland must have a mechanism of replenishing the lost structural components of the cell to maintain the cellular function.

VII. The Secretion of Nonelectrolytes by the Human Eccrine Sweat Gland

A. Lactate and Urea

Lactate and urea constitute the major organic compounds secreted in sweat. The concentration of these compounds in sweat usually depends on sweat rate. At low sweat rates lactate concentration is as high as 30–40 mM but rapidly drops to a plateau at around 10–15 mM as the sweat rate increases. However, sweat lactate concentration is not strictly dependent on the sweat rate. When the sweat rate is lowered after a period of moderate to profuse thermally induced sweating in man, the rise in sweat lactate concentration is much less than expected from the drop in sweat rate (SATO, K., unpublished data). Acclimatization is known to lower sweat lactate (KUNO, 1956) whereas arterial occlusion rapidly raises sweat NaCl and lactate concentrations and reduces sweat rate (SATO et al., 1973). Since serum lactate concentration is 1–2 mM and an isolated sweat gland produces lactate in vitro (SATO and DOBSON, 1973; SCHULZ et al., 1965), sweat lactate can probably be generated through the glycolytic activity of the secretory cell. The origin of sweat lactate and glycolytic activity of the sweat gland has been discussed in detail in Section F of this review. In contrast, the available evidence indicates that sweat urea is derived mostly from serum urea (SLEGERS, 1966). It is not yet known whether the sweat gland itself produces some urea through the urea cycle. Enzymes of the urea cycle have never been measured in the sweat gland. Sweat urea content is usually expressed as a sweat-plasma urea ratio (s-p urea). At a low sweat rate range s-p urea is high (2–4) but approaches a plateau at 1.2–1.5 as sweat rate increases. The mechnism of this higher than unity s-p urea has been debated repeatedly in the past (BRUSILOW and GORDES, 1967; SLEGERS, 1969). One school of thought favors the idea that s-p urea of higher than unity represents water reabsorption by the duct in excess of urea backflux (absorption), and the other favors the synthesis of urea by the sweat gland itself. Only direct approaches, such as microperfusion of the ductal system with urea, determination of osmotic water permeability of the duct, measurement of urea permeability across the secretory coil epithelium, direct determination of urea synthesis in vitro, if any, or the measurement of urea cycle enzymes will provide a definite answer to the mechnism of urea excretion by the sweat gland.

B. Ammonia

The sources of sweat ammonia also remain to be determined. Sweat contains 0.5–8 mM total ammonia (BRUSILOW and GORDES, 1967; EMRICH et al., 1967;

MORIMOTO and JOHNSON, 1967), which is 20–50 times higher than the plasma ammonia level. The concentration of sweat ammonia is inversely related to sweat rate and sweat pH (EMRICH and OELERT, 1966). In general, the ammonia buffer system in biological fluids consists of ammonia-free base (NH_3), which readily penetrates the cell membrane by passive nonionic diffusion, and poorly permeable ammonium ions (NH_4^+). The proportions of these two forms of ammonia are determined by the pH of the fluid according to the Henderson-Hasselbalch equation: $[NH_4]/[NH_3]=10^{9.15-pH}$. For example, if the pH of sweat is 1 or 2 pH U less than plasma pH, the s-p ammonia (more precisely ammonium ions, NH_4^+) ratio of 10 or 100 respectively can be explained only on the basis of nonionic diffusion and not by the metabolic generation or acitve transport of ammonia. EMRICH and OELERT (1966) maintain that free base NH_3 cocentration (or the partial pressure of NH_3) is always lower than that of plasma, and thus the high sweat ammonium concentration can be explained only by the nonionic diffusion of NH_3 from plasma and ionic trapping of ammonium ions in sweat. BRUSILOW and GORDES (1967) contend that $[NH_3]$ in sweat is not infrequently higher than that of plasma, especially in those sweat samples with high pH values, and, therefore, that ammonia generated metabolically by the sweat gland must also contribute to sweat ammonia under some circumstances. It seems that the disagreement between the two studies depends mainly on the accuracy of pH measurement under a strict equilibration with 40 torr CO_2 and the specificity of the method for total ammonia measurement (since $[NH_3]$ is a calculated value from these parameters). Despite the disagreement, it can be safely stated that ast least the major portion of sweat ammonia is derived from plasma ammonia by nonionic passive diffusion of NH_3 and ionic trapping of NH_4^+ in an acid sweat. The possibility of metabolic generation of ammonia by the sweat gland must be tested in the future using a direct enzymtic or metabolic approach.

C. Amino Acids

The presence of free amino acids in human sweat has been well-documented. Recently GITLITZ et al. (1974) conducted a comprehensive study on amino acids in sweat, plasma, and urine from 22 healthy volunteers using ion exchange chromatography. Serine, ornithine, citrulline, and aspartic acid were present in sweat in mean concentrations more than ten times those of plasma. Other amino acids whose concentrations in sweat are higher than those of plasma include: glycine (s-p ratio 6.7), alanine (2.3), threoninene (3.4), histidine (4.9), valine (1.2), leucine (1.5), tyrosine (2.3), asparagine (2.5), glutamic acid (6.2), isoleucine (2.3), phenylalanine (2.2), and methionine (1.2). In contrast, glutamine, cystine, α-aminobutyric acid, and α-amino adipic acid were present in sweat in mean concentrations that are less than 20% those of plasma. There was no correlation noted between sweat and urine amino acid concentrations, but their data seem to suggest an inverse relationship between amino acid concentration and sweat rates. In spite of the considerable variations reported in the literature of the absolute quantities of these amino acids, there is surprising consistency among them regarding the relative abundances of amino acids in sweat. The mechanisms of amino acid secretion in sweat are totally unknown. The s-p values of GITLITZ et al. (1974) are subject to overestimation

if there was any evaporative water loss during sweat collection; however, such an artifact appears minimal since an airtight polyethylene arm bag was used for sweat collection. Possible epidermal contamination from the macerated skin must also be carefully excluded.

D. Macromolecules in Sweat

Human eccrine sweat contains protein (including glycoprotein) and acidic mucopolysaccharides (PALLAVICINI et al., 1963). The concentration of sweat protein (CIER et al., 1963; PAGE and REMINGTON, 1967; JIRKA, 1969) may depend on the degree of evaporation of sweat during collection and contamination by epidermal protein, but it ranges from 20 mg % (SATO et al., 1973) to 77 mg % (PAGE and REMINGTON, 1967). The major proteins of normal human sweat are albumin, α-globulin, and γ-globulin (PAGE and REMINGTON, 1967). The immunoelectrophoretic study by PAGE and REMINGTON (1967) revealed the presence in sweat of IgG, IgA, IgD, transferrin, ceruloplasmin, orosomucoid, and albumine. In contrast to many other body secretions (e.g., saliva) in which IgA is present in higher concentrations than IgG, IgG is the major immunoglobulin in sweat (IgG/IgA ratio = 3.4). The demonstration in sweat of "secretory piece" probably indicates that the mechanism of secretory IgA formation is similar to that of salivary glands (PAGE and REMINGTON, 1967). These authors also showed the presence of antitetanus antibodies in volunteers who had previously received booster doses of that texoid, indicating that the IgG represents antibodies. The presence of IgM is not a constant finding. Recently, FÖRSTRÖM et al. (1975) reported the presence of IgE in sweat, especially in patients with atopic dermatitis.

E. Other Organic Compounds in Sweat

Other organic compounds reported in sweat include: histamine (GARDEN, 1966; STÜTTGEN et al., 1960), kallirein (FRÄKI et al., 1970), prostaglandin (FREWIN et al., 1973; FÖRSTRÖM et al., 1974), vitamin K-like substances (SEUTTER and SUTORIUS, 1971), amphetamine-like compounds (VREE et al., 1972), and esterases (HERRMANN and HABBIG, 1976). The presence of prostaglandin activity is of interest since prostaglandin itself is a strong stimulant of eccrine sweat secretion (Fig. 22). Sweat also contains a trace of pyruvate (EMRICH and ZWIEBEL, 1966) and glucose (KUNO, 1956), but the latter must be reexamined using a more refined techniqe.

VIII. Secretion of Monovalent Ions and Water by the Eccrine Sweat Gland

A. Secretory Rate: Some Factors Determining the Sweat Rate

The population density of the sweat glands varies considerably from one area of the body to another (SZABO, 1962; SATO and DOBSON, 1970a) or according to age (KUNO, 1956). There may also be considerable variation in the function of each

sweat gland even within the same area. Furthermore, the rate of sweat secretion depends on such factors as acclimatization (KUNO, 1956), water intake (CAGE et al., 1970), skin temperature (WUSTER and MCCOOK, 1969; FOSTER, 1971), sweat gland fatigue, and vascular supply (LOVE and SHANKS, 1962). Maximal sweat rate as estimated on a gland per hour basis in the human is as much as 600 nl (10^{-9}) after pharmacologic stimulation (EMRICH et al., 1967) to as much as 3000 nl after thermal stimulation (SATO and DOBSON, 1970a). However, human sweat glands cannot continue to sweat at this high rate in vivo because of so-called "sweat gland fatigue" (KUNO, 1956; SARGENT, 1962), which is still a poorly understood phenomenon. When comparing sweat rates in clinical research, it is imperative to define whether the observed rate of secretion is a maximal or a submaximal sweat rate. Despite the fact that the detailed mechnisms of sweat secretion are not yet fully clarified, it is convenient to separate the entire secretory processes into three major determinant steps: (1) the intensity of pharmacologic stimulations, i.e., the concentration of acetylcholine, (2) the capacity of mitochondrial energy metabolism, and (3) the capacity of the membrane pump at the luminal membrane or the membrane facing the intercellular canaliculi. The role of each of the three steps in the determination of the sweat rate must be analyzed kinetically, although such analyses may be extremely difficult in an in vivo condition. The secretory rate will probably show a Michaelis-Menten-type saturation pattern up to 10^{-6} M acetylcholine, which is the case in vitro (SATO, 1973b; SATO, K., unpublished data). In vitro, the sweat rate induced by 10^{-6} M acetylcholine may be defined as Vmax, and a further increase in acetylcholine concentration will not enhance the sweat rate. Vmax may be determined by the maximal pump activity or by the maximal capacity of energy metabolism, whichever is smaller. Thus, in cholinergic sweating, the secretory response, as expressed in %/Vmax, should also show a saturation kinetic, and, at least in theory, the so-called "sensitivity" of the sweat gland should be defined by the *Km* of acetylcholine concentration. This, however, has not been done in sweat gland research. Instead, the magnitude of the secretory response to a given acetylcholine concentration has often been mistakenly used as a measure of the sensitivity of the sweat gland (WARNDORFF, 1970). As already discussed in a previous section, the availability of glucose to the sweat gland must also be considered as part of the second step, since the secretory rate also responds to the glucose concentration in the medium (SATO, K., unpublished data). We may then ask whether the pump activity (step three) or the energy metabolism (step two) is the determinant of Vmax., or whether both activities are balanced. The fact that DNP further enhances both lactate and $^{14}CO_2$ production in maximally stimulated (by Mecholyl) isolated eccrine sweat glands (SATO and DOBSON, 1973) suggests that there is a reserve capacity in the metabolic machinery. If this interpretation is correct, then it follows that Vmax. represents the maximal capacity of membrane pump activity which is fueled by metabolic machinery equipped with reserve capacity.

B. Isotonicity of the Primary (Precursor) Sweat

Table 1 tabulates electrolyte composition of both skin surface sweat and precursor fluid in various animal species. In man, as well as in monkey, it is now established

that an ultrafiltrate of plasma-like isotonic precursor fluid is secreted by the secretory coil (SCHULZ et al., 1965; SATO, 1973b; BRUSILOW and GORDES, 1963; SLEGERS, 1963). As the precursor fluid flows through the duct, part of sodium chloride is reabsorbed in excess of water by the duct producing hyposmotic skin surface sweat. The major disagreement among the above investigators is whether the precursor fluid is exactly isotonic (SATO, 1973b) or hypertonic by 28 mosmol (SCHULZ et al., 1965; SCHULZ, 1969). SCHULZ used micropuncture techniques and collected ultramicro volumes of precursor fluid (0.1–1 nl) directly from the human secretory coil in vivo, whereas this author employed an in vitro sweat induction method utilizing an isolated monkey sweat gland secretory coil where the sample size is 10–100 times larger, and the comparison between the incubation bath and sweat sample does not require a correction for Donnan's equilibrium, since the medium was protein-free (SATO, 1973b) or contained less than 0.5 g % bovine albumin. Since the handling of 0.1–1 nl samples is an extrmely difficult procedure, the possibility of technical error (such as evaporative loss of sample even under water-saturated oil) must be meticulously ruled out (SCHULZ, personal communication) in future investigations. Another explanation for this disagreement is the possible difference between in vivo and in vitro periglandular osmolarity. Since the secretory coil is tightly coiled with the duct and thus is very often in direct contact with the tubule where sodium is avidly reabsorbed and released peritubularly, there is no proof that the NaCl concentration of the immediate peritubular interstitial fluid is isotonic to plasma. In fact, the cryoscopic study of SLEGERS (1963) showed that the periglandular interstitial space had an osmolarity higher than that of plasma. In contrast, an isolated secretory coil in vitro is always exposed to a well-stirred incubation medium. If this latter explanation is correct, then, at least in human and monkey sweat glands, isotonicity of the precursor fluid (with respect to periglandular fluid) remains the more likely possibility. Additional evidence in favor of isotonicity (in terms of Na, K, osmolarity, but not for Cl^-, lactic anion, HCO_3^-) of the precursor fluid is the high transepithelial electric conductance of the secretory coil (Figs. 45 and 46), which is in the range of that of the kidney proximal tubule, an epithelium that transports Na isotonicly. These will be discussed in more detail in a later section, together with the other electrophysiologic data.

C. Reabsorption of NaCl by the Sweat Duct: Indirect Methods of Estimation

Although the dependence of sweat Na^+ and Cl^- concentrations on sweat rate had been observed as early as 1944 (JOHNSON et al., 1944; LOCKE et al., 1951; ROBINSON and ROBINSON, 1954), it was not until 1955 (SCHWARTZ and THAYSEN, 1955; BULMER and FORWELL, 1956) that a satisfactory hypothesis was advanced to account for it. In the preceding year, THAYSEN et al. (1954) studied Na^+, K^+, Cl^-, and HCO_3^- secretion in human parotid saliva induced by Mecholyl and proposed that saliva was formed in two stages: (1) the secretion of plasma-like isotonic precursor saliva, and (2) reabsorption of NaCl by the salivary duct (THAYSEN hypothesis, THAYSEN, 1960). The work of SCHWARTZ and THAYSEN (1955) on the human sweat gland was in fact the extension of their study of the human

parotid gland. They induced sweating in man by injecting Mecholyl intradermally and collected sweat samples at regular intervals using a small gastight sweat collection chamber. When the concentration of Na in sweat (C_1) was plotted against the sweat rate (V), the plots gave a curvilinear distribution, but the maximal Na concentration did not exceed 104 mM. However, when the rate of excretion of Na ($E_{Na} = C_1 \times V$, expressed in mEq.) was plotted against sweat rate, a linear distribution of plots was observed at high rates of flow, giving a linear function $E_{Na} = C_o \cdot V - T_{Na}$, where C_o and T_{Na} are constants, indicating the slope of the line and intercept respectively. According to THAYSEN's hypothesis (THAYSEN, 1960), there is a limited capacity for Na reabsorption by the sweat duct, and, after the reabsorptive capacity is saturated (e.g., at high sweat rate), the further increase in E_{Na} (ΔE_{Na}) as a result of an increase in flow rate (ΔV) should bear the relation $C_o = \Delta E_{Na} / \Delta V$. In other words, the slope of the asymptote (C_o) must be equal to the concentration of the plasma Na, 140 mM. Nevertheless, the value of C_o as estimated by SCHWARTZ and THAYSEN (1955) was only 68 mEq./L, a hypotonic value, but reexamination of their original plots suggests that a much higher C_o value could have been derived.

The first investigators who explicitly postulated the two-stage hypothesis, based on indirect evidence, were BULMER and FORWELL (1956) from Great Britain. They plotted Na concentrations in sweat induced thermally against the reciprocal of the sweat rate and observed a linear distribution of the plots. Extrapolation of the straight line to the "y" axis (at infinite sweat rate) gave 140 mEq./L, which they theorized to be the concentration of the precursor fluid. BULMER and FORWELL (1956) state: "It is suggested that sweat is formed from a precursor fluid with the same sodium concentration as interstitial fluid and that sodium is retained by the sweat gland (duct) as this fluid passes through them." In 1965, CAGE and DOBSON repeated the earlier experiment of SCHWARTZ and Thaysen (1955) except that they induced sweat secretion by thermal stimulation and were able to obtain a much wider range of flow rates. They calculated the slope of the asymptote (C_o) to be 140 mEq./L. Illustrative examples of the plots of sweat Na concentration vs. sweat rate (E_{Na}) (Fig. 32) are shown from the author's old data on human eccrine sweat (SATO and DOBSON, 1970a). The insert in Figure 33 is the schematic illusttration of the SCHWARTZ and THAYSEN model (SCHWARTZ and THAYSEN, 1955; CAGE and DOBSON, 1965a). The line AB has a slope of 140 mEq./L and this type of distribution of plots should be seen when there is no ductal Na reabsorption. In fact, this was actually the case when ductal Na reabsorption was inhibited by ouabain in vivo (SATO et al., 1969) or in patients with cystic fibrosis whose sweat duct is defective in the capacity to reabsorb NaCl (CAGE and DOBSON, 1965a). The curve ACD is the normal distribution of plots as seen in three curves in the same figure. The slope of the line CD should be identical with that of AB, 140 mEq./L. CAGE and DOBSON (1965a) defined AG or FC as free water clearance (a conceptual value indicating the amount of precursor fluid from which Na has been removed completely by ductal reabsorption, or T_{Na} as defined by SCHWARTZ and THAYSEN, 1955) and regarded it as a measure of the ductal reabsorptive capacity. AH or CE is another measure of ductal reabsorptive function and is referred to as water free Na reabsorption (CAGE and DOBSON, 1965a). Thus, AH equals 140 mEq. (C_o)$\times$AG. Overall, it must be emphasized that

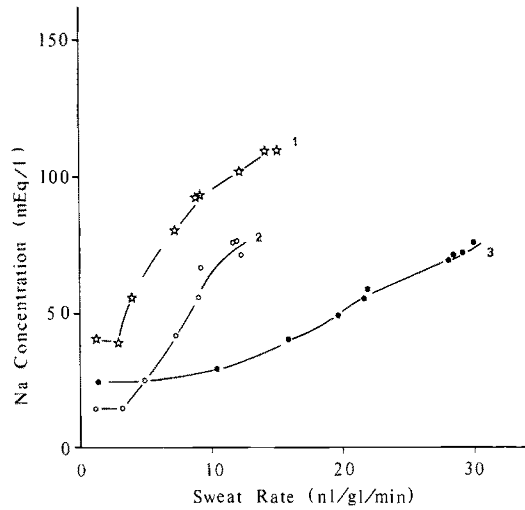


Fig. 32. Relationship between sweat rate and sweat Na concentration in vivo. Illustrative examples taken from SATO et al. (1971)

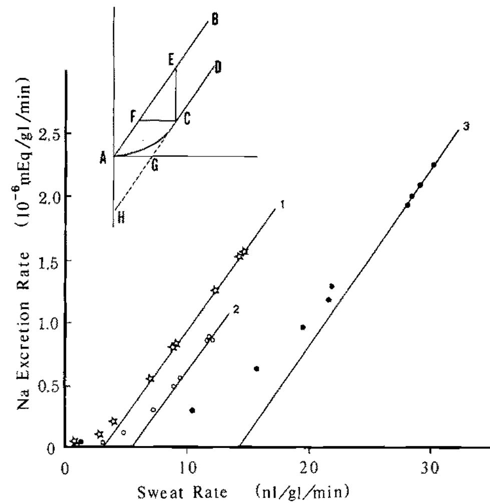


Fig. 33. Relationship between sweat rate and sweat Na excretion rate (Na contraction × sweat rate). The plots in Fig. 32 were replotted. The insert is SCHWARTZ-THAYSEN's analytic method as modified by CAGE and DOBSON (1965b). For more detail see text

SCHWARTZ and THAYSEN's (1955) or BULMER and FORWELL's (1956) plotting method is the only in vivo method available at present with which to indirectly evaluate the ductal function of the human eccrine sweat gland. This method, therefore, will continue to be useful in clinical research no matter how many other more sophisticated in vitro methods are introduced into sweat gland research in the future.

D. Mathematical Description of the Schwartz and Thaysen Method

Despite its theoretic innovation and practical applicability, SCHWARTZ and THAYSEN's (1955) plotting method was formulated rather empiricly. A mathemati-

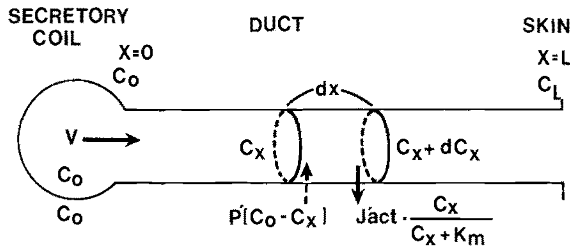


Fig. 34. Schematic model of ductal Na reabsorption. V. sweat rate, Co: Na concentration of interstitial fluid as well as the precursor sweat, X: any point along the length of the duct. See text for more detail

cal description of the method may help not only to uncover its pitfalls and shortcomings, but also to broaden its applicability based on more complete theoretical understanding. The mathematical techniques to be used here are the ordinary linear integration methods, and, in fact, similar techniques have been employed by SLEGERS (1967) for the sweat duct and by KNAUF and FRÖMTER (1970) for the salivary duct. In Figure 34, the eccrine sweat gland is schematicly illustrated as composed of the secretory coil and the duct. Since Na concentration of the primary sweat (C_o) is independent of the flow rate (Fig. 16), C_o is constant and simultaneously signifies Na concentration at the junction of the secretory coil and the duct ($x=0$), where x denotes any point along the length of the duct. At the skin surface ($x=1$), Na concentration of the oncoming sweat is C_1 (i.e., sweat Na concentration), and likewise at point x, Na concentration of the luminal fluid is expressed as C_x. Consider a thin disc of luminal fluid at x, whose Na concentration C_x changes to C_x+dC_x as the thin disc of luminal fluid moves by a small step, dx, to $x+dx$. The small change in luminal Na concentration dC_x must be the result of Na reabsorption, which is partially countered by Na entering the lumen from the interstitium by passive backflux. It is assumed that active reabsorption of Na may depend on C_x according to Michaelis-Menten's saturation kinetics as in the frog skin (CEREIJIDO et al., 1964) or gall bladder (DIAMOND, 1962). Then the Na pump activity per unit length of the duct at point x is expressed as $J'_{act} \cdot C_x/(C_x+Km)$ where J'_{act} is the maximal capacity for active transport per unit length of the duct and Km the Michaelis constant, the concentration of luminal Na which yields half the maximal pump activity (i.e., $^1/_2 \cdot J'_{act}$). The passive backflux is assumed to depend on Ficks' law and, therefore, is expressed as $P'\ (C_o-C_x)$, where P' is the permeability constant of Na per unit length of the duct and C_o-C_x is the concentration difference for Na between the ductal lumen and the interstitium.

Thus one can write

$$V \cdot \frac{dC_x}{dx} = J'_{act} \cdot \frac{C_x}{C_x+Km} + P'\ (C_o-C_x) \tag{1}$$

where V is the flow rate of sweat and thus $V \cdot dC_x/dx$ is the change in the amount of luminal Na between the points x and $x+dx$. Equation 1 can be integrated,

but its solution is far too complicated for practical application. At the boundary condition, where sweat rate (V) is infinitely high ($V \to \infty$), C_x approaches C_o and thus $C_x \gg Km$ if the Km for the sweat duct epithelium is small as in the frog skin (CEREIJIDO et al., 1964) or the gall bladder (DIAMOND, 1962). Then Equation 1 reduces to

$$V \cdot \frac{dC_x}{dx} = -J'_{act} + P'(C_o - C_x) \tag{2}$$

Rearranging and integrating one has:

$$\int_o^1 \frac{dC_x}{C_x - C_o + \frac{J'_{act}}{P'}} = -\int_o^1 \frac{P' \cdot dx}{V} \tag{3}$$

and thus

$$\left[C_x - C_o + \frac{J'_{act}}{P'}\right]_o^1 = \mathrm{K}_0 \cdot \left[\exp\left(-\frac{P'}{V} \cdot x\right)\right]_o^1 \tag{4}$$

From the boundary condition ($C_x = C_o$ at $x = 0$), one obtains $K_o = J'_{act}/P'$. Equation 4 can now be written as

$$C_1 = C_o - \frac{J'_{act}}{P'} + \frac{J'_{act}}{P'} \cdot \exp\left(\frac{-P' \cdot 1}{V}\right) \tag{5}$$

According to the assumption $V \to \infty$ and for mathematical reasons, Equation 5 reduces to

$$C_1 = C_o - \frac{J'_{act}}{P'} + \frac{J'_{act}}{P'} \cdot \left(1 - \frac{P' \, 1}{V}\right) = C_o - \frac{J'_{act} \cdot 1}{V} \tag{6}$$

Rearranging Equation 6 and defining $J'_{act} \cdot 1 = J_{act}$ (reabsorptive capacity of the entire duct) one has:

$$V \cdot C_1 = V \cdot C_o - J_{act} \tag{7}$$

It is now obvious that Equation 7 is none other than what SCHWARTZ and THAYSEN (1955) derived from their plots of Na excretion (E_{Na}) vs. sweat rate (V) or the line HGCD in the insert of Figure 33. Thus, the free water clearance is now expressed as J_{act}/C_o and the water-free Na reabsorption (y intercept) as J_{act}. At this point, however, those assumptions already made, as well as other factor(s) which might affect the practical application of Equation 7, must be briefly reevaluated. Among them, the sweat rate is the most critical; it must be sufficientyl high and the asymptote must be drawn according to the plots with the highest sweat rates. Theoretically, C_o or the slope of the asymptote can not be lower than the Na concentration of plasma or that of the immediate periglandular interstitial space. The question of whether plasma and immediate interstitial space are osmo-

tically equilibrated remains to be determined. However, if the data of SCHULZ (1969) are correct, then, at least in an in vivo condition, the immediate interstitial space is expected to have an osmolarity only 10% higher than that of plasma in general circulation. Another assumption to be discussed is the magnitude of Km; if it is too large to be neglected, then Equation 7 and the SCHWARTZ and THAYSEN (1955) method are no longer valid. However, as the plot in Figure 33 shows, the slopes of the asymptotes approach C_o at intermediate sweat rate ranges, indicating that Km may be rather small as in other epithelia. The third problem concerns the uncertainty that J'_{act} may change during a short experimental period, or it may show a flow dependency such as is seen in the kidney (BARTOLI et al., 1973), i.e., the faster the flow, the higher the maximal pump activity, although no undisputable experimental evidence exists to support or refute such a possibility in the sweat gland. The effect of backflux of water (from lumen to the interstitium) should also be considered. Although the backflux of water has been directly demonstrated by MANGOS (1973b) in an isolated human ductal system, this factor may affect Equation 7 relatively little since at the boundary condition, $C_o - C_x$ diminishes and so does the water backflux which depends also on $C_o - C_x$. The neglect of the amount of Na backflux into the lumen according to the potential difference $(P(ZF/RT) \cdot \Delta P.d. \cdot (C_x + C_o)/2$, where Z, F, R, T have their usual meaning in electrochemistry and $\Delta P.d.$ the potential difference) may result in the underestimation of J_{act} and/or the overestimation of P.

E. Other Mathematical Descriptions of Sweat Na Excretion

EMRICH and ULLRICH (1966) and EMRICH et al. (1967) observed a linear exponential correlation between $147 - \mathrm{Na}_x$ and the sweat rate (x) and thus experimentally arrived at the following equation:

$$\ln (147 - \mathrm{Na}_x) = -ax + \ln (147 - \mathrm{Na}_o) \tag{8}$$

where ln is natural logarithm, 147 is Na concentration of the precursor fluid (SCHULZ et al., 1965), a is the slope of the fitted line and Na_o is the extrapolated sweat Na concentration when the sweat rate (x) is zero. EMRICH and ULLRICH (1966) and EMRICH et al., (1967) induced sweating pharmacologically on the back of the finger in man, hence the obtained sweat rate was rather low. As shown in Figure 35, the author replotted the data already shown in Figures 32 and 33 according to the method of EMRICH et al. (1967) (Eq. 8). Interestingly, at the low sweat rate range, our data (which consists of thermally induced sweating) seem to fit Equation 8 reasonably well; however, the fitted lines then showed sharp bends as the sweat rate increased. When these steeper portions of the bent lines were extrapolated to the "y" axis, the "y" intercept $(145 - \mathrm{Na}_o)$ reached as high as 180 mEq./L. Since $(145 - \mathrm{Na}_o)$ should always be lower than 145, it is obvious that Equation 8 is not applicable in the intermediate to thigh ranges of sweat rate.

The effort by SLEGERS (1967) to describe ductal function mathematically deserves credit. In an attempt to differentiate the reabsorptive functions of the proximal and the distal duct, he analyzed separately the influx and outflux components in

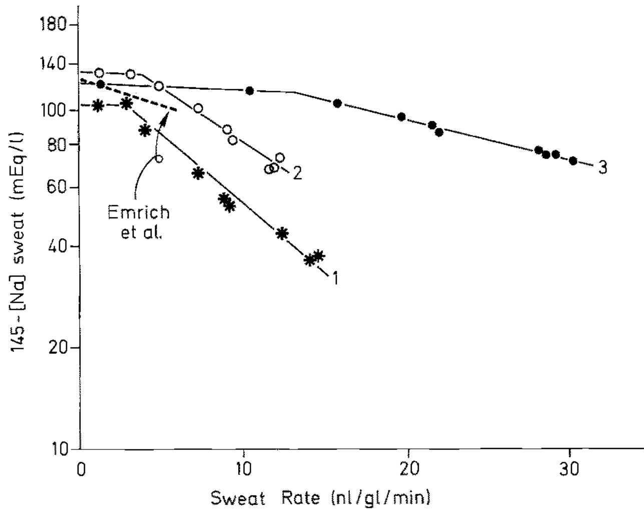


Fig. 35. Plots of sweat rate vs. ln $(145-Na_{sweat})$. The same data shown in Fig. 33 were replotted according to Eq. 8. For more detail see text

each of the duct. For the proximal duct, he assumed both influx (passive) and outflux (reabsorptive) coefficients and for the distal duct only the outflux component. The essential difference between the formulation of SLEGERS (1967) and Equation 2 is that he assumed that the Na outflux component is not a rate limiting capacity (J'_{act} in Equation 2) but increases linearly with luminal Na concentration ($K \cdot C_x$ where K is an outflux constant in his formulations). In effect, assumed that Km in Equation 1 is much larger than any values of C_x ($Km \gg C_x$ and $J'_{act} \cdot C_x/C_x+Km$) in Equation 1 reduces to $C_x \cdot (J'_{act}/Km)$). The term J'_{act}/Km thus corresponds to K in SLEGERS' (1967) formulation. Although Km for ductal Na reabsorption must be rather small because of the reasons discussed in a previous section, direct verification must await future studies using a microperfusion technique. However, the discrepancy between the calculated curve and the experimental curve (SLEGERS, 1967) is to be expected if a high Km value is assumed, since the saturation of the reabsorptive function is never reached in the formulation, whereas it may have already been reached in the middle sweat rate ranges in the actual experimental data. Furthermore, SLEGERS' (1967) formulation calls for complete reabsorption of sweat Na when the sweat rate approaches zero ($Na_o=0$ in EMRICH's equation) which, however, never occurs in actuality. This is because the passive component of the distal duct was omitted to make the mathematical formulation simpler. It should be noted, however, that none of the presently available mathematical descriptions are completely satisfactory. Equation 2 presumes uniformity of the ductal reabsorptive function, yet the distal duct is known to contain a much lower Na+K-ATPase than the proximal duct (SATO et al., 1971). Another innovation introduced by SLEGERS (1966) is the method of plotting between logarithm of sweat Na concentration and the perfusion (passage) time of sweat through the duct (which is proportional to the inverse of the sweat rate), although this method was an extension of the work of BULMER and FORWELL

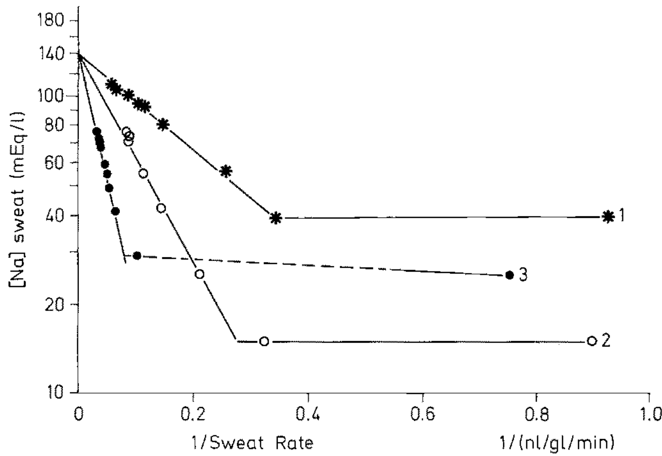


Fig. 36. Relationship between the inverse of sweat rate and logarithm of sweat Na concentration. The same data shown in Fig. 33 were replotted

(1956) which plotted Na concentration against the inverse of the sweat rate. According to the method of SLEGERS, the data in Figures 32 and 33 have been replotted as shown in Figure 36. Consistent with SLEGERS' (1966) observation, the fitted curve yields two components, the steeper and the horizontal, and the extrapolation of the steeper component cuts the "y" axis at around 140 mEq./L. Now consider this steeper component because only this component satisfies Equation 5, a linear exponential function. The extrapolation of this straight line toward Na_{sweat} (or C_1) $\rightarrow o$, also means $V \rightarrow o$ and Equation 5 becomes:

$$\lim_{V \to o} C_1 \simeq C_o - \frac{J_{act}}{P} \rightarrow o \tag{9}$$

where P is defined as $P' \times 1$ (permeability of the entire duct). Since C_o, J_{act} and P are constants, Equation 9 gives:

$$C_o = J_{act}/P = 140 \text{ mM} \tag{10}$$

Incorporating Equation 10 into Equation 5 we have:

$$C_1 = \frac{J_{act}}{P} \cdot \exp \frac{-P}{V} \text{ or } \ln C_1 = \ln C_o - \frac{P}{V} \tag{11}$$

or

$$\log C_1 = \log C_o - \frac{P}{2.3} \cdot \frac{1}{V}$$

Equation 11, therefore, represents the steeper component of the curves shown in Figure 36. From the tangent of this steeper component (slope), one can obtain the P value (tangent $\alpha = P/2.3$) and J_{act} can be calculated (see KNAUF and FRÖMTER, 1971)

from the "y" intercept (140 mM=J_{act}/P). At present we have no explanation or mathematical formulation for the horizontal component of the plot. Nor do we have any evidence that this component represents the function of the distal duct despite the proposal by SLEGERS (1967) that such might be the case. In fact, in an isolated sweat gland where sweat samples are collected directly from the open end of the proximal duct, we do observe a similar horizontal component (SATO, K., unpublished data). Our speculation at present is that it represents reduced pump activity at low sweat rates (note that a decrease in C_x no longer permits the assumption, $C_x \gg Km$, in Eq. 1) or the effect of reduced flow itself on the pump activity as in the proximal tubule of the kidney. The possible error that neglect of the transductal electric potential difference (p.d.) might have on the estimation of the above parameters has already been discussed in a previous section.

In summary, the new theoretical insight into SCHWARTZ and THAYSEN'S (1965) method as well as SLEGERS' (1963, 1967) and KNAUF and FRÖMTER'S method (1971) provides a new theoretical basis for the analysis and understanding of the biophysical parameters of eccrine sweat gland function and has proven that these sophisticated parameters are measurable, under some assumptions, by conventional simple in vivo sweat collection techniques.

F. Concentration in Other Electrolytes in Sweat

Potassium (K) concentration in the precursor sweat is also isotonic (SATO, 1973b) to plasma, i.e., about 5 mM, whereas that of final sweat is always higher than 5mM (SATO et al., 1970; EMRICH et al., 1967). In contrast to NaCl, sweat K concentration is higher (10–35 mM) at low sweat rates but approaches isotonicity (5 mM) as the sweat rate increases. Sweat calcium concentration shows the same trend. At very low sweat rates, it ranges from 3 to 10 mM but asymptotically approaches 1–2 mM at higher sweat rates (EMRICH et al., 1967). In addition to the above electrolytes, sweat also contains magnesium, iodide, phosphorus, sulfate, and metals such as iron, zinc, copper, cobalt, lead, manganese, molybdenum, tin, and mercury in trace amounts (KUNO, 1956; HARDEN and ALEXANDER, 1963; HOHNADEL et al., 1973; SEUTTER and SUTORIS 1972). Presumably because of technical difficulties, no study included the correlation between sweat rate and the concentrations of the above constituents. The variations in the concentrations of each constituent may be partially explained by differing methods of sweat collection, ie., the presence or absence of sweat evaporation or epidermal contamination.

Chloride (Cl) secretion in sweat generally follows that of Na except that its concentration is lower by 10–30 mM. The micropuncture studies by SCHULZ (SCHULZ et al., 1965; SCHULZ, 1969) have shown that Na and Cl concentrations in the primary sweat are 147–151 and 123–124 mM respectively, values identical to those of plasma. From these data, the anion deficit can be calculated to be approximately 30 mM (149 mM Na^+ 5 mM $K^+ - 124$ mM Cl^-). If lactate concentration is not modified by the duct, the secretory coil sweat should contain 15–20 mM lactic anion. Then the remaining anion deficit shrinks to 10–15 mM, which may be mostly occupied by bicarbonate (HCO_3^-). Since the secretory coil (but not the duct) avidly converts medium glucose to lactate (SATO and DOBSON,

1973), sweat lactate may be derived from the secretory coil but not from the duct, at least in significant amounts. Hence, if the micropuncture samples were absolutely free of contamination by the interstitial fluid, we cannot escape the conclusion that HCO_3^- concentration in the precursor sweat is approximately 10–15 mM, a value half that of plasma HCO_3^-. In the cat paw eccrine sweat gland, which consists of a long tubular secretory coil and a short duct (presumed but not directly proven to be nonfunctional), BRUSILOW and GORDES (1967) reported the total CO_2 content in the skin surface sweat to be 37.7 mM (range 29–45 mM, which mostly represents HCO_3^-). SLEGERS and MOON (1968) used the same VAN SLYKE method (VAN SLYKE and PLAZIN, 1961) as did BRUSILOW and GORDES (1967) but reported a somewhat higher value, 49 mM CO_2. Thus, the discrepancy in HCO_3^- secretion between human and cat sweat glands cannot be accounted for at present. HCO_3^- concentration in the skin surface sweat of the adult human ranges from 2 to 10 mM (BRUSILOW and GORDES, 1967) and tends to increase with the sweat rate (KAISER et al., 1974).

G. Sweat pH

Since the pH of primary sweat is not known, the problem of sweat pH shall be included in the discussion of ductal function. Human sweat, when equilibrated with 40 torr CO_2 (a partial pressure of CO_2 in the interstitial fluid), is slightly acid, and its pH value is dependent on sweat rate (EMRICH and OELERT, 1966). When sweat rate is low, its pH value is as low as 5, but it increases with increasing sweat rate toward a neutral pH. When sweat is exposed to air for sufficiently long, however, it shows a neutral to slightly alkaline pH (7–8) except at very low sweat rates when pH shows between 5 and 6. This indicates that the HCO_3^-–CO_2 buffer system is playing a major role in regulating sweat pH at medium to high sweat rate ranges. Other than HCO_3^-, the lactate-lactic acid system, phosphates, amino acids, ammonia, and perhaps also proteins in sweat may also be involved in determining sweat pH; however, their roles in quantitative terms are not clear. The site and the mechanism of sweat acidification are not known, although it is speculated that the duct acidifies the precursor sweat by reabsorbing HCO_3^- and/or secreting H^+ in exchange for Na^+ (KAISER et al., 1974). The fact that the ammonium ion (NH_4^+) and the acidity of sweat correlate does not necessarily justify the conclusion (MORIMOTO and JOHNSON, 1967) that the ammonium buffer system is the denominator of sweat pH, because the ammonium ion may simply be distributing according to nonanionic diffusion of NH_3 at low sweat rates (see Sect. G).

H. On the "Empty Duct" Theory of Lloyd

Is the ductal lumen empty of sweat during rest? The answer may be yes and no. LLOYD (1959a) put forth the empty duct theory based on the following observations:

1. There is a latent period between the start of nerve stimulation in cat foot pads and the first detectable beads of sweat on the skin surface.
2. The length of the latent period is proportional to the length of the preceding

rest period. Repetitive stimulation of the glands shortens the latent period to 2.5–3 s.

3. Skin impedance changes according to the rate of sweat secretion.

Since the sweat in the ductal lumen itself behaves as a conducting liquid, the impedance should be high and constant when the duct is filled with air, which is exactly what LLOYD observed.

In the days of LLOYD (1959a) when the basic principles for the movement of water were still poorly understood, the idea of sweat water reabsorption by the duct was still prevalent. In the light of recent information, LLOYD's empty duct theory can be reappraised. In the sweat duct, water moves according to the difference in osmolarity and the magnitude of the ductal water permeability. In cat foot pads, sweat is always slightly hypertonic (FOSTER, 1966). Thus, water tends to move into the duct rather than to be reabsorbed by it. If water evaporates from the ductal orifice, osmolarity will increase locally in the upper duct and more water will move in from the interstitium. However, since the ductal orifice is made up of a cornified cuticular border which is further surrounded and supported by epidermal cells, it is easy to assume that a very short length of the ductal lumen in the horny layer can become empty as a result of evaporation. This might give sufficiently high impedance to account for LLOYD's skin impedance measurement. Under a stereomicroscope, the ductal lumen of an isolated sweat gland can be observed to dilate and collapse according to variations in the hydrostatic pressure in the lumen. The fluctuation of skin impedance according to the repetitive stimulation of the cat sweat gland can be easily accounted for by changes in the diameter of the liquid-filled conducting channel — the ductal lumen. The long latent period in his experiments may be due partially to the sensitivity of the method of sweat measurement used and partly to the readiness of the secretory response of the gland itself, since the volume of the ductal lumen is so small in the cat that it may be filled instantaneously once profuse secretion sets in.

IX. Mechanism of Ductal Na Reabsorption in the Sweat Gland

The eccrine sweat duct is well developed both structurally and functionally in human and monkey. It consists of the proximal (or coiled) portion which coils intimately with the secretory portion and the distal duct which is straight to slightly helical and connects the coiled duct and the epidermal sweat duct unit. The absorption of NaCl by the duct due to active transport of Na ion appears to be firmly established. Firstly, ductal Na reabsorption is ouabain sensitive. Intradermal injection of ouabain in man was found by SATO et al. (1969) to completely inhibit ductal NaCl reabsorption as well as sweat secretion. The same effect of ouabain was observed also in an isolated eccrine sweat gland (SATO, 1973b). Secondly, the presence of Na+K-ATPase has been demonstrated in both the duct and the secretory coil (SATO and DOBSON, 1970c; SATO et al., 1971; GIBBS et al., 1967), which is also sensitive to ouabain. Thirdly, the transepithelial electric p.d. is

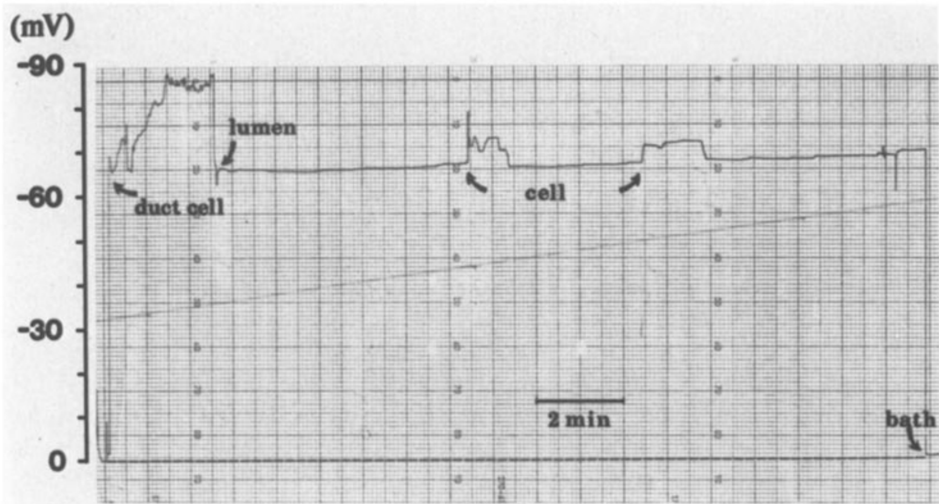


Fig. 37. The original tracing of the ductal cell membrane p.d. as well as transductal p.d. as measured by a glass microelectrode. In the middle of the tracing, the electrode was advanced into the ductal cells (arrows) from the ductal lumen

from 40 (SCHULZ et al., 1965, in the human duct) to 70 mV (Fig. 37, in the monkey duct at rest) lumen negative, indicating that Na^+ (but not Cl^-) must be transported against this electric p.d. in addition to the chemical concentration gradient of up to 140 mM (i.e., the lumen is always hypotonic). Thus, the total electrochemical potential gradient to be overcome by Na^+ may be as high as 130 mV since the luminal Na^+ can reach as low as 10 mM. As for Cl^-, the electric p.d. favors the movement of this anion, but it must be transported against the chemical gradient. Thus, a small electrochemical potential gradient remains in favor of passive movement of Cl across the ductal epithelium. In other word, Cl is most likely transported passively in the duct. The detailed mechanism of active Na reabsorption by the duct remains to be determined, although several speculative mechanisms must be discussed. The simplest and the most attractive one is the analogy to Na transport by the frog skin (USSING and ZERAHN, 1951; USSING, 1965) or the salivary duct (KNAUF and FRÖMTER, 1971). In these epithelia, Na is thought to diffuse passively from the lumen (outside in the frog skin) to the cell interior, and it is then actively pumped out from the cell to the interstitium (dermal side in the frog skin) at the peritubular cell membrane in exchange for K. At the luminal membrane, both the p.d. (as shown in Fig. 37, cell interior is electricly more negative than lumen) and the Na concentration difference favor the passive movement of Na into the cell. Since the luminal cell membrane (of the salivary duct) is more permeable to Na than to Cl^- or K, this membrane behaves like an Na electrode, whereas the contraluminal (peritubular) cell membrane behaves like a K electrode. Since the Na-K exchange pump at the peritubular cell membrane is assumed to be (but not proven to be) electroneutral, USSING and ZERAHN (1951) and KNAUF and FRÖMTER (1971) theorized that the transepithelial p.d. is the sum of

these two diffusion potentials. In the eccrine sweat duct, none of these parameters has been measured.

Recently a few interesting reports have been published pertaining to the mechanism of ductal Na reabsorption. KAISER and DRACK (1974) reported that the skin surface sweat of patients with cystic fibrosis (CF) has a higher pH than controls. Under PCO_2 tension of 40 torr, they measured sweat pH and calculated HCO_3^- concentration in sweat according to the Henderson-Hasselbalch equation. The concentration of HCO_3^- in CF sweat was thus calculated as 25–75 mM, in control children 25–35 mM, and in the adult control 0–20 mM. KAISER and DRACK 1974) did not measure total CO_2 concentration to corroborate their data. These data led KAISER and DRACK (1974) to speculate that in the normal sweat duct, a H^+-Na^+ exchange pump occurs since acetazolamide increases Na concentrations and the pH of skin surface sweat (KAISER and DRACK, 1974). They also speculated that the defect in the sweat duct of CF is the absence of the H^+-Na^+ exchange pump resulting in an increase in sweat Na^+ and a decrease in H^+ concentration (a hihger pH). Although KAISER and DRACK (1974) have never explicitly stated so, their observation that sweat HCO_3^- concentrations rise as high as 50–75 mM in the absence (in CF) of ductal HCO_3^- absorption (or H^+ secretion) implies that the primary sweat must contain at least that great a HCO_3^- concentration. If the primary sweat contains low HCO_3^- concentrations as deduced from SCHULZ's micropuncture data (SCHULZ et al., 1965; SCHULZ, 1969), or even if it is isotonic to plasma HCO_3^-, then KAISER's data must be interpreted as suggesting the ductal OH^- secretion (or H^+ absorption) in CF. The solution to this problem depends upon the use of in vitro perfusion techniques using single segments of the duct and the secretory coil. Previous reports on the effect of acetazolamide on the eccrine sweat gland in vivo are also at variance with KAISER's observation. RICHTERICH and FRIOLET (1963) did not observe an elevated Na concentration in sweat after intravenous administration of acetazolamide but did observe a decrease in sweat K concentration only in patients with CF. These investigators interpreted this as indicating that an increased ductal Na-K exchange occurs in CF and that this cation exchange might be inhibited by acetazolamide. SLEGERS and MOON (1968) reported a decrease in sweat rate, sweat pH, and sweat HCO_3^- concentration in cat paw sweat after intraperitoneal injection of acetazolamide. Cat paw sweat is regarded (but not proven) as representing primary sweat secretion unmodified by ductal absorption (BRUSILOW and MUNGER, 1962). Since, in their control cats, a gas mixture of a high CO_2 content (85% O_2+15% CO_2) was given, it is not clear how acetazolamide might have operated had the cats been given a gas mixture of atmospheric CO_2 content. Nevertheless, their study suggests that the secretion of primary fluid may also be influenced by an acetazolamide-sensitive transport system such as Cl pump, H pump, H^+-Na^+, or H^+-K^+ exchange, or Cl or HCO_3^- secretion. We have previously reported that acetazolamide or HCO_3^- in the incubation medium has little effect on glucose metabolism of isolated monkey palm eccrine sweat glands (SATO and DOBSON, 1973). Consistent with this, we now have data indicating that neither primary sweat secretion nor ductal Na reabsorption is affected by acetazolamide (Diamox 2 mM, Figs. 38 and 39). The removal of HCO_3^- from the incubation medium (replaced by 2 mM phosphate buffer and NaCl gassed with 100% O_2) also had no inhibitory effect (Fig. 40); however, as

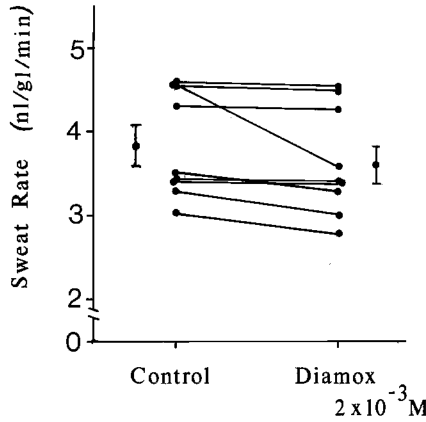


Fig. 38. Effect of acetazolamide (Diamox) on MCH-induced sweat secretion in vtitro

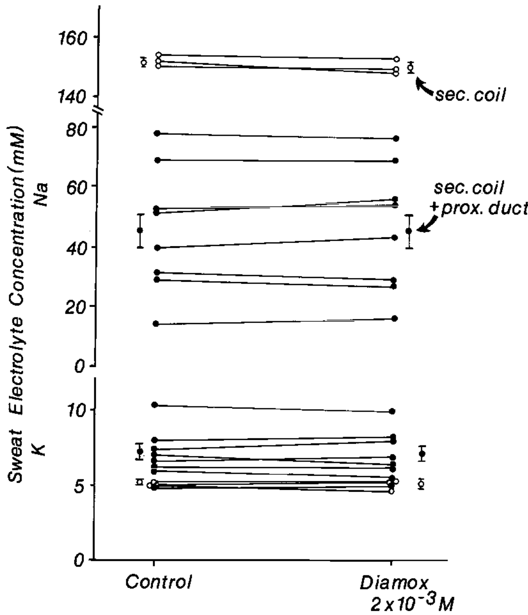


Fig. 39. Effect of acetazolamide (Diamox) on Na and K concentrations of secretory coil sweat and ductal sweat

shown in Figure 41, the ductal Na absorption was partially inhibited in the absence of HCO_3^-. Since the Na concentration of primary sweat is always isotonic (see also Fig. 39), the data in Figure 41 showing that sweat Na concentration was higher by 20 mM in the absence of HCO_3^- in the medium indicates, in fact, a partial inhibition of ductal Na reabsorption in the HCO_3^--free medium. Thus, despite the fact that the ductal function was insensitive to acetazolamide, the effect of the HCO_3^--free medium on Na reabsorption by the duct suggests (but does not prove) that either the H^+-Na^+ or the H^+-K^+ exchange mechanism is "partially" involved in the ductal reabsorptive function.

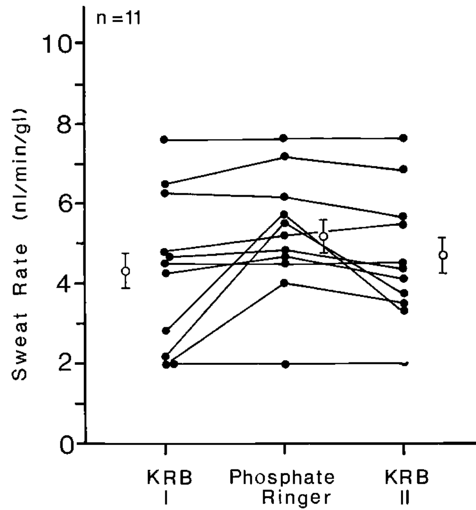


Fig. 40. Effect of HCO_3^--free phosphate Ringer on the secretory rate in vitro. Kreb's Ringer (KRB) contains 25 mM HCO_3^- and was gassed with 95% O_2 plus 5% CO_2, whereas phosphate Ringer was gassed with 100% O_2. pH of the medium was 7.4 in both solutions

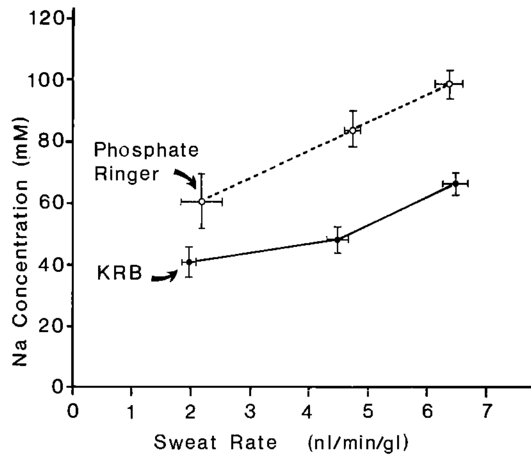


Fig. 41. Effect of HCO_3^--free phosphate Ringer on ductal Na reabsorption. The concentration of the precursor fluid was always isotonic to the medium (not shown)

MANGOS (1973b) recently modified the original BURG techniqe (BURG et al., 1966) for perfusing an isolated human distal sweat duct. He observed that the distal duct of the human sweat gland actively transports Na^+ but that the luminal perfusate must be sweat for Na to be reabsorbed. When he perfused Ringer's solution through the ductal lumen, ductal Na absorption was drastically suppressed, thereby suggesting that some factor(s) in normal sweat are involved in the regulation of ductal Na absorption. He was also able to confirm his previous observation that sweat in CF contains a factor(s) which inhibits Na reabsorption by the duct. In the salivary duct, however, Na^+ is normally reabsorbed from Ringer's solution used as a perfusate, but the presence of Ca^{++} in the perfusate was found to partially inhibit ductal Na absorption (SCHNEYER, 1974b). It is not known whether the difference between sweat and Ringer's solution as a perfusate in the sweat duct is the concentration of free Ca^{++}, which may be lower in sweat than in Ringer's

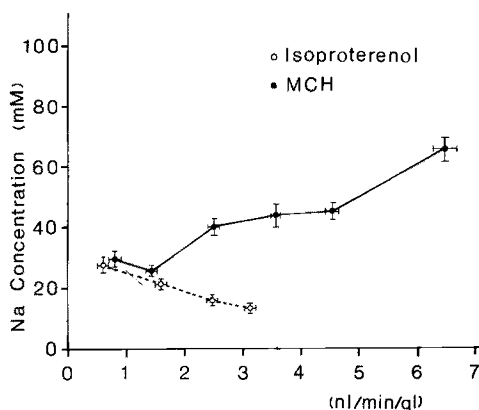


Fig. 42. Comparison of sweat Na concentrations between MCH-induced and isoproterenol-induced ductal sweat. Sweat samples were collected from the open end of the proximal duct

solution. The remaining question concerns the humoral control of the ductal reabsorptive function. In the cannulated rat submaxillary main duct, SCHNEYER (1973) observed that administration of isoproterenol to rats as a single intraperitoneal dose produced a sustained elevation in net reabsorption of Na^+ by the duct. The prior administration of the β-blocking agent, propranolol, suppressed the effect of isoproterenol. In keeping with SCHNEYER's observation, we have also found that isoproterenol-induced sweat obtained from the open end of the proximal duct contained a much lower Na concentration (Fig. 42). Since the Na concentration of the precursor fluid is always isotonic, the lower sweat Na concentrations in the same sweat rate range clearly indicate increased ductal Na reabsorption. cates that β-adrenergic stimulation might also have a similar stimulating effect on the eccrine sweat duct.

The inverse relation between sweat rate and Na concentration may be the result of stronger β-stimulation on the duct because a higher concentration of isoproterenol was required to induce a higher sweat rate. In order to verify the direct effect of β-stimulation on the ductal function, microperfusion of the ductal system will be mandatory.

X. The Effect of Aldosterone and Antidiuretic Hormone on the Ductal Function

A. Aldosterone

In 1948, CONN et al. first reported that aldosterone and other mineral corticoid hormones caused a reduction in sweat Na an increase in sweat K concentration, which was not accompanied subsequently by the so-called "escape phenomenon" usually seen in the kidney. CONN's observation has since been supported by a number of studies using more refined techniques or different approaches (GRAND et al., 1967; GRANDCHAMP et al., 1968; SATO and DOBSON, 1970b). Other than the

change in sweat Na/K ratio, aldosterone also reduces sweat rate (GRANDCHAMP et al., 1968; SATO and DOBSON, 1970b; DOBSON and SLEGERS, 1971). The effect of aldosterone begins to appear 6 h after a single local or systemic administration, reaches its peak at 24h (SATO and DOBSON, 1970b), and subsides in 48–72h. In reviewing or evaluating the sweat K concentrations reported in the literature, it is important to note that pharmacologically induced sweat (i.e., pilocarpine iontophoresis or intracutaneous injection) has a much higher K^+ concentration than does thermally induced sweat (SATO et al., 1970). Since K^+ concentration changes with sweat rate, any studies not taking this into account must be accepted with caution. The site of action of aldosterone on the sweat electrolyte composition is probably the duct cells. The decrease in sweat Na/K ratio suggests the presence of Na^+-K^+ exchange in the duct as in the distal tubule of the kidney. However, the decrease in Na concentration and the increase in K concentration in sweat is by no means one to one. Occasionally, only Na concentration decreased, whereas there were no changes in K concentration and vice versa (SATO et al., 1970). It is also possible to speculate that an increase in sweat K^+ concentration of 5 or 10 mM could also be achieved by the change in tubular permeability to K and/or an increase in luminal potential negativity, if any, due to enhanced Na transport after aldosterone. The mechanism for enhancement of the sweat gland function in acclimatization is not yet clear. The study by BAILEY et al. (1971) confirmed previous observations that Na deprivation stimulates both renin and aldosterone secretion and observed furthermore that high thermal stress, per se (a single 1 h exposure to a humid temperature of 105° C), is a potent stimulator of renin and aldosterone secretion in both the presence and absence of Na deprivation. Nevertheless, direct evidence is lacking that the enhancement of sweat gland function (a higher maximal sweat rate, Vmax, and reabsorptive capacity, J_{act}) is mediated solely by aldosterone. Observations such as evidence of dual innervation in the eccrine sweat gland (UNO and MONTAGNA, 1975), activation of the pentose cycle by the adrenergic β-stimulation (SATO, 1973a), and acute stimulation of ductal Na reabsorption by isoproterenol (Fig. 42) suggest that sympathetic stimulation of the gland is also involved in the regulation of the sweat gland function in addition to aldosterone.

B. Antidiuretic Hormone

Antidiuretic hormone (ADH) does not appear to directly influence the water and electrolyte secretion by the sweat gland in man (PEARCY et al., 1955–1956; RATNER and DOBSON, 1964). In an isolated monkey sweat gland in vitro, neither sweat rate nor sweat electrolyte concentration was affected even by a massive dose of ADH (2 U/mol medium) on a fresh gland or on a gland stored at 8° C for 24 h (SATO, K., unpublished data). There are, however, several reports of both reduction in sweat rate and increase in sweat Na concentration after local administration of ADH (FASCIOLO et al., 1969; QUATRALE and SPIER, 1970; SLEGERS and VAN'T HOT-GROOTENBOER, 1971). AHD is known to cause vasoconstriction when injected subcutaneously. These authors claim, however, the effects of ADH were observed

even with a dose of ADH which did not appear to have caused vasoconstriction. The absence of vasoconstriction or blanching of skin, which results predominantly from constriction of the capillaries of dermal papilla, does not necessarily rule out the reduction in blood flow in middermal arterioles.

XI. Electrophysiologic Approach to an Isolated Monkey Eccrine Sweat Gland

The application of electrophysiologic techniques is indispensable at this point in order to acquire more information on the movement of ions and the permeability of the cell membrane before and after stimulation of the eccrine sweat gland with various secretagogues. The electric data presented here are preliminary and unpublished. Nevertheless, it was felt that a brief presentation of such data might provide deeper insight into the mechanisms of eccrine sweat secretion. The transepithelial p.d. of the secretory coil was measured as whown in Figure 43. A special pipet was constructed according to BURG and ORLOFF (1970). The cup-like space at the end of the pipet was filled with liquid dielectric Sylgard 184 to insulate the lumen of the secretory coil or the duct from the bath. The luminal electrode was filled with Ringer's solution in order to eliminate the liquid junction potential and was connected to the differential amplifier by way of a calomel half cell. The bath was connected to the ground as well as to the other input of the amplifier in the same way. Figure 44 is an illustrative example of transepithelial p.d. of a secretory coil. When Mecholyl (or acetylcholine) was added to the incubation bath, a sharp negative spike-like potential was recorded, which was followed by a transient shift of the p.d. toward zero. A steady lumen negative p.d. (range −2–10 mV) was then developed in about 1 min after the addition of acetylcholine. The transepithelial p.d. so developed was inhibited by atropine, cyanide, or ouabain. The lumen negative p.d. was somewhat unexpected because

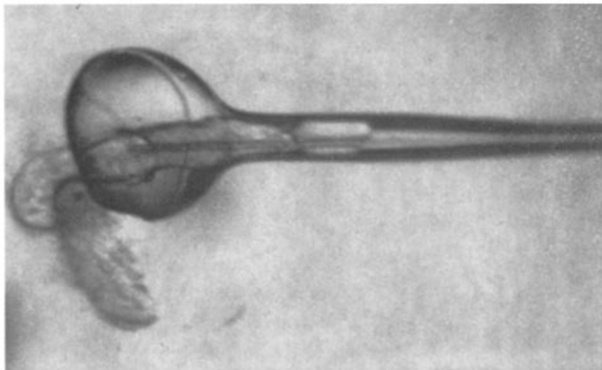


Fig. 43. Method of measuring transepithelial p.d. using a coaxial double pipet. A segment of the secretory coil is held in an outer pipet and a thin inner electrode (capillary) is inserted into the tubular lumen. SYLGARD 184 is used to achieve electric insulation. For more detail see text

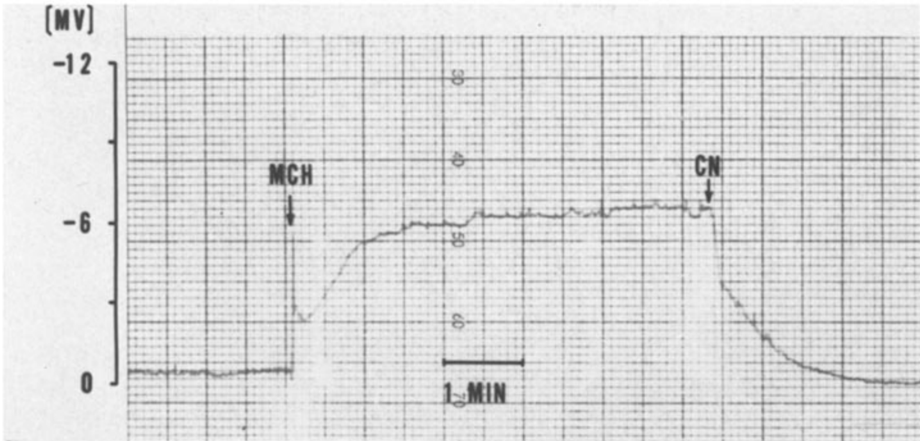


Fig. 44. An illustrative example of the transductal p.d. CN: cyanide 1 mM

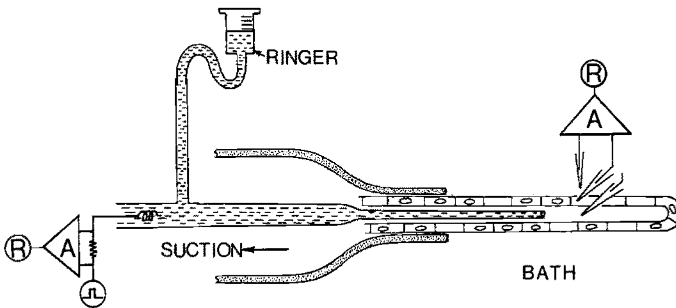


Fig. 45. Schematic illustration of the method for measuring the change in the basal membrane resistance and the transepithelial resistance of the secretory coil. A small hydrostatic pressure was applied to the lumen to prevent the collapse of the secretory coil lumen. A long segment of the secretory tubule (coil) drawn by suction into the pipet was several times the length constant (83μm) of the secretory tubule. A: differential amplifier

we had anticipated a lumen positive p.d. if the Na+K-ATPase mediated Napump is the primary driving force and if Cl^- moves passively according to the electric coupling. The transepithelial impedance was measured as illustrated in Figure 45. The luminal diameter was kept constant by applying low hydrostatic pressure to the lumen through the current injection electrode. Square pulses, $5–10 \times 10^{-7}$ A were injected into the lumen and the voltage deflection across the tubular epithelium was recorded differentially by means of two Ling-Gerard-type glass microelectrodes. The distance between the measuring electrodes and the luminal current electrode was changed by sliding the latter electrode back and forth along the lumen (Fig. 46). As was expected from the isotonic nature of the primary secretion by this epithelium, the transepithelial impedance of the unstimulated secretory coil, as calculated from the length constant (mean 83 μm) (Fig. 46), was extremely low, 2.2 ohm cm^2, which is about one-half that of the rat kidney proximal tubule (FRÖMTER and DIAMOND, 1972). The validity of the cable analysis

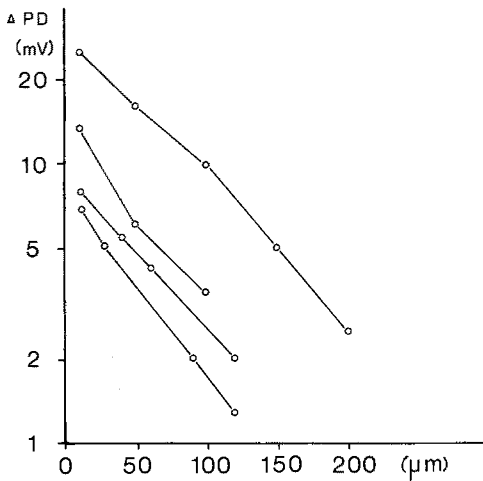


Fig. 46. Cable analysis of the secretory coil epithelium. Each line indicates the potential decay as a function of distance from the site of current injection. The mean length constant was 83 μm; effective resistance: 6.5×10^3 ohm

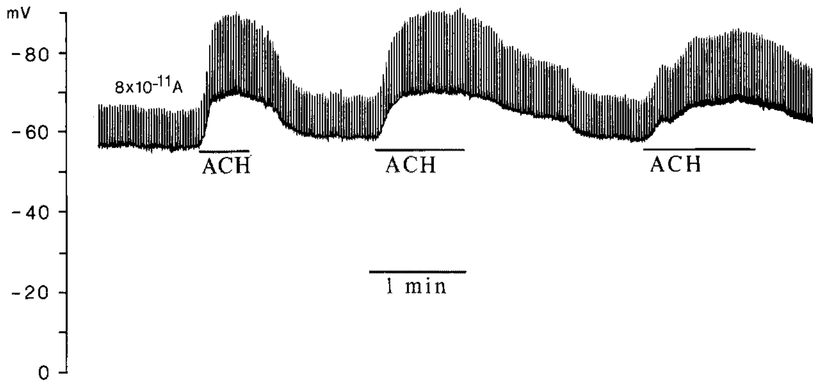


Fig. 47. A rare type of cell p.d. — possible myoepithelial cell p.d. Note the slowly rising hyperpolarization on exposure to locally superfused acetylcholine. The superimposed potential pulses are due to injection of square current pulses (8×10^{-11} A) through a bridge-balanced glass microelectrode

was proven by the agreement between the actual and the calculated core (luminal) diameter, 9 μm (also see Figs. 27 and 28). The cell p.d. was measured across the basal cell membrane by impaling the cell with a Ling-Gerard electrode, the shaft of which was made very flexible by replacing the midportion of the glass shaft with a length of silicone rubber rubing. Square pulses with $1\text{–}10 \times 10^{-10}$ A, 0.4 s duration, were continuosly injected through the cell electrode, and the potential drop due to the tip resistance of the electrode was balanced by means of a bridge circuit before impaling the cell; the maintenance of the balancing was checked again after withdrawing the electrode from the cell.

Two types of cell p.d.'s were observed. The first type p.d. showed a stable, noiseless baseline (−50–70) with a high input impedance (50–100 megohm). This type of cell p.d. was encountered very infrequently but tended to be observed when the electrode was advanced along the surface of the secretory coil. The p.d. was slowly hyperpolarized on exposure to acetylcholine (Fig. 47). The other type of

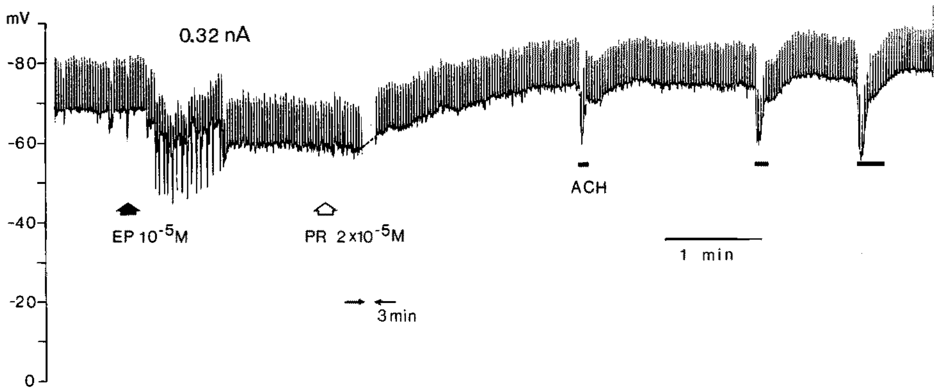


Fig. 48. Secretory cell p.d. as measured with a glass microelectrode. The same method is used as in Fig. 47. For detail, see text. PR: propranolol, ACH: local superfusion of acetylcholine. Superimposed pulses are the measure of input impedance of the impaled secretory cell

cell p.d. was seen more frequently and ranged from −40–80 mV (mean −63 mV) with a noisy baseline and with superimposed depolarizing spikes. The input impedance of these cells was 20–30 megohm. Because this latter type of cell p.d. could be seen consecutively by moving the electrode along the tubular wall, we reasoned that it represents that of the secretory cells. Furthermore, since the myoepithelial cells are discontinuous cells and the cell is slim and spindleshaped, the first type of cell p.d. probably belongs to the myoepithelial cells. The secretory cell p.d. showed a gradual but transient depolarization (by 5–10 mV) on exposure to epinephrine, whereas the local perfusion of acetylcholine produced a sharp transient depolarization, but the p.d. spontaneously returned to the resting level despite the continuous local perfusion of acetylcholine (Fig. 48). In sharp contrast to pancreatic acinar cells (NISHIYAMA and PETERSEN, 1975) in which acetylcholine drasticly reduces membrane input impedance, that of the eccrine sweat gland shows no significant decrease, whether acetylcholine is added directly to the incubation medium or is administered by local superfusion. Since the input impedance method is not a way of specifically measuring change in the specific site of the cell membrane (e.g., luminal or basal membrane), we injected square pulse currents ($1–10 \times 10^{-7}$ A, 0.45 s duration) into the lumen with a luminal electrode (see Fig. 45) and picked up the current-induced voltage deflection differentially across the basal cell membrane using two glass microelectrodes. Since it is assumed that acetylcholine works primarily at the cell membrane, any decrease in membrane resistance due to this drug may be reflected in a decrease in the height of the voltage pulses. Nevertheless, the change in the height of the voltage pulse (and the voltage divider ratio) was not significant after the addition of acetylcholine (Fig. 49). The effect of the calcium ionophore A23187 was also studied on the p.d. and on both input impedance and basal membrane resistance since, as shown in the pharmacologic section of this review, this ionophore is a strong stimulant of sweat secretion comparable in magnitude to acetylcholine. A high concentration (10^{-4} M) was used to elicit a quick sudorific response as shown in Figure 49 (see also Fig. 25). This ionophore produced no depolariza-

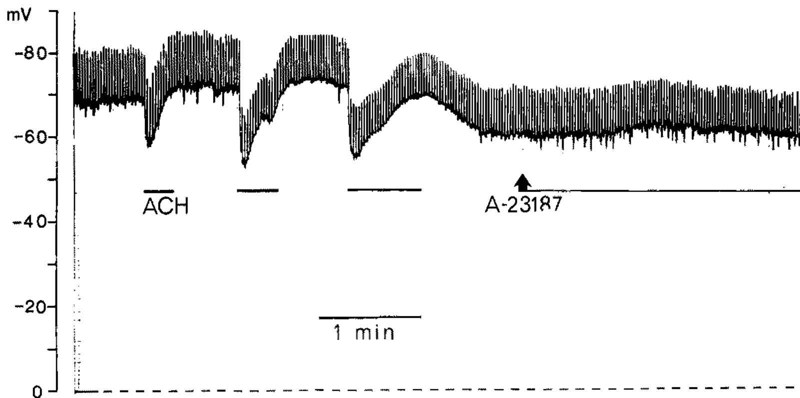


Fig. 49. Secretory cell p.d. and its response to acetylcholine applied by local superfusion and A23187 (10^{-4} M) which was directly added to the incubation medium. The superimposed voltage pulses are the qualitative measure of the basal membrane resistance. The method in Fig. 45 was used

tion, no change in input resistance, and no change in the basal membrane resistance, although prolonged exposure to this drug tended to cause a gradual decrease in the cell p.d.; however, such a slow effect, if any, is practically indistinguishable from the natural decay of the membrane potential.

If the results of our series of electrophysiologic observations are indeed correct, then the widely held view of the action of acetylcholine on the exocrine cells does not apply to the monkey eccrine sweat gland. For example, in the salivary gland (KAGAYAMA and NISHIYAMA, 1974) and in pancreatic acinar cells (NISHIYAMA and PETERSEN, 1975; PETERSEN, 1974) abundant evidence is already available which indicates that acetylcholine drasticly increases the basal cell membrane permeability to Na^+ and causes an influx into the cell of Na^+, which is then pumped out electrogenically by the Na pump located in the luminal cell membrane. In the salivary gland, isotonicity of secretion is achieved since Cl passively follows Na^+ by electric coupling and water follows both cellularly and paracellularly according to the difference in osmolarity (PETERSEN, 1973). Thus, in section L of this review, some of the possible mechanisms of sweat secretion will be briefly discussed based on the assumption that our preliminary electrophysiologic observations are correct.

XII. The Mechanisms of Electrolytes and Fluid Secretion by the Secretory Coil of the Eccrine Sweat Gland

The more information we accumulate on the secretory mechanism of the eccrine sweat glands, the more strongly we come to realize that the study of the eccrine sweat gland must be conducted as part of an overall effort by the membrane researchers in elucidating the mechanism of epithelial transport such as occurs in other mammalian and insect exocrine glands, kidney tubules, gall bladder, intestinal mucosa, choroid plexus, amphibian skin, urinary bladder, gastric mucosa,

and cornea. Admittedly, however, the eccrine sweat gland is handicapped as a membrane model because of its small tissue size, the difficulty in isolating a single tubular segment, and the extreme difficulty of impaling the cell with a glass microelectrode, etc. Nevertheless, due to its simple (although coiled) tubular structure in which serous secretory function predominates, it has an advantage over the mixed mucous plus serous secretion found in other exocrine glands. FRÖMTER and DIAMOND (1972) tentatively classified the transporting epithelia into two groups, the leaky and the tight epithelia. The leaky epithelia transport Na^+ and water isotonicly and are characterized by low transepithelial resistance, high values for osmotic water permeability (Lp), close to zero transepithelial p.d., relatively high transport rate, low limiting concentration ratio, and a low osmolarity ratio (FRÖMTER and DIAMOND, 1972). FRÖMTER (1972) postulated that these characteristics of the leaky epithelia derive from the leakiness of the terminal bar (i.e., so-called tight junction) to small molecules and water since approximately 96% of the transepithelial current bypasses the cells. The secretory epithelium of the monkey (and perhaps also human) eccrine sweat gland can now be classified as leaky epithelium because it satisfies most of the criteria defined by FRÖMTER and DIAMOND (1972). Thus, in constructing a hypothetic scheme for the routes of ions and water movement during sweat secretion, the information available about these epithelia and the application of currently accepted theories of membrane transport are vital in supplementing the scarce information available on the eccrine sweat gland. Unfortunately, however, even in such a well-studied epithelial membrane as the gall bladder epithelium, we have witnessed in recent years changes in concepts in terms of the mechanism of isotonic transport, from the standing gradient model and a neutral NaCl pump theory of DIAMOND and BOSSERT (1967) to electric coupling through a high conductance paracellular shunt (FRÖMTER, 1972), and recently, a coupled NaCl influx theory (FRIZZELL et al., 1975). This indicates that in none of the well-studied membranes is the ultimate mechanism of membrane transport definititely established, either at the membrane or at the molecular level.

A. Possibility of the Transepithelial p.d. as a Driving Force for Na Transport

As discussed in a previous section, our preliminary estimate of the transepithelial impedance of the secretory coil is 2.2 ohm cm^2 in the resting state. Although the transepithelial resistance has not yet been estimated during stimulation, the transepithelial input impedance does not significantly change after stimulation with acetylcholine (SATO, K., unpublished data). Thus, assuming a transepithelial resistance of 2.2 ohm cm^2 during stimulation and a mean transepithelial p.d. of −6 mV as a driving force, we can calculate the theoretical maximal transfer of Na across the coil epithelium according to: ${}_{d}J^{Na}_{net} = G_{Na} \cdot \Delta \text{p.d.}/F$ or ${}_{d}J^{N}_{net} = G_{Na} \cdot F \cdot \Delta \text{p.d.}/RT$ (FRIZZELL et al., 1975), where J^{Na}_{net} is the net secretion of Na (expressed as $\mu mol/cm^2 \cdot h$), G_{Na} the transepithelial Na conductance, and tentatively, $G_{Na} \simeq G_{Cl} \simeq (^1/_2) \cdot G_{total} = 1/(2.2 \text{ ohm cm}^2 \times 2) = 0.23 \text{ mho cm}^{-2}$. Thus, ${}_{\alpha}J^{Na}_{net}$ is calculated to be 48 $\mu mol/cm^2 \cdot h$. On the other hand, the actual secretory rate J^{Na}_{sweat} is estimated to be 51 $\mu mol/gl \cdot h$ or 138 $\mu mol/cm^2 \cdot h$. The para-

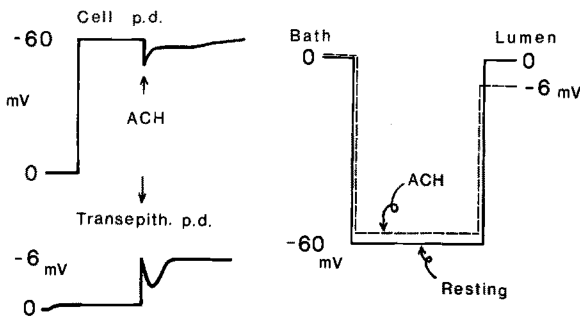


Fig. 50. Schematic illustration of the membrane potential change and the potential profile across the secretory cell

meters used for calculation are: sweat rate, 360 nl/gl · h; tubular length, 1.3 mm (SATO, 1973c); tubular electric diameter $\simeq$ luminal diameter, 9 μm. Although the partial conductance for each ion (G_{Na^+}, G_{Cl^-}, $G_{HCO_3^-}$, G_K, etc.) is not known and the effect of changing the transepithelial p.d. on ${}_dJ^{Na}_{net}$ has not been studied, there is a possibility that as much as one-third the total Na secretion can be ascribed to the diffusional Na flux according to the transepithelial electrochemical potential gradient for Na^+. If this is indeed the case, then as in the gall bladder (FRÖMTER, 1972) or the intestinal mucosa (ROSE and SCHULTZ, 1971), the above diffusional flux will take place mainly through the terminal bar (paracellular shunt pathway) rather than through the cell also in the sweat gland, since the cell membrane resistance may be rather high (as expected from the high input impedance). In order for the above pathway to continue to operate, however, there need be some mechanism(s) which maintain the transepithelial p.d. during sweat secretion. As shown in the potential profile of the secretory cell during the steady state of acetylcholine stimulation (Fig. 50), the lumen negative p.d. during sweat secretion seems to be achieved mainly by the depolarization of the luminal cell membrane. Several possible mechanisms can be raised to account for this potential profile, and they will be briefly discussed.

B. Active Transport of Na^+ or Cl^- as a Primary Driving Force for Sweat Secretion

Several possible models of Na and Cl transport, which simultaneously accompany the depolarization of the luminal cell membrane, have been depicted in Figure 51. Model A is the combination of a neutral Na-K exchange pump and an increase in membrane permeability to Cl^- during secretion (presumably due to intracellular Ca^{++}) at the luminal membrane. A similar mechanism has been assumed in the "neutral NaCl entry" theory in the gall bladder (FRIZZELL et al., 1975) and in the salivary gland of the blow fly, except in the latter epithelium, an electrogenic K pump was assumed in the place of a Na-K pump (BERRIDGE and PRINCE, 1971; this insect gland secretes K instead of Na). In order for Cl to move passively across this membrane to the lumen, the concentration of Cl^- in the cell $[Cl^-]_c$ must satisfy $(RT/F)\cdot([Cl^-]_l/[Cl^-]_c) < 60\,\text{mV}$, where R, T, F have their usual meaning, $[Cl^-]_l$ is Cl^- concentration in the lumen, and 60 mV is

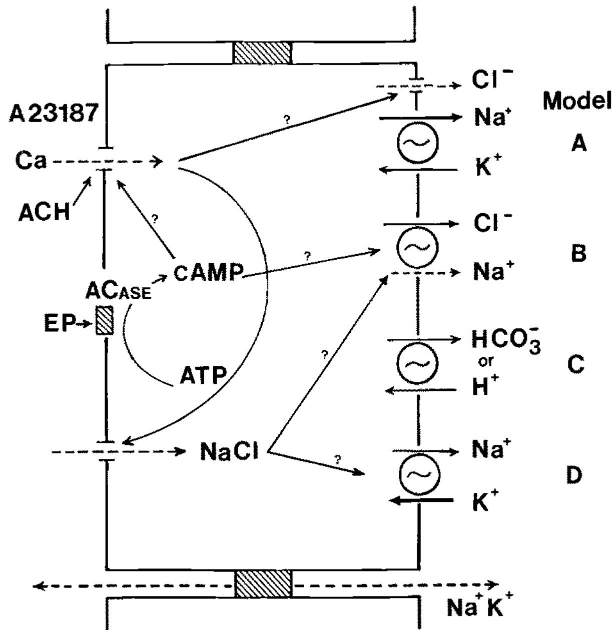


Fig. 51. A "working" model for Na and Cl transport by the secretory cells of the eccrine sweat gland. See text for detailed discussion. ACase: adenylcyclase. The straight arrows across the membranes with interrupted lines indicate passive movement and those with solid lines, active transport

the measured p.d. across the luminal membrane. In other words, this model may work if $[Cl^-]_c$ is higher than 12 mM assuming $[Cl^-]_l$ to be approximately 120 mM. Although $[Cl^-]_c$ is not known in the sweat gland, it may be higher than 12 mM since in the gall bladder (FRIZZELL et al., 1975), $[Cl^-]_c$ has been measured to be 90 mM. For continued secretion, $[Cl^-]_c$ of higher than 12 mM must be maintained by some other mechanism(s), for example by passive influx of NaCl from the contraluminal side of the cell. The presence of Na+K-ATPase in the secretory coil and the inhibitory effect of ouabain on sweat secretion (SATO and DOBSON, 1970c; SATO et al., 1971, 1969) are in keeping with this model; however, they are not necessarily indicative of the involvement of Na^+-K^+ exchange pump as a driving force for secretion.

Furthermore, our data that input impedance did not change significantly after acetylcholine stimulation (Fig. 48) and that furosemide at 5×10^{-6} M strongly but reversibly inhibited sweat secretion (Fig. 52) cannot be explained by model A. Furosemide does not inhibit Na+K-ATPase or the sodium pump but inhibits carbonic anhidrase in the rat kidney proximal tubule (RADTKE et al., 1972). In the thick ascending limb of Henle's loop (of the rabbit kidney), an active Cl pump has been shown to be the primary driving force for NaCl transport (BURG and GREEN, 1973). BURG et al. (1973) further demonstrated that furosemide (10^{-5}–10^{-6} M) specifically inhibited active Cl transport in this segment of the kidney tubule. Based on these studies, model B has been hypothesized (Fig. 51). The presence of ouabain-sensitive Na+K-ATPase in the sweat gland may not

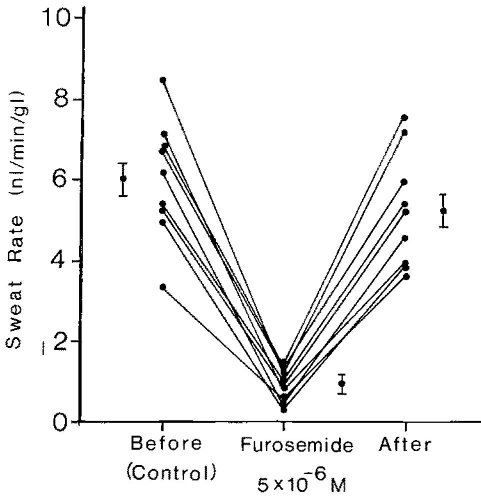


Fig. 52. Effect of furosemide on sweat secretory rate in vitro. Reversibility of the effect of furosemide was tested by changing the medium before and after the addition of furosemide

argue against this hypothesis since the same enzyme is also present in a high concentration in the thick ascending limb of Henle's loop (SCHMIDT and DUBACH, 1969), and, in fact, the role of Na+K-ATPase on the Cl pump, if any, has not been clarified. However, as has been already discussed above, if $[Cl^-]_c$ is higher than 12 mM, which is a likely possibility, the electrochemical potential gradient for Cl is downhill for Cl efflux from the cell, and hence it is not essential to invoke an active Cl transport across the luminal membrane. In contrast, Na movement across the luminal membrane is definitely uphill; it must overcome both electric and chemical potential gradients. Thus, from the energy viewpoint, model B works only when there is an intimate coupling between Cl^- and Na^+, but the coupling ratio is such that Cl^- movement tends to slightly dominate the movement of Na in order to make the lumen electrically negative. Model C must be taken into consideration if HCO_3^- concentration in cat paw sweat of 37–49 mM (SLEGERS and MOON, 1968; BRUSILOW and GARDES, 1967) indeed represents that of human, monkey, and cat primary sweat. Our observations that neither acetazolamide nor HCO_3^--free incubation medium reduced the secretory rate in an isolated monkey sweat gland (Figs. 38–41) may appear to disfavor model C but cannot completely rule out this model unless the presence of HCO_3^- secretion of H^+ absorption is directly disproved in the secretory coil. That the transepithelial p.d. is partially suppressed by acetazolamide (Fig. 53) is in line with model C; however, it is somewhat puzzling since acetazolamide does not significantly reduce the secretory rate. Model D is included simply as an alternative possibility to explain the lumen negative p.d. In this model the Na^+-K^+ exchange pump is electrogenic and directed inward. The continuous supply of K^+ may be achieved by influx of K across the terminal bar (tight junction) according to the electrical p.d. In other words, K^+ recycles in and out of the lumen through a paracellular pathway. One major problem with this model is how Cl^- is secreted under this condition. The paracellular route is less likely because of the lumen negativity of the transepithelial p.d. The only possibility left is, therefore, to assume the coupling

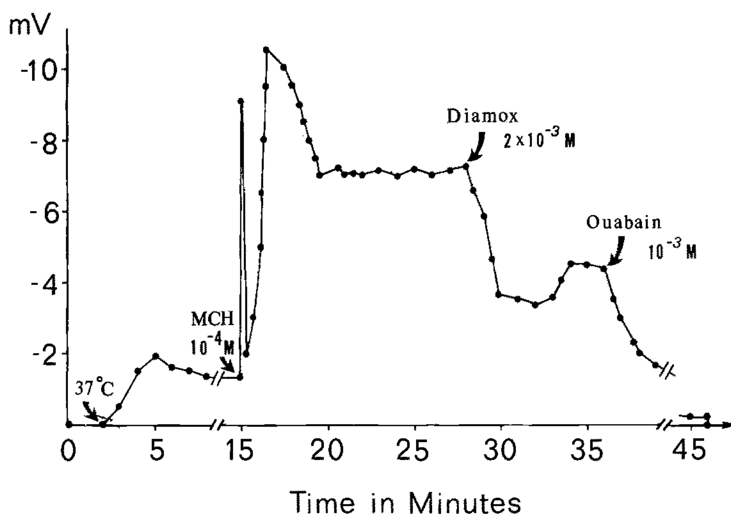


Fig. 53. Effect of acetazolamide (Diamox) on the transepithelial p.d. of an isolated secretory coil

of Cl^- with Na^+ pumped out by this Na^+-K^+ exchange pump. Then it is no longer necessary to invoke the inwardly directed electrogenic Na^+-K^+ pump and model D now simulates model A. At present, the available information does not allow us to favor one model over the other. Alternatively, it is always possible that two or more of the above mechanisms are simultaneously involved in the process of secretion.

C. The Roles of Ca^{++} and cAMP in the Stimulus-Secretion Coupling

In the pharmacologic section of this review, indirect evidence has been presented that influx into the cell of Ca^{++} constitutes a critical initial step in the overall processes of sweat secretion by the secretory cells. However, the sequence of cellular events intervening between the influx of Ca^{++} across the basal membrane and the active cation (or anion) transport at the luminal membrane (including the cell membrane forming the intercellular canaliculi) are totally unknown. As has been already discussed, the involvement of an exocytotic mechanism is least likely to play a major role in sweat secretion, a feature which distinguishes the eccrine secretory cells from other protein or hormone secretory cells (DOUGLAS, 1968). For the sake of future studies, several possible sites where intracellular Ca^{++} might act must be discussed in the absence of any supporting data. These are:

1. Ca^{++} modifies from inside the cell the membrane permeability to NaCl across the basal membrane.
2. Ca^{++} increases the permeability of the luminal membrane to Cl^- as in model A.
3. Ca^{++}'s effect is mediated by another intracellular second messenger, such as cGMP or cAMP.
4. Ca^{++} directly activates cation transport at the luminal membrane, etc.

Among the above, the fourth possibility apperars the least likely because Ca^{++} is a strong inhibitor of Na+K-ATPase in an in vitro enzyme preparation as well as in red cell membrane transport (SKOU, 1965). In the insect salivary gland (PRINCE et al., 1973), introduction of Ca^{++} into the cell by A23187 rather lowered the tissue level of cAMP;[3] hence, at least in this insect gland (where A23187 is also a stimulator of fluid secretion as in the monkey sweat gland), the effect of Ca^{++} does not apperar to be mediated by cAMP. If this is also the case in the mammalian sweat glands, the third possibility can be excluded. Indirect evidence has also been presented in this review that adrenergic sweat secretion may be mediated by intracellular cAMP. Since fluid secretion induced by adrenergic stimulations is also inhibited by removal of Ca^{++} from the medium (SATO, K., unpublished data), it is possible to speculate that the sudorific effect of cAMP is also mediated by influx of Ca^{++} into the cell. Such a possibility must be verified or ruled out by studying the Ca^{++} uptake by the tissue in an in vitro system.

D. Effect of Acetylcholine on Membrane Permeability

Puzzling electrophysiologic data have already been presented in this review which show no significant change in membrane resistance as measured by current-induced voltage changes. Firstly, our technical error, if any, must be ruled out, or our experimental data must be confirmed by others. The secretion of ions in the secretory coil at the rate of 138μmol/cm^2 · h (in Na^+) is an extremely high rate of ionic movement, and it is quite natural to expect that there should be a drastic decrease in the membrane permeability as measured electrophysiologically to allow the passage of ions across the membrane. However, implicit in this assumption is that when the cell membrane is stimulated by acetylcholine, Na traverses the cell membrane through its own channel, which in turn causes a change in the membrane p.d. favorable for the counter ion (Cl^-) to enter the cell via the different channel (i.e., electric coupling), and also that the same channel for each ion species is used for these ions to move when potential difference is applied artificially to measure the membrane resistance. It should be remembered, however, that no solid experimental evidence exists to support or refute this contention in this or other transporting epithelia. FRIZZELL et al. (1975) recently suggested, based on theoretical reasoning as well as experimental observations, that in the gall bladder Na^+ and Cl^+ enter the cell as a neutral complex (i.e., cotransport of Na^+ and Cl^-) through a nonconductive pathway and that such a neutral particle can easily cross a membrane possessing even an infinite electric resistivity. These investigators also contend that isotonic transport by the gall bladder is achieved by influx of neutral NaCl across the mucosal membrane and an active Na pump and passive Cl^- efflux across the serosal membrane.

Therefore, if our electrophysiologic data are indeed correct, the theory of FRIZZELL et al. (1975) is most likely to explain how a massive influx of NaCl can be achieved in the absence of any change in the electricly measured membrane resistance in the eccrine sweat gland. The unpublished observation that sweat

[3] Our most recent unpublished data indicate that neither A23187 nor acetylcholine increases the tissue level of cAMP but isoproterenol does.

secretion completely stops if Na^+ is replaced by choline or Cl^- and HCO_3^- are replaced by SO_4^{--} is also suggestive of, although not indicative of, a neutral NaCl entry mechanism in the sweat gland, such as in the gall bladder epithelium. A further mechanism whereby the influxed NaCl turns on the membrane pump is unknown. A tracer flux measurement may be mandatory to verify such a new mechanism in the exocrine gland.

In summary, an attempt has been made to construct as many hypothetic models of ion transport in the secretory cell as can be conceivable, based on the limited amount of information available. The basis for all the hypothetic schemes is that Ca^{++} influx into the cell induced by acetylcholine must cause a massive influx into the cell of NaCl without an accompanying change in membrane electric resistance and that active transport, presumably at the luminal membrane, must produce a luminal electric potential negativity with respect to the interstitium. Each model has been discussed in regard to its plausibility and shortcomings.

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H^+ Transport by a Non-Electrogenic Gastric ATPase as a Model for Acid Secretion

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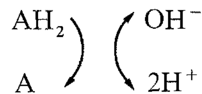
The primacy of H^+ transport in endergonic and exergonic processes in biology seems to be becoming part of scientific dogma. One might expect the secretory process of the mammalian perietal cell to be the ultimate expression of the proton transport process and thus to serve as a model for other H^+ translocases.

Accordingly, this review will discuss some recent findings relating to transport by the gastric mucosa, in particular the transport of H^+.

I. Mitochondrial H^+ Transport

Although the role of a proton gradient in chloroplast and mitochondrial phosphorylation and in active transport by bacteria or yeast is well established, we do not have a detailed picture of the structures constituting the H^+ pump. The original chemiosmotic hypothesis whether correct in detail or not, made the important contribution to biological thought that the mode of energy conservation by mitochondria was in the form of ion gradients [1], especially that of H^+.

Redox reactions catalyze the transfer of H^+ and electrons between donors and acceptors, the terminal acceptor in mitochondria being O_2. A vectorial arrangement of such a reaction may be written as:



thus an H^+ and OH^- gradient is set up.

Equally the hydrolysis of ATP can be written as:

The equilibrium for this reaction can be written as:

$$\frac{ADP \cdot Pi}{ATP \cdot H_2O} = K.$$

If H_2O is reduced to very low levels, then ATP synthesis from ADP and Pi may occur. This was essentially the original core concept of the chemiosmotic hypothesis. However, although in a hydrophobic environment, the $[H_2O]$ can be low, it is very difficult to conceive of a mechanism acting directly to abstract H_2O.

An alternative way of writing the synthesis or breakdown of ATP is:

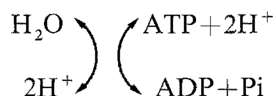
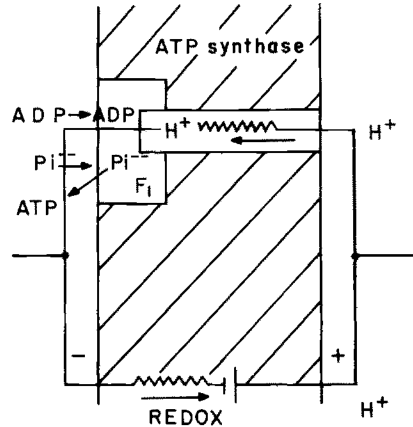


Fig. 1. Schematic representation of the chemiosmotic hypothesis, showing electrogenic redox pump generating an H^+ gradient. Return limb for the H^+ pump is ATP synthase reaction. This involves movement of H^+ across the membrane and reaction with bound ADP and Pi forming ATP



This illustrates the reversible translocation of $2H^+$ with the formation or breakdown of ATP. In this scheme, if the nexus of the arrows is considered to be a phase boundary, such as a membrane, the breakdown of ATP results in the net transfer of $2H^+$ as charged species. Hence, in considering the reversibility of the reaction, the electrochemical gradient of the proton, as well as the phosphorylation potential is significant. This results in:

$$n\left(\frac{RT}{F}\Delta pH - \Delta\psi\right) = \Delta G' = \Delta G^\circ + RT \ln \frac{ATP \cdot H_2O}{ADP \cdot Pi}$$

where R, T, F have their usual meanings, n is number of H^+ transferred, ΔpH is the pH gradient across the membrane, $\Delta\psi$ is the electrical potential difference, $\Delta G'$ is the actual free energy of hydrolysis, ΔG° is the standard free energy of hydrolysis. The term in brackets has been called the proton motive force (pmf). It has been the major contribution of the chemiosmotic hypothesis to recognize the equivalence of the electrochemical gradient of H^+ and the phosphorylation potential [1]. The required value of the pmf for ATP synthesis is, therefore, a function of the phosphorylation potential ATP/ADP · Pi and the value of n. The greater the value of n, the lower the required pmf.

Conversely, at zero potential the maximum gradient of H^+, ΔpH, is determined by the $\Delta G'$. Notably, the ΔG° of ATP is strongly pH-dependent above pH 6.

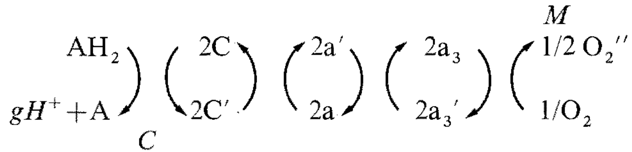
The critical component, therefore, of an ATP synthetase are a proton conductor across a membrane and a sensor of the H^+ concentration, a reversible ATPase. This is illustrated schematically in Figure 1.

A. Development of H^+ Gradients

It has been amply established that H^+ gradients can drive ATP synthesis [2], or that ATP hydrolysis can produce H^+ gradients [3]. It has also been well documented that redox reactions, or photolytic reactions may generate H^+ gradients [4].

Thus, there are three types of electrogenic mechanisms involved, redox, photolytic and ATP driven.

In primitive systems such as bacteria or mitochondria, in addition to an ATP generator of an electrochemical H^+ potential, there are also redox generators of such a potential. These are due to an oriented transmembrane complex of electron acceptors.



This illustrates schematically the oxidation of a substrate at complex IV of the redox chain where $2H^+$ is released on the cytoplasmic side of the mitochondrial membrane (C) and electrons to O_2 on the matrix side (M). Thus an H^+ gradient is generated electrogenically by such a complex. This is considered to happen at three sites in the mitochondrial redox chain. The disposal of the H^+ electrochemical gradient is by the ATP synthesis complex.

An H^+ gradient may also arise by coupling a proton conductance directly to a generator of potential such as electrogenic anion pump. In this case movement of H^+ is dependent on active movement of another ion.

Finally an electroneutral process may be considered, such as an exchange of H^+ for K^+, both ions participating in the translocation mechanism.

It is intriguing that all these processes must be, and actually have been, considered in the mechanism of H^+ secretion by gastric mucosa.

Because it has been shown that purified mitochondrial ATP synthetase complex may act on its own as an H^+ translocase, and because a gastric ATPase also can translocate H^+ ions, a brief digression into the electrogenic H^+ ATPase is worthwile.

In fact, because of the state of development of knowledge in gastric mucosa, our major concern will not be with the redox involvement in H^+ gradient generation, but with ATP and ATPase mechanisms. The evidence relating to redox involvement has recently been reviewed [5].

B. Electrogenic H^+ ATPase

The ATP synthetase present in mitochondria or chloroplasts contains an ATPase of complex subunit structure (F1) [6]. The function of these subunits is at present not understood. Additional components are associated with the ATPase such as an F_0 complex [7] or oligomycin sensitivity conferring factor (OSCF), a DCCD binding protein and an ATPase inhibitor [8]. Of particular interest is the role of F_0. A suggestion has been made that F_0 is the coupling site for F_1 on the mitochondrial membrane, and that the F_0 complex is responsible for the H^+ conductance of the mitochondrial membrane [9]. Thus, oligomycin or DCCD interacts indirectly with F_0 by combining with other factors inhibiting H^+ movement and in this configuration, (i.e. the combined F_1F_0), the ATPase activity is inhibited. The

coupling between oxidation and phosphorylation being due to the pmf across the membrane, oligomycin prevents interaction of the pmf and ATP synthetase. The F_0 path is not specifically required for respiration hence the addition of protonophores such as DNP or TCS allows respiration to continue [10]. However, H^+ flux through F_0 is specifically required for ATPase activity, since oligomycin, which blocks H^+ conductance through F_0, inhibits ATPase activity even in the presence of uncouplers.

In addition to the oligomycin site, there is a N, N′ dicyclohexyl carbodiimide binding protein [11]. Binding of DCCD to this protein has the same functional result as oligomycin, i.e. inhibition of phosphorylation but not F_1 ATPase. The binding protein is a low M.W. protein of about 10,000 mol wt soluble in chloroform-methanol.

If this type of model is accepted, the F_1 complex must be capable of sensing the change in $[H^+]$ or EMF at the F_0-F_1 interface. This has to be a specific region of proton translocation otherwise uncouplers would be able to reverse the oligomycin or DCCD inhibition of the F_1F_0 complex. It is therefore not adequate to propose a simple proton conducting circuit between F_1 and F_0.

Rather there could be a proton binding site on F_1 and a proton acceptor site on F_0 in the ATPase. Uncouplers would neither be able to accept H^+ from F_1 nor interfere with the translocation of H^+ from F_1 to F_0. H^+ movement across the mitochondrial membrane, in the absence of uncoupler, would then require dissociation of H^+ from the binding site on F_1 and translocation through F_0. ATPase inhibitors such as DCCD or oligomycin would then block either dissociation of H^+ from the binding site on F_0, or translocation from F_1, or translocation of the dissociated H^+ through the F_0 complex. In no case could uncouplers interfere with the reaction. This shielded proton movement implies the existence of channels rather than carriers, as illustrated in Figure 2. Hence, uncouplers will not be able to penetrate the channel region.

The reverse of the process, ATP synthesis by an electrochemical H^+ gradient would then require movement of H^+ through F_0 complex to the binding site and transfer of this H^+ to the acceptor site on F_1. Uncouplers would bypass this process by providing a shunt conductance for H^+ elsewhere in the mitochondrial membrane. Acceleration of ATPase activity with uncouplers then results from the reduction of the back EMF across F_0 complex.

The substantive point of this hypothesis is that there is a chemical group

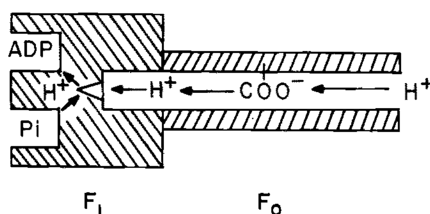
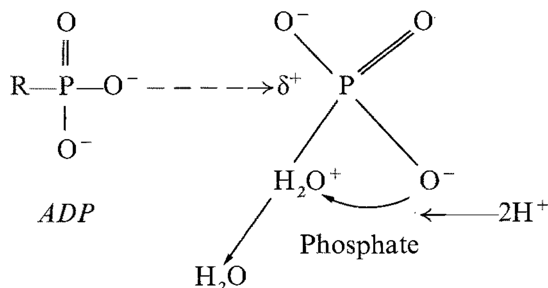


Fig. 2. More detailed representation of electrogenic H^+ ATPase of mitochondria. The representation is in the direction of ATP synthesis. COO^- represents DCCD binding protein F_0 (the H^+ conducting channel). Potential across the membrane is considered to move bound Pi to the H^+ site on F_1, the ATPase complex, and these react with ADP forming ATP. Reverse reaction generates an H^+ gradient and a potential

on F_0 which binds H^+ in response to a gradient (chemical or electrical). This interacts directly with an equivalent group in one of the F_1 subunits resulting in protonation of that subunit. This model has thus translated an ion gradient into a chemical change in F_1, the ATP, ADP and Pi binding complex.

A mechanism must also be present to sense the E.M.F. across the mitochondrial membrane (Fig. 2). If the translocation of Pi through the ATPase is in the charged form, then in the ATP synthetase reaction, direct application of a potential, mitochondrial interior negative, will move phosphate into the H^+ region (the F_0F_1 interface). The reaction at this site could be written [12]:



where the protonation of phosphate increases the electrophilic reactivity of the phosphorus. Reaction with O^- of ADP results in transfer of an electron and expulsion of H_2O. The H^+ gradient is responsible for the protonation and the EMF for translocation of the phosphate. Hydrolysis reverses the sequence, also producing a potential and H^+ gradient. The key reactions are therefore H^+ and Pi binding site translocation (Fig. 2).

II. Plasma Membrane Cation Transport

There appear to be two types of cation translocating ATPase. One already discussed is the mitochondrial F_1F_0 complex. No evidence exists for the cation translocation ability of F_1 alone. The other type is exemplified by the $Na^+ + K^+$ ATPase [13] or Ca^{++} ATPase [14] and probably by the $H^+:K^+$ ATPase [15] of gastric mucosa. These differ in type of subunits and in reaction mechanisms. Thus both the Na^+K^+ and Ca^{++} ATPase contain a 100,000 M_r (relative molecular weight) subunit which is phosphorylated by ATP on a γ-aspartyl group [16, 17] during enzyme activity. In the case of Na^+K^+ ATPase at least, there appears to be a complex series of reaction steps namely:

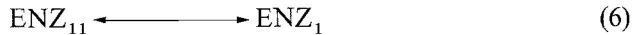
$$\text{ENZ}+\text{ATP} \xleftrightarrow{Mg^{++}} \text{ENZ}-\text{ATP} \quad (1)$$

$$\text{ENZ}-\text{ATP} \longleftrightarrow \text{ADP}\cdot\text{ENZ}\cdot\text{Pi} \quad (2)$$

$$\text{ADP}\cdot\text{ENZ}\cdot\text{Pi} \xleftrightarrow{Na^{+}} \text{ENZ}\cdot\text{Pi}_1+\text{ADP} \quad (3)$$

$$\mathrm{ENZ \cdot Pi_{1}} \longleftrightarrow \mathrm{ENZ \cdot Pi_{11}} \quad (4)$$

$$\mathrm{ENZ \cdot Pi_{11}} \overset{K^+}{\longleftrightarrow} \mathrm{ENZ_{11} + Pi} \quad (5)$$



$$\mathrm{ENZ_{11}} \longleftrightarrow \mathrm{ENZ_{1}} \quad (6)$$

These steps have been dissected by the use of inhibitors such as ouabain [18] or N-ethyl maleimide [19], separate addition of Na^+ and K^+ and by ion gradient effects [20]. Kinetics as well as radiation inactivation cross section suggest a dimer form for activity of the enzyme [21]. Although, with the exception of red cell ghosts, vesicles containing this enzyme have been too leaky for transport studies, reconstituted vesicles have recently been described which do transport Na^{++} with ATP addition [22]. Little success has been reported for planar bilayer incorporation of this type of pump, although fragments may have been incorporated [23]. Hence the electrical characteristics of these pumps are not precisely known.

It seems that the isolated sarcoplasmic vesicles containing the Ca^{++} ATPase have a large anionic shunt conductance so that in vitro there is little likelihood of a potential developing with transport [24]. Ion pathways of the NaK ATPase have been measured by flux studies (which do not reveal the electrical characteristics) in red cells, resealed red cell ghosts, and in reconstituted vesicles. There appears to be at least five modes of operation of this pump, or five classes of cation flux. (1) Na^+K^+ exchange, (2) pump reversal, (3) Na^+-Na^+ exchange, (4) K^+K^+ exchange, (5) Na^+ efflux [25]. The explanation for these activities must reside in the location and type of sites responsible for the ion movements.

It should not be considered that there is a fundamental difference in the thermodynamics of this type of ATPase and the electrogenic H^+ ATPase discussed above. Thus, this type of ATPase is also reversible with respect to ion gradients. In red cell ghosts which translocate Na^+ outwards and K^+ in, it was found that loading with K^+ and placing these in high Na^+ medium resulted in net synthesis of ATP. More recently the addition of Na^+ to non-vesicular preparations of $Na^+ + K^+$ ATPase has been shown to result in synthesis of ATP from ADP and Pi. Similarly binding of Ca^{++} can reverse the Ca^{++} ATPase.

The differences between these ATPases and the electrogenic H^+ ATPase may therefore be more apparent than real. That a phosphorylated intermediate is formed may only be coincidental in that a carboxyl group may react with the "activated" phosphate to form an isolatable "intermediate" in the plasma membrane ATPases. In the mitochondrial type a binding site may hold the phosphate for nucleophilic attack by ADP.

III. Photolytic H^+ Transport

The recognition of the proton pump capability of lumirhodopsin [4] has particularly interesting implications about H^+ transport mechanisms. A retinylidene protein (26000 daltons) moves H^+ in response to a light flash. Presumably a

reaction occurs in the retinal to change conformation of the protein. This in turn shifts the pK_a of a group in the protein from a high to low pK_a. This low pK_a form then donates H^+ across the membrane in one or several stages to another low pK_a group which donates H^+ to the medium.

It is not unreasonable to conceive of H^+ translocation to be via a similar mechanism. Thus interaction of a protein with ATP results in phosphate transfer. The acceptor group phosphorylation or binding results in a pK_a shift downward, translocation of H^+ across the membrane, and formation of an H^+ gradient. A similar mechanism with changing cation affinities in a site mobile across the membrane could account for cation translocation as has often been suggested.

IV. Nature of Ion Movement

Two types of structures may be responsible for ion translocation across bilayers. The simplest would be a waterfilled pore of dimensions so that hydrated ions could cross the bilayer (i.e. the cation selectivity sequence would correspond to sequence 1 of Eisenman). The other type involves substitution of water of hydration by binding groups such as polar oxygen and hence would alter the selectivity sequence. This type could be further classified into mobile and fixed sites (i.e. carriers or channels). These can be distinguished readily in a low conductance bilayer since a single channel event can significantly alter the background conductance of a bilayer, the channel "size" being to the order of $10^{-11}\Omega^{-1}$. A carrier binding one or two ions will show smooth conductance changes with incorporation into a low conductance bilayer. With the possible exception of the H^+-K^+ ATPase to be discussed below, no electrical evidence is available to establish whether ion conductance through pumps is by channel or carrier mechanisms.

The mitochondrial H^+ pump discussed above is electrogenic, i.e., the pump is capable of generating a potential or short circuit current across a membrane directly without the presence of diffusion potentials. The reasons for considering a channel mechanism have been discussed.

The rate of pumping (i.e. ATPase activity) will be a function of the gradient and the potential difference across the membrane. Accordingly, an increase of membrane conductance will serve to reduce the potential difference and hence accelerate the pump. The absence of a conductance or return limb will inhibit pump activity. The potential difference generated by a "perfect" electrogenic pump will then be 60 mv for each 10-fold increase in gradient generating capability for a univalent ion. If other conductances are present in the membrane, the potential difference will decrease as a function of the equation:

$$E_M = \frac{g_A E_A}{g_A + g_B}$$

where g_A, E_A are the conductance and E.M.F. of the active pump and g_B is the lumped conductance term for all other conductances. From this if $g_B \gg g_A$, the potential

difference generated by an electrogenic pump could be very small and not necessarily detectable. At $g_B=0$, E_A is 60 mv for a ten-fold gradient developed by the pump.

The alternative to an electrogenic pump is a neutral pump, with forced exchange between cations or anions or forced cotransport of cation and anion. Such a pump could only secondarily generate a potential due to a diffusion potential dependent on the concentration gradients of the ions transported, and the membrane selectivity.

V. Determination of Electrogenicity of Pumps

In intact tissue, the electrogenicity of a pump can be determined in several ways. The measurement of a potential difference in excess of that calculated based on diffusion potentials is perhaps the most direct. Alternatively, demonstration of linear relationships between ion flux and current generated or between potential difference and ion flux seems conclusive. In vesicles the evidence is more indirect, but knowledge of ion gradients across the vesicle membrane is easier to obtain.

In vesicles, measurement of changes in potential difference can be achieved by measuring the distributions of a radioactive or fluorescent lipid permeable cation. Thus,

$$E=\frac{RT}{nF}\ln\frac{A_i}{A_o}$$

where A_i is the internal and A_o is the external concentration of ion. It is often difficult to be exactly sure of A_i (especially in fluorescent studies), hence a calibration technique has been developed whereby the membrane is made K^+ selective by means of valinomycin and then a known K^+ gradient is applied.

An alternative approach in terms of distinguishing neutral and electrogenic pumps is to modify membrane conductance and measure changes in pump rate (i.e. ATPase activity). In general, an electrogenic pump will increase rate with increase in conductance and decrease rate with decrease in membrane conductance. If however, the non-specific conductance g_B is very high as discussed above, it may be very difficult to modify g_B sufficiently to achieve any change in rate or potential. In such vesicles a pump could therefore be effectively non-electrogenic rather than neutral. If however, it can be shown that in vesicles of low conductance, no potential develops and that changes of conductance do not affect pump activity, it may be safe to assume that the pump is neutral.

VI. Measurements of H^+ Gradients

In the special case of H^+ gradient formation by vesicles, it is possible to measure the magnitude of the H^+ gradient in three ways. The simplest is the use of a pH electrode and knowing vesicle volume the pH gradient can be calculated from the

appearance or disappearance of H^+ in the external medium. Alternatively, the trapping of a weak acid or weak base can be used on the supposition that the charged form of these are impermeable and the neutral form is freely permeable. Hence the ΔpH for trapping of a base

$$\Delta pH = \log_{10} \frac{AH^+}{A} = \log_{10} \frac{A_i}{A_o}$$

A technical problem arising is the degree of impermeability. Using a radioactive marker, centrifugation or filtering and washing could result in a considerable underestimate of the gradient if the charged form had finite permeability. Thus, rapid flow dialysis may be the method of choice for estimating uptake of base or acid.

Alternatively, pH indicator dyes can be used with color or fluorescence changes in the appropriate range. The drawbacks in this case are the site of binding of the dye (if bound) and changes in spectrum with binding as well as absorbance changes. Hence, quantitation of the change of pH may be difficult.

These would seem to be the possibilities available and with the measurement of ion movements, the use of potential probes and ionophores, and variation of the conditions of study of transport, a decision as to the type of pump and whether H^+ is transported is in general feasible in isolated vesicles.

With this brief background discussion of a complicated field, we can now approach the problem of H^+ transport in gastric vesicles due to $H^+ + K^+$ ATPase activity.

VII. $H^+ + K^+$ ATPase

The first indication that gastric mucosa might contain a transport ATPase similar to the $Na^+ + K^+$ ATPase was the finding of a K^+ activated p-nitrophenylphosphatase [26] or acetyl phosphatase [27]. The $Na^+ + K^+$ ATPase is capable of catalysing similar partial reactions [28, 29] but in contrast to the gastric phosphatase activity, the $Na^+ + K^+$ partial reaction is ouabain inhibited.

Subsequently a K^+ activated ATPase was shown to be present in rabbit, frog, dog and hog mucosa [15, 30, 31]. In contrast to the $Na^+ + K^+$ ATPase, there was no inhibition by ouabain nor was there any requirement for Na^+.

A. Purification

The K^+ enzyme is localized exclusively in the microsomal fraction of the gastric mucosal homogenate. This microsomal fraction can be further divided into two membranes and one mitochondrial band on density gradient centrifugation using sucrose or ficoll sucrose gradients [32, 33]. At this stage, there is a 10-to 20-fold purification of the ATPase or pNPPase in terms of the original homogenate

Table 1. Purification of vesicles

	μmoles Pi mg^{-1} h^{-1}			
	5′-AMPase	Mg^{++}-ATPase	K^+-ATPase	K^+-ATPase + val
Total homogenate	0.5±0.3	6.2±0.6	2.0±1.0	3.6±1.8
Microsomal fraction	1.5±0.6	15.9±1.5	7.1±4.3	16.7±4.0
Light membrane fraction	4.6±0.1	6.4±1.3	32.5±3.2	62.9±1.7
Electrophoretic fraction I (F1)	0.6±0.2	2.7±0.6	64.1±3.9	87.6±2.5
Electrophoretic fraction II	14.7±1.2	16.6±2.0	17.3±1.2	19.4±1.7

The activity of 5′ AMPase and ATPase in the total homogenate, microsomal fraction (100,000 g precipitate of the post 20,000 g supernatant) the light membrane fraction on the 7.5% ficoll to 37% w/w sucrose gradient and the 2 fractions derived from the latter by free flow electrophoresis. The K^+-ATPase activity is the difference between activity in the presence or absence of K^+. The K^+-ATPase+valinomycin activity is the difference between the activity in the presence of K^+ and valinomycin and in the presence of Mg^{++} alone

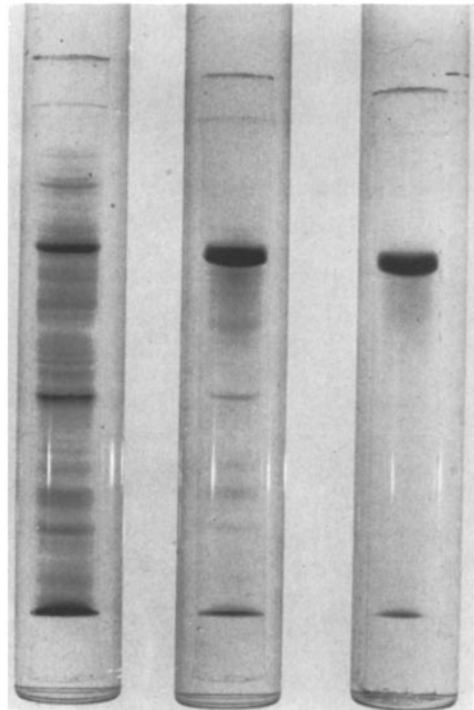


Fig. 3. Gel pattern on 10% acrylamide gels of microsomal (*M*) zonal gradient peak (*G*) and free flow electrophoretic fraction (*FI*) showing progressive reduction of the number of peptides in each fraction and increase in relative amount of the 100,000 peptide region

(Table 1). The two membranous bands differ somewhat in their content of 5′ nucleotidase and considerably in their ion permeability. The material not entering the gradient, i.e., at 7% ficoll is considerably less permeable to H^+. The gel pattern observed is fairly typical of membranes showing a complex pattern with some prominent high mol wt Bands.

Free flow electrophoresis which takes advantage of variations of surface charge separates either membranous band into several fractions. Maximum separation is

obtained in the presence of ATP. The calculated surface charge alteration based on the ATP dependent band displacement is equal to the number of phosphorylation sites per vesicle measured using γ ^{32}P-ATP [33].

This electrophoretic fraction is now 40-fold enriched with respect to $H^+ + K^+$-ATPase and is free of 5′ nucleotidase and Mg^{++} ATPase. Moreover, gel electrophoresis shows that a peptide region of 100,000 mol wt accounts for more than 75% of the protein [33] (Fig. 3). At this stage of purification there is no detectable NADH or NADPH oxidase activity.

B. Properties of the Enzyme

Work on this enzyme has been relatively recent hence details of its action are not nearly as well known as those of the $Na^+ + K^+$ or Ca^{++} ATPase. However, since it bears a remarkable resemblance to these ATPases, rapid progress has been made in defining some of its key features [30].

A phosphorylated intermediate which is acid stable, alkali and NH_2OH labile is formed upon incubation with γ^{32}-ATP and Mg^{++}. A peptide of 100,000 mol wt is phosphoryltated both in the crude membranes and the purified electrophoretic fraction [34]. The pH optimum of phosphorylation is broad, being between 6 and 6.8 (RABON, unpublished observations). The enzyme also catalyzes an ATP-ADP exchange [35], a characteristic reaction of energy conservation by ion gradients or covalent intermediates [13]. Phosphorylation may be enhanced by inhibitors of the overall reaction such as F^-, Zn^{++} [15] and decreased by inhibitors such as pCMBS and DCCD [35].

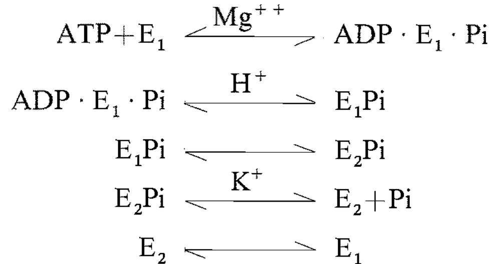
The overall ATPase reaction requires the presence of alkali metal cation, as does the pNPPase or acetyl phosphatase. The cation accelerates dephosphorylation of the protein. In our laboratory there is an unexplained anomaly in the relative effectiveness of the various cations in terms of the overall ATPase activity and dephosphorylation. Thus, the selectivity sequence of cations for ATPase activity if K 100; Rb 76; Cs 16; Na 7; Li 4. For dephosphorylation the sequence was found to vary in terms of Cs^+ or Na^+ whereas for pNPPase activity the selectivity sequence was identical to the sequence for ATPase. In contrast to the $Na^+ + K^+$ ATPase, no additional effect is found for combinations of cations. NH_4^+ and TI^+ are also effective stimulating ions, TI^+ being more effective than K^+, and NH_4^+ having about equal activity. The pH optimum for dephosphorylation is 7.4 and the apparent K_A for cation is a function of both pH and temperature. For K^+, at pH 7.4 and 37° C the K_A was found to be 0.5 mM.

The dephosphorylation is not Mg^{++} dependent since CDTA does not block the K^+ enhancement of the reaction. Based on the ATP-ADP exchange and loss of phosphoenzyme with CDTA the first step of the reaction is reversible [35]. The presence of a K^+ gradient allows phosphorylation of the protein from inorganic phosphate, but does not result in an ATP-Pi exchange. This finding implies that there are two forms of the phosphoprotein, a K^+ sensitive form and an ADP sensitive form, as has been suggested for the $Na^+ + K^+$ ATPase [36].

The substrate specificity of the enzyme is ATP 100, GTP 12, CTP 15 ITP 0. β-γ methylene ATP is not hydrolysed by the enzyme, but acts as a competitive

inhibitor for ATP. The K_M for ATP is 10^{-4} M at pH 7.4, 37° C [30, 35]. ATP also is a competitive inhibitor for the pNPPase [30].

From these considerations one may suggest a tentative scheme such as [26, 36] for the $Na^+ + K^+$-ATPase (see above).



This scheme will be discussed later in relation to the possible dimeric structure of the active form of the enzyme.

C. Vesicular Structure of the ATPase Particle

There are several properties of the ATPase which appear to be explained by the vesicular structure in which the ATPase finds itself. Most microsomal preparations from a variety of tissue appear vesicular, but little evidence from $Na^+ + K^+$ ATPase studies suggest that these vesicles are capable of maintaining ion gradients even in the presence of ATP [37].

In contrast, the gastric vesicles seem relatively ion impermeable, as would be expected from the known conductance properties of the mucosal surface of the stomach [38]. A key experiment demonstrated that ATPase activity was enhanced by the addition of ionophores such as valinomycin [39]. Table 2 illustrates the ionophore enhancement of the ATPase activity in a ficoll-gradient preparation from hog mucosa. The stimulation by valinomycin is much less than the stimulation by gramicidin. This can be explained in one of two ways. Valinomycin is K^+ selective and does not transport H^+ and requires an associate conductance path for net K^+ movement. Gramicidin is a channel former which can exchange K^+ for H^+ hence does not require an associate conductance. Hence, the greater stimulation by gramicidin could be due to facilitation of K^+ entry relative to valinomycin or be due to dissipation of a limiting H^+ gradient or both.

Freeze-drying the preparation essentially abolishes the ionophore enhancement of the enzyme. Preincubation with K^+ reduces but does not abolish activation. NH_4^+ stimulation is independent of vesicular form or gramicidin due to the high permeability of NH_3. Protonophores or lipid permeable ions do not stimulate ATPase activity.

Table 2. Effect of ionophores on ATPase mole Pi mg^{-1} h^{-1}

Mg	K^+	NH_4^+	K^+ valinomycin	K^+ gramicidin	NH_4^+ gramicidin
3.6	5.4	85.6	9.8	96.7	80.1

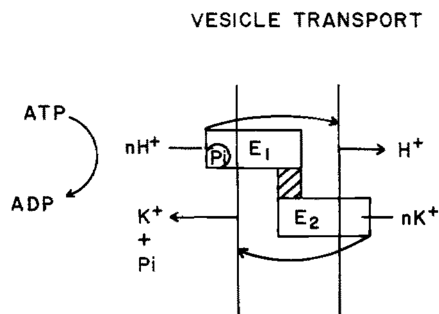


Fig. 4. Scheme illustrating a dimeric model for $H^+ + K^+$ ATPase. Here a H^+ and K^+ site exchange occurs, sites being mobile across the membrane. The movement of H^+ is obligatorily coupled to the E_1 to E_2 conformational change. In turn, because of dimeric nature of the enzyme, this is coupled to the E_2 to E_1 conversion of the other subunit which is K^+ dependent. Irradiation of E_1E_2 form will then inactivate ATPase and H^+ transport whether E_1 or E_2 is destroyed. pNPPase inactivation requires that both E_1 and E_2 are inactivated. Antibody against E_1 will inhibit ATPase, but only partially inhibit pNPPase since both E_1 and E_2 can hydrolyze pNPP but only E_1 can react with ATP

The most reasonable explanation of these findings is that the vesicular permeability to K^+ is low. This explains the K^+ ionophore enhancement of activity. If protonophores or lipid permeable ions also enhanced activity, the explanation could be that there was a potential difference developed which restricted ATPase activity. Since preincubation with K^+ did not abolish but reduced ionophoretic enhancement of activity, the implication is also that K^+ is transported out of the vesicle during ATPase activity.

Accordingly the K^+ site for ATPase, activation is on the opposite side of the membrane compared to the ATP site and K^+ is probably transported during enzyme activity.

In contrast to ATPase activity, pNPP hydrolysis, although K^+ dependent, is insensitive to any of the ionophores. Accordingly, the K^+ site for pNPP hydrolysis is on the external face of the vesicle. Since pNPP hydrolysis is a partial reaction of the ATPase, either the K^+ site for pNPP hydrolysis is different from the ATPase K^+ site, or the site is mobile across the membrane.

The simplest model is, therefore, that the K^+ site is mobile across the membrane (Fig. 4).

D. Active Form of ATPase

The rationale has been given for the involvement of a phosphorylated intermediate in ATPase activity, and for the presence of a mobile K^+ site. The phosphorylated peptide may be the only subunit of the enzyme, or the carbohydrate staining region on the gel may contain a second subunit. Based on the Ca^{++} and $Na^+ + K^+$ ATPase, the 100,000 dalton unit may well be the functional transport unit.

The enzyme may function as a monomer, a dimer, or a higher polymeric form. There are various ways of distinguishing monomer as opposed to functional dimer.

Kinetically, the Hill coefficient for pNPPase activity at a Mg^{++}/pNPP ratio of 1 is 1.8, suggestive of a dimer. With ATP as substrate, the Hill coefficient is 1. This may suggest that for every ATP site on the functional enzyme, there are 2 pNPP sites.

Inactivation of the enzyme by irradiation can be described by:

$$A/A_0 = e^{-V \cdot D}$$

where A is activity at time t, A_0 is initial activity, V is apparent volume of target and D is dose of radiation. V is obtained from the slope of ln A/A_0 against D (in time of irradiation). The ATPase activity is inactivated at twice the rate of the pNPPase. This can be explained if the ATPase is a dimer, with half site reactivity, but either half of the dimer can hydrolyse pNPP.

Recently, it has also been possible to generate antibodies against this ATPase. With this antibody, the inactivation of the ATPase was about twice that of the pNPPase [64]. This may be explained also by half site reactivity, if the antibody is directed against the ATP site (and pNPP site) on half of the enzyme. The pNPP site on the other half of the enzyme is not affected (Fig. 4).

In subsequent sections we shall develop this dimer concept to explain electroneutral H^+ and K^+ exchange.

VIII. H^+ Transport

The addition of ATP to a suspension of dog microsomes [40] or purified hog membrane vesicles [41] results in a transient alkalinisation of the medium (Fig. 5).

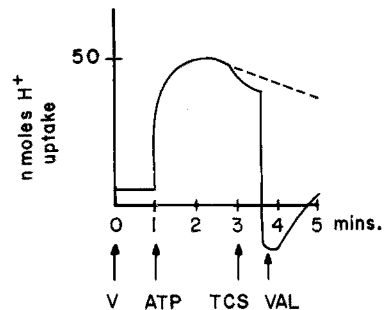


Fig. 5. An experiment where ATP is added to a vesicle suspension in the presence of 150 mM KCl. There is rapid uptake of H^+ which spontaneously dissipates with consumption of ATP. Addition of tetrachlorsalicylanilide (TCS) has only a slight effect. Subsequent addition of valinomycin results in dissipation and overshoot of gradient

A. Detection of H^+ Gradient

This may be done using a pH electrode since the intravesicular space is shielded from the electrode. Disappearance of H^+ may be due to binding, or to uptake into

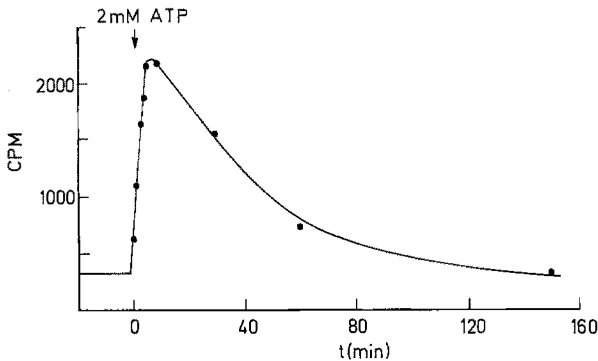


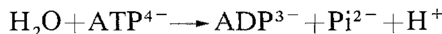
Fig. 6. Effect of ATP on uptake of ^{14}C-imidazole at pH 7.4 and 75 mM RbCl, 125 mM sucrose and 2 mM MgCl with 2 mM MgATP. Wash solution was at pH 6.1 to minimize imidazole efflux and in separate experiments a constant baseline was observed in the absence of ATP additions

an intravesicular space. The effectiveness of ionophores, such as nigericin or a combination of valinomycin and tetrachlorsalicylanilide (TCS) in reducing the alkalinisation, shows that a gradient is probably responsible [40, 41]. The reduction in H^+ uptake obtained by increasing the tonicity of the medium [42] (i.e., decreasing the intravesicular volume) also argues for H^+ uptake.

An alternative method is the use of pH indicator dys and measuring O.D. changes with ATP addition. It is best to use an indicator the color change of which is in the pH range either outside or inside the vesicles and is suitably distributed. This latter point is perhaps the most problematic. The use of such an indicator gives an estimate of the pH range attained in the vesicles. As with the pH electrode techniques, variation in vesicular volume and the use of ionophores allows direct proof that a gradient is produced. A dye which has proved suitable is bromcresol green.

Finally, one may use trapping of a base on the assumption that the charged form is not permeable. The addition of radioactive imidazole at pH 7 followed by filtration and counting allows the distribution ratio to be measured. With this compound the addition of ATP results in a considerable enhancement (35-fold) of the quantity of imidazole trapped (Fig. 6). Unfortunately, there may be sufficient efflux of imidazole to make this a low estimate of the actual pH gradient developed. As before, nigericin prevents the ATP dependent accumulation of imidazole.

From these lines of evidence, gastric vesicles are capable of energized H^+ uptake. Parenthetically, the pH of study of any ATPase reaction is important because of the reaction:



$$H_2O + ATP^{4-} \longrightarrow ADP^{3-} + Pi^{2-} + H^+$$

which at pH 7.4 results in a release of H^+ for every mole ATP hydrolysed. At lower pH, depending on the Mg^{++} concentration [43], one can find a pH at which no H^+ is released upon hydrolysis due to maintained protonation of the products of hydrolysis. Since Mg^{++} affects the pK_a of all the reactants, its concentration is important in determining the pH at which no net change of $[H^+]$ occurs during hydrolysis. Hence, at a pH of around 6.12 for 2 mM Mg^{++}, changes in medium pH are due to transport.

B. Substrate Specificity of H^+ Uptake

Except for ATP no other nucleoside triphosphate gave any indication of H^+ transport [42]. Para nitrophenylphosphate was also inactive in the test system consisting of gastric vesicles suspended in KCl at pH 6.1. Here also there is no net change of pH due to the hydrolytic reaction itself.

Redox substrates such as lactate, NADH, NADPH, ascorbate, reduced cytochrome c and $FADH_2$, were inactive in generating a pH gradient in the purified fraction. These data are in contrast to those obtained in the less purified microsomes where there is a positive change of pH in the medium with the addition of NADH even in the presence of amytal [41] due to H^+ consumption. It would seem, therefore, that the H^+ transport is a property of the ATPase.

C. Cation Requirement

There is an absolute requirement for alkali metal cation in the H^+ transport process. If vesicles which have been prepared directly on the ficoll gradient are added to 150 mM KCl at pH 6.1, there is virtually no H^+ utake with the addition of ATP. After some minutes of incubation, the H^+ uptake is observed following ATP addition. From this it is clear that internal K^+ is required for H^+ uptake. This is futher confirmed by the increased rate and maximal uptake observed with vesicles equilbrated with K^+ or vesicles in an outward K^+ gradient experiment. In fact, the outward gradient results in the largest K^+ uptake.

The cation selectivity of the process is identical to the selectivity of the ATPase. Thus the sequence is, when K^+ is set to 100; K^+ 100; Rb^+ 80; Cs^+ 4.5; Na^+ 0.24; Li^+ 0.1; choline 0.

The requirement for cation is not met by lipid permeable cations, hence is a requirement that is specific and not due to provision of a nonspecific conductance. Accordingly, the cation requirement is for ATPase activation or as a counterion for H^+ uptake or both.

D. Effect of Inhibitors

Various inhibitors of ATPase activity such as Zn^{++}, F^-, pCMBS or DCCD also inhibited H^+ uptake to the same extent as the ATPase.

E. Effect of Ionophores

The use of these agents allows conclusions to be drawn in relation to the inherent membrane conductance as well as to the conductance requirements for transport.

Assuming an outward H^+ gradient is present across the vesicle membrane, generated by ATP and an inward K^+ gradient is present due to the conditions of study (i.e. addition of vesicles to KCl, or generation of a K^+ gradient by ATP),

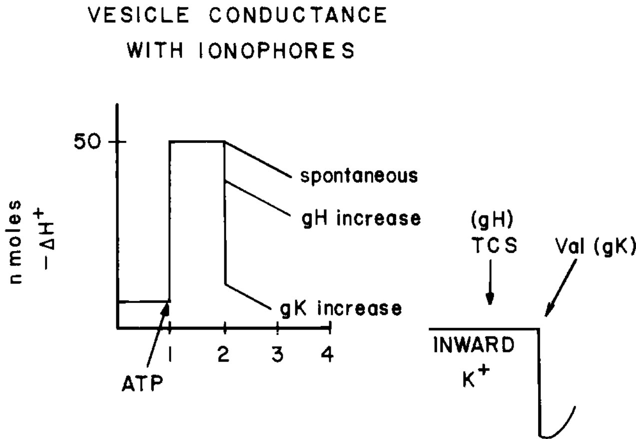


Fig. 7. A schematic representation of the effect of ATP and ionophores on vesicle H^+ gradient. ATP generates an H^+ gradient, and there is a spontaneous decay of the H^+ gradient. Addition of a protonophore rapidly decays gradient by about 20% and then continued decay returns to spontaneous rate (gH increase). Addition of a K^+ ionophore is more effective in dissipating gradient (provided there is an inward K^+ gradient) (gK^+ increase). Second half of figure shows the effect of ionophores on the vesicles in the absence of ATP but with an inward K^+ gradient present. A protonophore has no effect on H^+ gradient. A K^+ ionophore, with or without protonophore, induces an efflux of H^+

then the continued existence of the H^+ gradient is due to a limiting conductance in the membrane, either for H^+, K^+ or Cl^-. If the H^+ conductance is rate limiting, then the addition of an H^+ conductance, as with a protonophore (TCS, CCCP), should effectively dissipate the gradient. If this is not the case, then another conductance may be limiting. The addition of valinomycin which induces a K^+ conductance results in dissipation of a large fraction of the H^+ gradient (Fig. 7). From this we can conclude that the major conductance is for H^+ and not for K^+ (42).

Nigericin which acts as a cation exchange ionophore [44] dissipates either an H^+ or K^+ gradient. In the absence of an initial K^+ gradient, the H^+ gradient is converted to a K^+ gradient with the addition of nigericin. Nigericin dissipates the gradient in gastric vesicles.

The ionophores may also be used to estimate the conductance requirements of the membrane during pump activity. In general, as discussed in relation to the electrogenic H^+ ATPase, increase of membrane conductance will increase pump activity if the pump is electrogenic. No increase in ATPase activity is found with addition of protonophores, nor is there much ($<15\%$) inhibition of H^+ uptake. Valinomycin enhances H^+ uptake with ATP addition under inward K^+ or zero K^+ gradient conditions. This is explained by the requirement of the system for internal K^+, rather than due to any conductance change. Lipid permeable cations do not substitute for K^+ ionophores, which is additional evidence for the specific internal K^+ requirement, rather than K^+ providing a cationic conductance to satisfy an electrogenic H^+ pump.

The major conclusion from this is that the H^+ transport by these vesicles is likely to be non-electrogenic. Accordingly, there is a contransport of anion or

countertransport of cation which is a pump requirement. This can be studied by investigation of the transport of other ions by these gastric vesicles.

F. Stoichiometry of H^+ Uptake

The ratio of H^+ movement for each high energy phosphate in mitochondria and chloroplasts varies between 2 and 4 depending on the conditions of the experiment [45, 46]. Measurement of ATP hydrolyzed at the same time as H^+ uptake gives a ratio of moles H^+ transport per mole ATP hydrolyzed of 4, when only the K^+ activated component of the ATPase is used. From this, since the H^+ gradient achieved by the mucosa is 10^6 or better, the H^+/ATP ratio has to vary from 4 to less than 2 as the H^+ gradient increases. It can be calculated that 9 kcal/mol are required for H^+ transport at a 10^6 gradient. The $\Delta G'$ for ATP hydrolysis is about the same hence the maximal molar ratio of H^+/ATP should be about 1.

IX. Cation and Anion Movement

The uptake of H^+, whether electrogenic or neutral must be accompanied by cotransport of Cl^- or countertransport of K^+, since K Cl is the only added salt. Two types of studies can therefore be performed, uptake and efflux [47].

A. Rb Transport

Using $^{86}Rb^+$ and $^{36}Cl^-$ it was shown that both ions were taken up into an intravesicular space of about 2 μl mg^{-1} protein. The $T_{1/2}$ was temperature sensitive, being several hours at 4°, and 40 min at room temperature. The addition of ATP transiently reduced Rb^+ uptake but had no observable effect on Cl^- uptake.

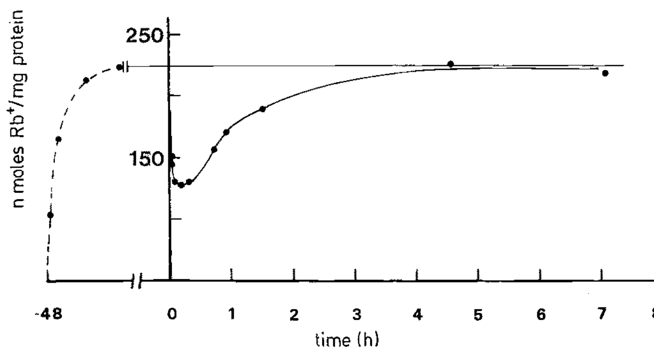


Fig. 8. Efflux of Rb^+: vesicles were preequilibrated for 48 h as detailed in the text and 2 mM ATP added to the suspension at zero time on graph. It can be seen that there is a transient rapid efflux of Rb^+ to about 50% of the measured equilibrium value (horizontal line) and with consumption of the ATP there is reequilibration of the Rb^+

When the vesicles were allowed to equilibrate with either Rb^+ or Cl^-, and ATP added to this suspension, there was a transient efflux of Rb^+, but no effect on Cl^- (Fig. 8). The movement of Rb^+ therefore develops a chemical gradient of Rb, and could be due to an energized $H^+:Rb^+$ exchange, or due to a change of potential across the vesicle membrane, the interior of the vesicle becoming positive with ATP addition.

Two means are available for distinguishing these possibilities, by the use of conductance modifying agents and determining their effects on Rb^+ transport and by the direct measurement of vesicle potential. The latter will be discussed in a subsequent section.

B. Effects of Membrane Active Agents

An ionophore with Rb^+ specificity, such as valinomycin, would be expected to increase the rate of Rb^+ efflux with the Rb^+ movement being due to a potential. In fact no change in Rb^+ efflux was noted in the presence of valinomycin. The reuptake of Rb^+ after ATP had been consumed was accelerated.

Lipid permeable cations, such as triphenylmethyl phosphonium or dimethyl dibenzyl ammonium should, at adequate concentration, substitute for Rb^+ if the movement were due to a potential and reduce Rb^+ efflux as well as themselves show similar efflux characteristics. Neither phenomenon occurs, lipid permeable cations being without any effect on Rb^+ movement and showing no redistribution themselves with ATP addition.

A lipid permeable anion, such as SCN^-, should enter the vesicle in response to a positive internal potential and thereby reduce the potential, and hence reduce Rb^+ movement that is dependent on potential. Again SCN^- is without effect on Rb^+ efflux.

Accordingly, modifying membrane conductance by adding alternate electrogenic pathways does not effect Rb^+ efflux induced by ATP therefore Rb^+ movement is not due to a potential difference across the membrane of the vesicle.

C. Relationship to ATPase Activity

Inhibitors of ATPase activity inhibit Rb^+ movement (e.g. pCMBS, DCCD, Zn^{++}, F^-). The nucleoside triphosphate substrate specificity for Rb^+ efflux is the same as for the ATPase as is the pH optimum and the K_M. The Rb^+ to ATP ratio in terms of moles transported is 3.4 considering only the cation activated component of the ATPase.

X. Measurement of Vesicle Potential

In the previous sections arguments have been put forward that the transport process catalyzed by the ATPase is essentially a nonelectrogenic $H^+ + K^+$

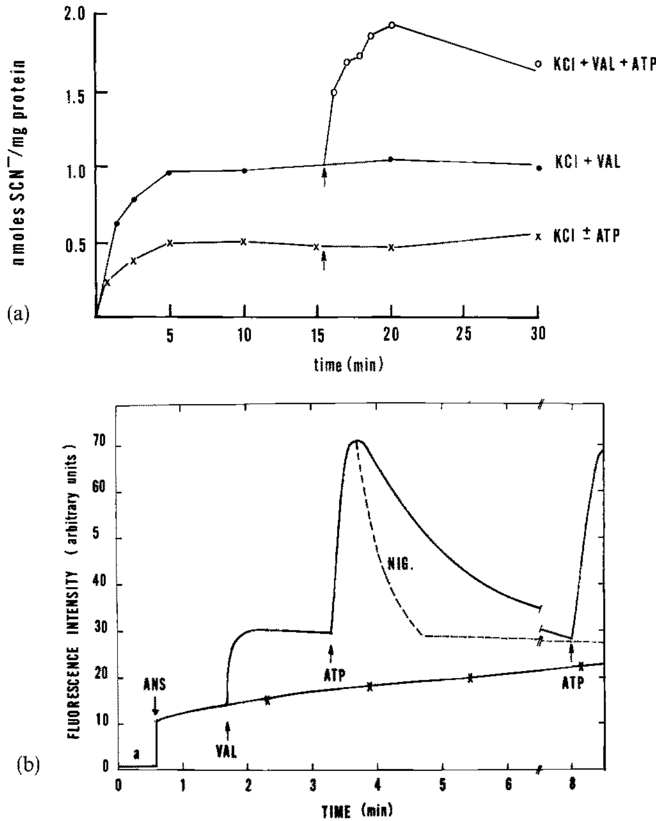


Fig. 9. (a) Uptake of ^{14}C SCN^- with time when added to vesicles alone (*lower* ×———× *curve*) or vesicles in the presence of valinomycin (*upper* ●———● *curve*). ATP was added to both sets of tubes at the time indicated, but enhanced SCN^- uptake only in the presence of valinomycin (○———○). (b) ANS fluorescence events of gastric vesicles. Initial addition is that of membranes to cuvette, followed by ANS. It can be seen that there is an initial rapid rise with ANS addition followed by a slow increase of fluorescence (×———×) which eventually reaches the valinomycin level (not shown). Addition of valinomycin before the plateau results in a further rapid increase in fluorescence, and subsequent addition of ATP (1.7×10^{-5} M) results in a transient fluorescent enhancement, which can be repeated with a second addition of ATP. Also shown is the effect of nigericin on ATP-induced fluorescence, giving immediate inhibition of the fluorescence and inhibition of the effect of subsequent ATP addition

exchange. To further establish this, radioactive or fluorescent lipid permeable anions or cations can be used to measure potentials as discussed above.

Whether ANS or ^{14}C-SCN^- are used, although uptake is observed, no change in fluorescence, or uptake of SCN^- is found with ATP addition. This might be so because the techniques are too insensitive to measure potentials, although these ions have been used with success elsewhere [48, 49].

Since with ATP addition to equilibrated vesicles there is a K^+ gradient developed due to the $K^+:H^+$ exchange, the presence of valinomycin should theoretically result in the development of a positive internal potential due to the inward K^+

gradient. This should then result in enhanced SCN^- uptake and ANS^- fluorescence with ATP addition in the presence of valinomycin. Both phenomena are found [42, 50]. Accordingly, these techniques are sufficiently sensitive in these vesicles to measure a potential but the only potential detectable is due to the presence of valinomycin and the ATP dependent K^+ gradient (Figs. 9a, b).

Accordingly, these data confirm that the gastric vesicles contain an $H^+ + K^+$ ATPase catalysing a neutral $H^+ + K^+$ exchange, and that this transport differs from that catalyzed by mitochondrial or bacterial H^+ ATPase.

It should be noted that these changes are measured with a considerable lag phase, about 3–5 s with the ANS technique and about 15 s with the $S^{14}CN$ method. Hence it is possible that a voltage transient is generated during this transport but is rapidly shunted by development of a pump related conductance. To exclude this, stop flow fluorescent methods are required.

XI. Incorporation of ATPase into Bilayer

In general, incorporation of plasma membrane ATPase into planar bilayers has not been convincingly demonstrated, or has not been reproducible. In general, there are two forms in which attempts have been made to incorporate such pump, in the purified form and in vesicular form. In the latter experiments Ca^{++}, the use of phosphatidyl serine (PS) in the bilayer and elevated temperatures improve the incorporation and fusion process.

Some success has been achieved with the gastric membrane fractions [51]. The addition of the ATPase enriched fraction to a PS bilayer results in a reproducible conductance increment in the bilayer ranging from one to three orders of magnitude. This conductance is cation selective, with the same selectivity sequence as for the ATPase activation, or for H^+ transport by the vesicles.

In addition, the addition of ATP to the bilayer with the vesicles incorporated results in the development of a sizable P.D. (Fig. 10). Interestingly, the characteristics of the potential response are a function of the cation composition of the medium.

Thus the presence of Na^{++} results in the development of a potential which is little affected by the nature of the anion. Indeed the potential in Cl^- is greater than that in SO_4^{--}. This is similar to the reduced H^+ and Rb^+ movement found with SO_4^{--} as compared to Cl^- but is contrary to what would be expected from the relative bilayer conductance of these anions. Substantial ancillary evidence exists that the bilayer phenomena are pump properties. For example, the reaction is ATP specific and it is blocked by pCMBS, as is the pump in natural vesicles.

In the presence of K^+, the potential develops initially with the same characteristics as with Na^+. In a time-dependent manner however, there is a fall of potential. This time dependence is characteristic of a bilayer thickness dependent phenomenon, as occurs using malonyl digramicidin. It seems reasonable to explain this by postulating a thickness-dependent K^+ conductance developing to shunt the H^+ potential.

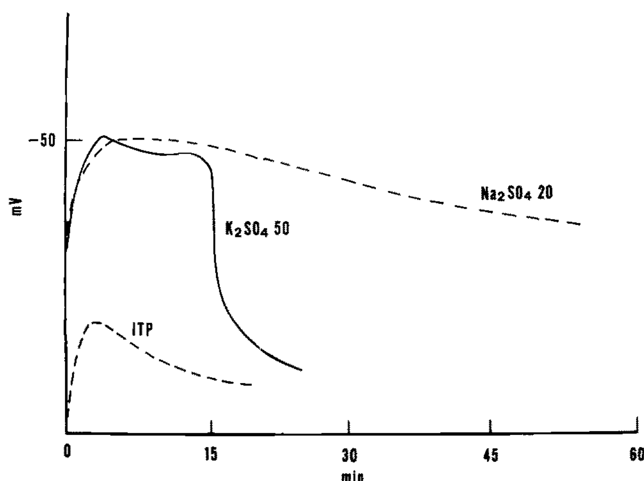
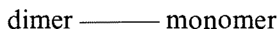


Fig. 10. Effect of ATP addition (*upper two curves*) and ITP addition (*lower curve*) on one side of the bilayer with incorporated vesicles. Addition of ATP at zero time resulted in development of a potential of −50 mv which was well maintained in the presence of Na_2SO_4 (– – – – – –). ITP produced only a fraction of the potential. Results of addition of 50 mM K_2SO_4 to both sides of bilayer showed that ATP produced essentially the same potential, but after 15 min there was a sharp decline in potential to about 10% of the peak value (————)

If this interpretation is correct this implies that the K^+ site not only is anatomically distinct from the H^+ site, but is so located that it is buried in a bilayer which is capable of moving protons due to pump incorporation.

In one sense therefore, this may explain the absence of an observed potential in intact vesicles and argues for equal and opposite electrogenicity of the two transport processes, the H^+ and K^+ transport in the vesicles. However, an alternative explanation may be that the form of the pump is different in natural vesicles and in a bilayer. With dilution or change in lipid environment it may be that a dissociation reaction is formed thus:



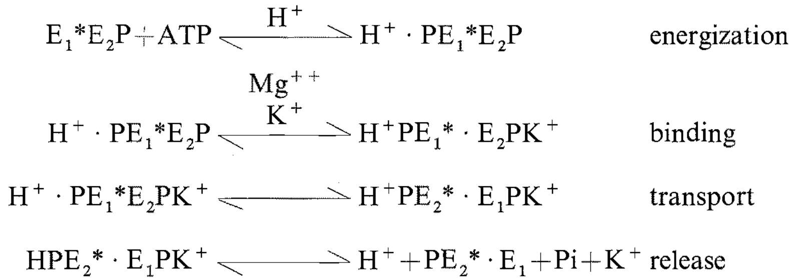
dimer ——— monomer

and that monomer transport has different electrical characteristics.

XII. Dimer-Monomer Transport Model

As mentioned above, there is some evidence that the $Na^+ + K^+$-ATPase may function as a dimer, or higher polymer of the catalytic subunit [21, 25]. If one makes the assumption as suggested in a recent review [25] that the functional dimer is composed of two different conformations E_1 and E_2 of the catalytic subunit, i.e., E_1E_2 and does not exist as E_1E_1 or E_2E_2, the reaction sequence

may be written thus:



$$E_1{*}E_2P + ATP \underset{Mg^{++}}{\overset{H^+}{\rightleftharpoons}} H^+ \cdot PE_1{*}E_2P \qquad \text{energization}$$

$$H^+ \cdot PE_1{*}E_2P \overset{K^+}{\rightleftharpoons} H^+PE_1{*} \cdot E_2PK^+ \qquad \text{binding}$$

$$H^+ \cdot PE_1{*}E_2PK^+ \rightleftharpoons H^+PE_2{*} \cdot E_1PK^+ \qquad \text{transport}$$

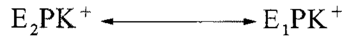
$$HPE_2{*} \cdot E_1PK^+ \rightleftharpoons H^+ + PE_2{*} \cdot E_1 + Pi + K^+ \qquad \text{release}$$

Here the * is used to designate the same monomer. As written there is obligatory H^+ and K^+ exchange that is neutral. To convert this to an electrogenic mechanism, $2H^+$ can be translocated in exchange for 1 K^+ (Fig. 4).

When the reaction is written as the monomeric reaction, as in section 7b above, the translocation of H^+ is now distinct from the movement of K^+. The reaction:

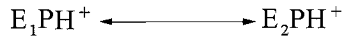
$$E_2P \longleftrightarrow E_1P$$

which may occur in the absence of K^+ allows the transport to be electrogenic. The transformation:



$$E_2PK^+ \longleftrightarrow E_1PK^+$$

then represents a K^+ conductance. In the dimer form, this latter transformation depends on the simultaneous:



$$E_1PH^+ \longleftrightarrow E_2PH^+$$

which is energized, and hence cannot formally be represented as a conductance.

XIII. Localisation of the ATPase

Histochemical techniques for ATPase localization are, in general, unsatisfactory. Using a double antibody fluorescent technique, where antibody was raised against the free flow electrophoresis fraction, provided firm evidence that the transport fraction derived exclusively from parietal cells. Moreover, the fluorescence was localized to the apical region of the cytoplasm as would be expected of the localization of the tubulovesicles in the resting parietal cell [64].

General

It appears that the $H^+ + K^+$-ATPase is capable of catalysing a $K^+ : H^+$ exchange and separable H^+ and K^+ conductances are present in the pump mechanism.

The transport mechanism of this ATPase remains obscure as does the mechanism for any other transport ATPase. It seems evident that the mechanism is closely similar to that of the Na^+-K^+ and Ca^{++}-ATPases in that we have a reversible ATPase with a partially electrogenic mechanism and a reaction mechanism operating via a covalent phosphate.

In the discussion on the mechanism of the mitochondrial ATPase, it was pointed out that the protonated intermediate could interact with ADP and Pi binding sites increasing the electrophilic reactivity of the phosphorus. This could result from a direct protonation reaction, or from a conformational change, or both.

In the K^+-ATPase, H^+ and K^+ both play a role in the interconversion of chemical and ion gradient energy forms. We have presented some evidence that the hydrolytic site which forms the covalent intermediate is mobile across the membrane or that the K^+ site is mobile. Binding of K^+ or H^+ at either side of the membrane would serve not only to activate formation or hydrolysis of the intermediate, but also to provide the energy for the conformational change resulting in translocation of the phosphate or the K^+ site on the protein.

In the introduction some detail was presented on the mechanisms of the H^+ ATPase and the plasma membrane type of ATPases. Considering the data available on the gastric ATPase, the mechanism of action of this enzyme seems clearly more closely related to the plasma membrane type of ATPase than the electrogenic H^+ ATPase of mitochondria. This does not mean, that when the molecular mechanism is understood, entirely different reaction paths will be found. For example, if the gastric ATPase functions to transport H^+ by the energized downshift of a group pK_a, this would make an analogy to the photolytic H^+ transport by lumirhodopsin. Mitochondrial FI ATPase may also alter the pK_a of a protonatable group as a function of binding of Pi or ADP. The additional subunit complex of F_1, the F_o with its attendant binding sites which serve to conduct H^+ may be incorporated into the peptide backbone of the gastric ATPase. The nonelectrogenic character of the latter may be determined by the dimeric nature of the transport form, rather than by the characteristics of the ion translocation process itself.

XIV. Relationship of Vesicles to Intact Tissue

In this section, the properties of acid secretion by intact mucosa will be discussed entirely in relationship to the data obtained for hog gastric vesicles. Intact tissue secretion has been well defined only for amphibian mucosa hence the comparison will be between amphibian secretion and mammalian vesicles.

A. Cl^- Transport

As mentioned, gastric vesicles have a low Cl^- permeability, but a Cl^- space equivalent to the cation (Rb^+) space, as well as equivalent uptake rate for Cl^- ($T_{1/2}$=36 min at 22° C). No evidence could be obtained for active Cl^- transport in these vesicles.

The short circuited amphibian mucosa actively transport Cl^- in that there is a net S $\longrightarrow$ M flux of Cl^- under these conditions (52). Removal of Na^+ abolished the Cl^- component as well as the potential difference across the tissue [53]. The addition of amytal inhibits H^+ and Cl^- secretion but adding ascorbate and mena-

dione [51] restores 80% of the ATP level and Cl^- transport, but not H^+ secretion. It would appear that Cl^- transport may therefore be ATP dependent, but complex I is critical for H^+ secretion.

The vesicles discussed above lack the Cl^- transport system. In the absence of Cl^- there is a 80% decline in acid rate if SO_4^{--} is the substituting anion in the medium bathing the mucosa [54]. Removal of Cl^- and addition of SO_4^{--} also reduced H^+ transport and Rb^+ transport in the vesicles but by about 40% (42). Addition of SCN^- did not restore the H^+ uptake in the vesicles, so the reduction in H^+ rate is not due to the development of a potential in SO_4^{--}. This is confirmed by the lack of change of SCN^- uptake or ANS fluorescence with ATP addition in the absence of valinomycin. The reduction by 40% of these parameters in the presence of valinomycin is consistent with the expected fall in the K^+ gradient dependent potential.

Thus, the anion effect in vesicles is reasonably consistent with the data on the intact tissue, but as will be seen later the interpretation has to be different to what has been thought correct for the intact stomach.

B. Metabolic Data

Over the years there has been considerable controversy over the primary energy source for acid secretion, i.e., whether an ATP or directly redox linked process was responsible [5]. The massive quantity of mitochondria in the parietal cell has made a decision particularly difficult. The problem is also complicated by the fact that secretion and metabolism are both stimulated by secretagogues, hence transients that are present due to secretion may be observed by those due to stimulation of metabolism.

The most detailed study of ATP and related nucleotide levels as well as that of phosphocreatine gave only marginal evidence of an ATP-related transport phenomenon [55]. These results in dogs with a large change in secretory rate are in fair agreement with the results in frogs with much smaller changes in secretion [56].

The data obtained by spectroscopic observations of intact frog mucosa [5] have been interpreted as substantiating a redox energy source for acid secretion. As discussed in detail elsewhere, however, [65] this data can be interpreted differently. However, when the observations on metabolite levels made on intact dog mucosa are combined with the spectroscopic data on frogs, it appears that a crossover does occur with onset of secretion between NAD^+ and FAD in the mitochondrial respiratory chain, with reduction of cytoplasmic NAD^+. This suggests that an additional component may be involved in gastric acid secretion other than the $H^+ + K^+$ ATPase.

C. Morphological Data

It seems well established that at rest, the cytoplasm of the parietal cell is filled with tubulo-vesicles the contents of which are not in continuity with the luminal bathing solution [57]. These specialized structures fuse with the apical surface of the cell and vastly increase its surface area upon stimulation of secretion [58]

and then revert to the cytoplasmic form with cessation of secretion. This provides a rationale for two of the major findings discussed above; first a unique and simple structure present in fractions of this tissue and second a degree of ion impermeability usually found only in preformed vesicles. It would seem intuitively obvious, althoug not proven, that the vesicular structures contain a component essential for H^+ secretion, as for example the $H^+ + K^+$ ATPase.

It is therefore possible to develop the hypothesis that at least part of transport regulation by gastric mucosa is due to incorporation of the pump into the plasma membrane of the cell. As such, this is a relatively unique means of transport regulation.

D. The Requirement for K^+

That H^+ secretion had a requirement for K^+ in the bathing solution was shown almost 20 years ago [59]. Removal of K^+ rapidly inhibited secretion, at a time when tissue K^+ had only been slightly reduced [60]. Addition of K^+ to the mucosal solution rapidly restored secretion [60]. Hence, it would appear that a small K^+ compartment is essential for secretion. This was confirmed in experiments using Rb^+ to restore insulin inhibited secretion in the dog [61]. Moreover, the J_{SM} for K^+ is much less than the acid rate [62]. This finding is consistent with a recycling mechanism for K^+ if involved in $K^+:H^+$ exchange. As for the vesicles, Rb^+ and Cs^+ can partially substitute for K^+, but not Na^+ or Li^+.

The data for the vesicles are therefore quite compatible with the findings on intact mucosa.

E. Electrogenicity of H^+ Secretion

One of the most striking results in amphibian mucosa is the finding that while acid secretion continues in SO_4^{--} containing, Cl^- free solutions, the P.D. across the mucosa is inverted, i.e., the lumen is positive [54]. Moreover, the P.D. is linearly related to the acid rate [63]. This has been interpreted as compelling evidence for electrogenic H^+ secretion. In Cl^- solutions, the data are much less convincing. Based on this, one would anticipate that, at least in SO_4^{--} solutions, evidence would have been obtained for electrogenic H^+ uptake in vesicles.

However, an alternative interpretation of the SO_4^{--} effect on acid secretion may be proposed. In SO_4^{--} solutions, in contrast to Cl^- solutions, the mucosal surface of the tissue demonstrate a K^+ selective conductance [38].

If the inverted potential is then due to a K^+ diffusion potential across the mucosal surface of the tissue, then the magnitude of the potential will be a function of the concentration of K^+ at the luminal surface.

In the presence of an $H^+ + K^+$ exchange that is non-electrogenic, the K^+ concentration at this surface will be reduced in proportion to the acid rate, hence the potential will also be proportional to the acid rate.

Thus, although electrogenic H^+ secretion may be the correct interpretation of data from the intact mucosa, alternate explanations may be correct (Fig. 11). Such explanations are certainly necessary to reconcile vesicle data with those of intact organs.

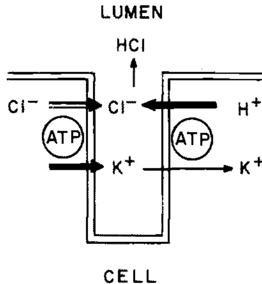


Fig. 11. Schematic representation of secretory surface of parietal cell. H^+ secretion is visualized as being due to the $H^+ + K^+$ ATPase, hence due to an $H^+ + K^+$ exchange. K^+ is visualized as diffusing into the lumen accompanying electrogenic active Cl^- transport that is also ATP dependent. Site of energization of Cl^- transport may not be at the apical membrane

XV. Summary

It would appear, therefore, that hog gastric mucosa contains a particularly interesting system which in many respects is compatible with a model for H^+ secretion. It is quite different, at current levels of analysis, from the H^+ ATPase of mitochondria. Indeed the stomach appears to have developed a unique method of developing H^+ gradients. The magnitude of this gradient is such that perhaps this ATPase is only partially responsible, and that a second pump, in series with the one discussed in some detail here, is required for the complete process.

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