ELECTROCHEMICAL ASPECTS OF PHYSIOLOGICAL AND PHARMACOLOGICAL ACTION IN EXCITABLE CELLS[1]

PART I. THE RESTING CELL AND ITS ALTERATION BY EXTRINSIC FACTORS

ABRAHAM M. SHANES

National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Public Health Service, Department of Health, Education, and Welfare, Bethesda, Maryland

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I. NOTATION

C_m	Membrane capacitance, specific
E_i	Injury potential
E_m	Resting potential
E_K, E_{Na}, E_{Cl}	Equilibrium potential (of K, Na, Cl)
G_m (also G_K, G_{Na}, G_{Cl})	Membrane conductance, specific (also potassium, sodium, chloride conductance)
I_K, I_{Na}, I_{Cl}	Influx (of K, Na, Cl)
O_K, O_{Na}, O_{Cl}	Outflux (of K, Na, Cl)
P_K, P_{Na}, P_{Cl}	Membrane permeability (to K, Na, Cl)
[ ], *e.g.* $[K]_o$, $[K]_i$, $[Na]_o$, etc.	Concentration, *e.g.* of extracellular K, intracellular K, extracellular Na, *etc.*
R_i	Protoplasmic resistance, specific
R_m	Membrane resistance, specific ($1/G_m$)
Ω	Ohm

II. INTRODUCTION

It has become increasingly clear in the past decade that certain features of cellular functioning—bioelectrical potentials, ion distribution, and ion movement—and the influence of some physiological and pharmacological agents on these, are more readily understood in terms of interactions involving physical properties of ions and cellular membranes which are relatively nonspecific, rather than from the standpoint of specific enzymatic reactions alone. Enzymatic reactions, included under the general term metabolism, are by no means unimportant. They are necessary at the very least for the establishment and maintenance of the basic structure of protoplasm and cellular membranes as well as for the maintenance of an ionic composition peculiar to protoplasm. And in the case of muscle, of course, they play an important part in contraction processes.

Here we shall be concerned with the "electrochemical" characteristics of certain excitable cells and the general role of metabolism in their maintenance. By electrochemical characteristics are meant the electrical potential differences, the ion distributions and movements which appear to be related to them, and the physical properties (electrical conductivity or resistance, capacitance, permeability) of the phases present, namely, the membranes, protoplasm, and ex-

tracellular "spaces". Muscle contraction is necessarily excluded. These will be discussed in Part I for the condition of "rest", *i.e.*, for excitable cells in which the physiological state is relatively constant or in which slow, small, or graded changes are induced experimentally. Cells that have been altered experimentally must be considered since it is otherwise impossible to elucidate the mechanisms involved. The alterations to be described are the changes of cellular electrical potentials, membrane resistance, ion distribution, and ion movement in response to ions, drugs, metabolic inhibitors and substrates, and junctional transmitters.

Part II, to appear later, will deal with the condition of "activity", the situation associated with the all-or-none impulse or action potential, including excitation and recovery processes. This division does not imply a basic difference in the mechanisms. Actually, both aspects—rest and activity—are complementary, for it is now clear that principles governing one contribute to an understanding of the other.

Biological systems will be dealt with for which measurements are available of both electrical and chemical (ionic) characteristics: Invertebrate and vertebrate nerve; striated (vertebrate and invertebrate), cardiac, and smooth muscle; and junctions, including the myoneural junction, motoneuronal synapses, and invertebrate sensory receptors. Such a comparative approach has the advantage that general principles as well as special peculiarities of different cells are more likely to be recognized. Only brief reference will be made to certain other systems, particularly frog skin and red cells, which have provided important clues to mechanism.

The extensive work on the electrical properties and the action of drugs on electric organs has been thoroughly reviewed recently by Grundfest (185); since the associated ionic characteristics, especially with respect to ionic movement, remain to be worked out in detail, the important studies on these organs are mentioned only briefly here.

The pharmacological and physiological agents and conditions to be discussed are those for which both electrical and ionic data are available. They will include the inorganic ions, sodium, potassium and calcium; local anesthetic and other compounds, such as cocaine, procaine, urethane, antihistaminics, and veratrine and its component alkaloids; substances active at junctions, like acetylcholine, γ-aminobutyrate, and epinephrine; and compounds or conditions which act as metabolic substrates or as inhibitors of metabolism.

This review will describe results obtained chiefly in the past ten years. Papers not available by July, 1957 are not included unless advance information had already been incorporated. It is not intended to be exhaustive but rather deals with findings on nerve and skeletal and heart muscle cells *in vitro* and/or under conditions which provide (a) control of or a reasonable evaluation of environmental and experimental factors and (b) precision of measurement. Such selection is absolutely essential since procedural artifacts or secondary effects may otherwise easily be confused with cellular properties. Of course, more subtle artifacts may remain, but certainly those currently recognized should be eliminated or taken into account. Cautionary sections are included in connection

with various techniques and measurements to indicate some of their limitations.

The studies which are carried on in this field generally represent efforts to accumulate information sufficient to set up a working hypothesis or, very often, simply to test such hypotheses. A discussion of the details of hypotheses which have appeared in recent years, and their variants, is far beyond the scope of this review.

For convenience, a quantitatively formulated hypothesis, subsequently shown to be oversimplified, serves as a useful basis for the organization of the facts. According to this hypothesis, the relatively steady potential difference between the interior and exterior of each cell—the resting membrane potential—is a consequence of the selective permeability characteristics of the membrane and of the concentrations of the ions bathing the extracellular and intracellular surface. If this is correct, one should be able to collect electrical and chemical (ionic) data which conform to the equation. With this in mind, first resting potential then chemical measurements are described.

The relative effectiveness of sodium, potassium, and other ions in changing the resting potential is given in detail and it is noted that multivalent ions and a variety of drugs classified as "stabilizers" *reduce* the electrical effectiveness of these ions; attention is called to other conditions or agents which are "labilizers" and *accentuate* the ionic effects on membrane potential. The action of the drugs alone on the resting potential is considered and, finally, the consequences of metabolic inhibition on the resting potential, as modified by drugs, substrates, ions, and certain other experimental conditions, are described.

A formula derived from the working hypothesis is given. It shows that the relative magnitude of the *change* in resting potential with a change in ion concentration provides a measure of the "permeability" to the ion, *i.e.*, the ease with which this ion enters and tends to pass through the cellular membrane. Decrease of monovalent cation effects by stabilizers is shown to indicate reduced permeability whereas labilizer action indicates increased permeability. These conclusions are shown to be consistent with the action of the physiological and pharmacological agents directly on the membrane potential and on the membrane electrical conductivity as well as on the changes in resting potential with metabolic inhibition. Stabilizer effects are noted to be more striking when they prevent an increase in permeability, but the decrease in permeability noted above, while small, is usually evident.

Data which have accumulated on ion distribution and net ion movements under the previously described conditions are then compared with the electrical results and the working hypothesis. The correlations found agree well with hypothetical expectations. This is followed by a more detailed analysis of the electrochemical relationships obtained with radioisotopes on unidirectional ionic fluxes. These findings reveal that "permeability" of living membranes to ions involves processes more complicated than those which appear to be present in familiar artificial porous or solvent-type membranes.

Nevertheless, the concept of ion permeability as an overall measure of ion penetration has been found to be particularly useful for integrating electrical

potential *changes* with membrane conductivity changes and ionic movement as brought about by ions and stabilizer and labilizer drugs. This is further exemplified by the recently acquired data on electrical (membrane potential and conductance) changes and on ion movements connected with the action of transmitters, related drugs, and antagonists at a variety of junctional membranes, as distinguished from the membranes previously discussed.

In conclusion, several suggestions are made to account in more detail for the permeability characteristics of living membranes as revealed by the literature which has been surveyed. These are designated a "pore-solvent" hypothesis because certain factors involved in ion uptake by solvents, such as hydration energy of ions (*i.e.*, the difficulty involved in removal of water from ions), as well as pore size limitations, appear to govern ion entry. Also, it appears not unreasonable from recent studies that agents which alter permeability or permeability changes act on the region *between* the pores, stabilizers by "dissolving" in this region (by displacing membrane molecules) and thereby increasing the "lateral pressure" or spreading tendency. Such lateral pressure would tend to decrease channel size (and permeability) but especially to interfere with an increase in channel size during an increase in permeability. Membrane "rigidity" is also noted as a probable factor. Solvation (interaction with the membrane components) may in some cases be involved in ion penetration, and labilizers may act to reduce lateral pressure by being adsorbed on rather than dissolving in the interchannel region; however, an experimental basis similar to that for stabilizers remains to be established for these proposals.

In addition to the strictly physical (electrochemical) factors described above (*viz.*, membrane potential, permeability, and ion concentration), data on the relation of metabolism to them are presented. In general, interference with metabolism has little or no effect on membrane potential, although potassium uptake and either intracellular sodium "extrusion" or extracellular sodium "exclusion" are markedly depressed. Metabolism therefore contributes directly to ionic movement, designated as "active transport", but not directly to membrane potential. Cardiac glycosides characteristically suppress such active transport, whereas a typical stabilizer, such as cocaine, does not but acts instead on ionic movement that is independent of active transport, *viz.*, the "passive" fluxes, governed by the physical (*i.e.*, electrochemical) factors already discussed. Physical characteristics of active transport are noted (*e.g.*, independence of membrane potential, dependence on ion concentration) which require movement of at least sodium as part of an uncharged complex. A scheme involving the escape of acid metabolites is pointed out to provide some of the characteristics of ion transport.

This review concludes with a summary, based on the facts described, of a current outlook of the resting excitable cell and of the nature of its electrochemical response to physiological and pharmacological agents. It is a picture inescapably colored by some of the author's opinions and will be recognized as a greatly refined version of the classical view of the cell. There is the familiar description of an aqueous phase of protoplasm, with a high concentration of potassium but

with low concentrations of sodium and chloride, bounded by an ion-selective but labile membrane such that the interior is usually electrically negative to the outside, surrounded in turn by supporting structures and by an aqueous phase, the bathing medium, with ion concentrations the reverse of those in the protoplasm (*e.g.*, 44, 200, 215, 219, 271, 411, 428). One important difference is the enormous advance which has been made in the techniques for actually measuring permeabilities to specific ions and the changes in these permeabilities. Another is the possibility of distinguishing the contribution of metabolism to ion transport and ion distribution and its contribution thereby to cellular electrochemistry. Still another is a recognition experimentally of the complexity of the physical factors governing passive ion transfer across living membranes.

III. THE RESTING POTENTIAL, E_m

A. General principles

Much of recent research has been directed towards accurate measurements of the membrane potential difference and its changes, made possible chiefly by the use of microelectrodes with sufficiently small tips. This is of great importance for a full analysis of the electrochemistry of the system and will be discussed later at length. But valuable qualitative and semiquantitative information of importance to the more refined measurements as well as to the concepts involved has continued to accumulate with the familiar gross techniques associated with nerve trunks and small nerve bundles and entire muscles, that is, those employing external electrodes, usually in conjunction with the moist chamber (66, 68, 310, 311, 317, 319, 320, 412).

It is customary to speak of potentials although reference actually is to *differences* of electrical potential, either the exterior relative to the interior, or one region of the surface relative to another. Microelectrode techniques have led some to the use of the external medium as the zero reference. This introduces the necessity of minus signs for all figures since the interior now determines the sign. More serious is the possibility of confusion when comparisons are made with results obtained prior to the microelectrode era, all of which refer to the external potential relative to the interior and hence are positive. The latter convention, which will be continued in this review, has the additional important advantage that a decrease (*i.e.*, a negative change) in membrane potential is consonant with a decrease in the potential difference across the membrane, whereas the reverse is true for the other convention. Certainly, this is no more serious than the long-preserved convention of indicating current flow in good conductors from plus to minus, although we have known for some time that electrons flow in the opposite direction.

"Membrane potential," E_m, will henceforth refer to the best estimate of the potential difference across the cell membrane. This is determined in most cells with the intracellular electrode. "Injury potential," E_i, will designate the potential at an intact region relative to a cut or otherwise damaged or treated end of one or more fibers; this is roughly proportional to the resting membrane potential, and provides a measure of the changes in membrane potential brought about

by experimental conditions applied to the intact region. When qualitative comparisons are made, no distinction will be drawn. Myelinated nerve fibers, which do not withstand too well impalement with an electrode (55, 476), must still be studied with the injury technique; several procedures have been developed which give promise of providing the full magnitudes of the membrane potential despite the use of external electrodes, for example, by balancing out external currents with a potentiometer (246) or by electronic feedback (155) or by greatly increasing the external resistance (383, 456, 458, 479). The terms "depolarization" and "hyperpolarization" will be used in the customary qualitative sense to indicate a decrease or increase in the potential difference across the membrane.

The potential of a treated, intact region, relative to another intact region which serves as a reference, is designated the demarcation potential. This difference of potential will, in general, equal the *change* in injury potential brought about by the same experimental conditions. Small changes are detected with greater precision as demarcation potentials, since drift in the potential difference prior to treatment, *i.e.*, the baseline, can be much smaller or absent than when the injury potential is used.

B. Cautionary comments

1. Magnitudes of potentials. a. Multifiber preparations. Under moist-chamber conditions, injury potentials and demarcation potentials actually represent the product of the external resistance and the current flowing longitudinally outside the fibers. Since the external resistance is in series with the protoplasmic and membrane resistances, the proportion of the total membrane potential change actually detected is determined by the ratio of the external resistance to the total, chiefly the internal and external resistances. Obviously, application of hypertonic or hypotonic solutions, involving changes in either external or internal resistance as well as in membrane potential, will alter the magnitude of the potential changes; hence quantitative estimates require the use of suitable control solutions (319).

When experimental agents are locally applied, as frequently is the case in moist chamber experiments, slow augmentation of effects with time may be the consequence of longitudinal diffusion from the site of application. Conversely, when the regions adjacent to that on the electrode undergo change, their electrical behavior will be detected by the same electrode.

This can be seen when nerves are deprived of oxygen on either side of a region kept in solution. The segment in solution may undergo little electrical change (402, 413), but depolarizations are observed nevertheless due to the adjacent segments in the gas phase (434). The slow rise in potential seen when glucose is applied to segments of sciatic nerves with their intermediate regions in mercury (516), appears to be due to the lateral diffusion and utilization of the glucose to restore the membrane potential of the segments made anoxic by the mercury (402). The slow depolarizations seen with the application of KCl to sheathed nerves in a moist chamber (310) may be due in part to such lateral diffusion.

These limitations of the moist chamber technique can be circumvented by removing electrolyte from the interstitial spaces by a continuous flow between

electrodes of an electrolyte-free solution such as isotonic sucrose, as described by Stämpfli (456). By this means Straub (469) has been able to obtain injury potentials practically identical with the membrane potential of single medullated fibers as estimated by the potentiometric procedure (246). This will be referred to as the sucrose-gap technique.

b. Single fiber preparations. Measurements on single fibers depend chiefly on (a) a capillary electrode containing usually KCl (preferably 3 M) inserted into the axoplasm of larger fibers, and a suitable electrode system outside the preparation, or (b) two electrodes outside the smaller fibers, one on a normal region, the other on a segment treated with isotonic KCl. While estimates of E_m are improved by these procedures, neither can be considered to be without limitations.

1) Microelectrodes. There are at least four sources of error. While diffusion potentials are minimized by the high (3 M) KCl concentration in the internal electrode, they cannot be taken as zero even under optimum conditions since the mobilities of potassium and chloride are not identical, as originally believed (36, 328); the possibility of at least several millivolts error, in a direction reducing the measured potential, must be allowed for from this source, especially in vertebrates, where much of the internal negative charge probably is immobile. If this is assumed for vertebrate muscle, we can easily show that 3 M KCl will give a diffusion potential of 5 mV while 0.1 M KCl will produce 22 mV; the difference between these calculated values compares favorably with the 13.6–17.6 mV difference in E_m obtained for frog muscle using these solutions in microelectrodes (305).

Adrian (2) has called attention recently to the possibility of a much larger error resulting from exaggerated differences in K^+ and Cl^- mobility possibly brought about by charge developed on the glass of the microelectrode or by protoplasmic plugs at the electrode tip. Large variability in measured potentials, such as has been reported for giant axons (186) or for muscle fibers (437, 438), may be attributable to this; certainly, only until precautions are taken such as outlined by Adrian, preferably supplemented by studies of E_m values with different KCl concentrations in microelectrodes to evaluate diffusion potentials, can other factors be sought to account for apparent discrepancies from theoretical relationships such as will be discussed later.

Another source of variability is the extent of "sealing-in" of the electrode at the site of puncture. Where possible, this should be followed, as can be done by observing the rise in membrane resistance after entry of the microelectrode (83). In larger cells, the negligible change of E_m with the entry of a second microelectrode nearby has been considered an index of membrane integrity (344). Usually, simply the lack of change in membrane potential, or more usually in action potential, following the puncture by the single electrode used for measurement, has been considered adequate (305, 499). The last approach led to the important discovery by Ling and Gerard (305), confirmed by others (*e.g.*, 523) that, electrode tips below one micron in diameter, preferably not exceeding one-half, are satisfactory microelectrodes. However, their applicability particularly to small fibers is limited in two ways: (a) the size of the damaged region relative to surface area may be so large as to cause an appreciable shunt of E_m so that small fibers tend to give smaller measured membrane potentials (25, 27, 55, 145, 476, 499) and (b) leakage of electrolyte from the electrode into a small cell may alter the membrane potentials (51, 52). Some control of the latter is now possible by application of small potentials to the tips, a procedure still not in general use.

A fourth source of error in microelectrode determinations of E_m may be present under *in vitro* conditions, *viz.*, potassium accumulation around even single or superficial fibers because of leakage, so that $[K]_o$ at the surface of the fibers is somewhat larger than in the medium (158, 305, 306, 307). This should be checked by determining that rate of flow or stirring of solution does not alter the potential (*cf.* 366, 469).

2) External electrodes. Estimates of E_m with external electrodes depend on the abolition of membrane potential at one electrode with KCl. Regions so treated do not necessarily have zero membrane potential. Potassium concentrations within vertebrate cells may exceed that of isotonic KCl, while those of invertebrates are less (see Tables 1, p. 71 and 2, p. 72); moreover, the membrane may give rise to a KCl diffusion potential even near a cut end (434). In vertebrate nerve, isotonic KCl alone may cause irreversible damage over longer periods of time (209, 426), although not when KCl is added without removal of NaCl from Ringer (426); but even if KCl action were prolonged in single fiber studies to minimize membrane effects, the possibility of a diffusion potential in the axoplasm remains as an element of uncertainty.

A special aspect of single medullated fibers is their longitudinally heterogeneous structure resulting from the alternate succession of nodes and internodes. The special role played by the nodes, and the modifying influence of the heavily medullated internodes, have been summarized in several recent excellent reviews (455, 457, 477). It should be emphasized that in all studies of medullated nerve, including those of single nodes of Ranvier, substantial lengths of internode are necessarily involved in the techniques employed since the nodes are less than one micron long and the internodes at least 1000 times longer. And since the internodes can undergo changes, for example, in resistance with changes in the salinity of the medium (477) or with the addition of drugs (229, 478, 481), and in potential with KCl (246, 477), their contribution to the phenomena observed in most cases remains to be evaluated.

In conclusion, while studies with single fibers offer the best opportunity for measurements of E_m, allowance must be made for the several sources of error now recognized and for an element of uncertainty, in the neighborhood of mV, in the absolute values.

2. Diffusion limitations. The rate with which substances act on the nerve fibers depends in part on the speed with which they arrive at the susceptible locus. The exit of physiologically active cellular components (*e.g.*, potassium or lactate) may also contribute to observed phenomena if there is a restraint to their escape which causes accumulation around the fibers (5, 6, 158, 305, 409, 413, 415, 434); this is observed even with single fibers located at the very surface of a tissue unless good irrigation is provided (305, 307).

Passage through the interstitial region of nerve and muscle is governed not only by the usual factors of viscosity and electrochemical gradients but by the lengthening of the mean free path and the reduction of the effective diffusion area by the fibers themselves, acting as impervious rods (48, 200, 386). Calculations for tissues under *in vitro* conditions based on this model can describe most but not all of the entry or exit even of substances like sucrose or inulin, which seem not to penetrate the cells themselves (60, 256, 423, 428). These deviations probably arise from inhomogeneities within the tissue, such as (a) external or internal perineuria or perimysia which surround the nerve trunk or muscle and separate it into several bundles of fibers, (b) variation in the packing of the fibers which has the effect of creating a number of extracellular compartments, with different degrees of stasis, between contiguous fibers of each bundle, and (c) membranes surrounding individual cells, particularly in nerve fibers. The reality of the membranes separating the tissues into smaller bundles is indicated by experiments in which mineral oil, containing a suitable dye, was injected into toad sciatic nerves through their thick, superficial sheath; the oil persistently penetrated only part of the trunk diameter, and invariably moved longitudinally into only one of the two distal branches despite attempts to build up enough pressure to force it into the other branch (425). The probability of the second type of inhomogeneity is indicated by the time course of the loss of electrolyte from an agar-coated, multistranded wire; this

loss fails to become the single exponential process expected at later times (232), much as in nerve (428) and in muscle (256). Evidence that superficial membranes around individual fibers exert a resistance to diffusion is very limited; it will be described in Part II in connection with after-potentials.

The considerable furor created by a vehement denial that the peripheral connective tissue sheath, the perineurium-epineurium surrounding vertebrate nerve trunks, is a barrier to diffusion of ions and polar substances (310–313), has subsided with general agreement that the older evidence, demonstrating its interference with penetration (127), is valid. Establishment of this point was important inasmuch as most experiments are performed *in vitro* and deal with application of substances to, or their removal from, the surface of the trunk. Sheaths around nerve trunks of herbivorous insects, such as the locust, have been found to perform the important function of providing protection from the high potassium content and low sodium levels of hemolymph (239). Evidence for the diffusion resistance of the peripheral sheath of vertebrate nerve is as follows:

(a) Rates with which physiological effects are induced by experimental substances of polar character applied to the trunk are greatly increased upon removal of the sheath (68, 69, 130, 132, 283, 443) and reduced again upon return of the sheath (68, 132).

(b) Penetration of stains and of radioisotopes in the intact nerve trunk is governed largely by the sheath (298, 423); in the absence of the fibers, emergence of radioisotopes from the sheath exhibits the same kinetics as in the intact nerve (417).

(c) In spinal roots, where the thick sheath is absent, penetration is similar to that in desheathed nerves (132, 319); also, in invertebrate nerve the absence of dense connective tissue or epithelial-like layers (299) is associated with rapid penetration (*e.g.*, 434).

(d) Physiological changes by vascular perfusion of intact nerve with experimental agents are rapid and similar to those of desheathed preparations (282).

(e) The sheath exhibits the electrical properties of capacitance and high resistance to be expected of a highly impermeable structure and contributes to the electrical characteristics of peripheral nerve trunks (363, 373); these properties are weak or absent at spinal roots (318, 372).

(f) The effects of potassium accumulation during potassium leakage, as with anoxia (411, 414) or azide inhibition (146), are reduced by sheath removal, especially with good circulation of the medium (146, 425).

Whenever possible, this sheath must be routinely removed to facilitate penetration, for not only does it delay attainment of equilibrium distribution, but penetration may be so slow as to obscure effects readily demonstrable when the sheath is absent. Thus, the action of acidification of the medium in lowering excitability is readily shown in the absence of the sheath but is not evident when the sheath is present (58).

A difficulty with the removal of the sheath from the trunk for studies with frog nerve is the swelling which follows (313, 413, 416).

This involves the interstitial space, possibly the connective tissue, rather than swelling of and damage to the fibers, for the potassium content and functional activity of frog nerve remains intact (416), individual fibers do not show such swelling (159), and the diameter increase of the nerve is at a minimum at a pH of 5, which suggests the isoelectric point of connective tissue (350). In cat nerve, however, the weight gain following sheath removal is accompanied by substantial potassium loss (71). The pH effect merits further study in view

of the large variability of the data; preliminary studies in our laboratory indicated no marked dependence of Ringer uptake on pH. However, oncotic agents—bovine albumin and dextran–reduce the amplitude and rate of swelling (420). The viscosity of the solutions is an important element in swelling; strong concentrations of high-molecular-weight dextran (*e.g.*, 20%w/v, of Swedish "F" dextran, with an average molecular weight of 260,000) can prevent uptake of Ringer solution by desheathed frog nerve for three hours (420).

The negligible weight gain of the desheathed nerve of the giant tropical toad (*Bufo marinus*) in Ringer makes it the preparation of choice for studies involving both electrolytes and electrical measurements on trunks (418) and perhaps for single fibers as well. This species may also be preferable with the sucrose-gap procedure, for the weight gain of desheathed bullfrog nerve in isotonic sucrose (with calcium at the same concentration as in Ringer) is even faster and larger than in Ringer (420). On the other hand, if such swelling in sucrose involves only the interstitial region, it may have the advantage of facilitating the removal of the electrolytes.

Other alternatives are to use preparations in which diffusion is a less limiting factor, *e.g.*, spinal roots, invertebrate nerves, or, better still, single fibers, or to employ perfusion techniques. Each raises its own questions, such as the physiological state of the preparation, the adequacy of control of perfusion through the tissue, the consequences of non-uniform distribution of blood vessels (60), and the applicability of results from the invertebrates to vertebrates. A combination of techniques is undoubtedly the best approach.

C. Results

1. Ion effects and interacting ions and compounds. a. Potassium. 1) Effects. The early demonstration with crab nerve that, at lower potassium concentrations, increments in external potassium are relatively less effective than at higher concentrations in decreasing the injury potential, and at high concentrations E_i varies as the logarithm of external potassium (61), has been confirmed with solutions in which KCl is added without removal of sodium from the medium (434). The potassium-excess solutions were selected because KCl diffuses rapidly into the crab nerve fibers and therefore is not osmotically effective (212, 399); replacement of K for Na therefore can cause swelling of the fibers and irreversible damage (399). Similar E_m-$[K]_o$ curves have been obtained for desheathed frog and toad nerve, but with potassium (or rubidium) replacing sodium (131). This type of solution is necessary for frog nerve fibers because KCl does not penetrate at moderate (*i.e.*, ⅓ isotonic) concentrations (423); at isotonic concentrations entry occurs with damaging swelling (209, 423).

The same general relationship between E_m and extracellular potassium concentrations, $[K]_o$, has been obtained more recently with single medullated and invertebrate nerve fibers. These studies also provide absolute values for E_m, the most reliable of which are tabulated in Table 1. Except for crab fibers, which have appreciably higher membrane potentials, E_m for the nerve fibers lies between sixty and seventy mV. In frog (247), *Loligo* (70), and *Sepia* (226) axons the slope of the linear portion of the E_m-log $[K]_o$ curve is 50 mV for a ten-fold change in

TABLE 1

Electrical and potassium characteristics of excitable cells

Animal	Fiber	Reference	Diameter	R_i	R_o^*	R_m	C_m	E_m	$[K]_i$	$[K]_o$	E_K
			μ	Ωcm	Ωcm	Ωcm^2	$\mu F/cm^2$	mV	mM	mM	mV
Squid (*Loligo*)	Hind-most Stellar nerve	44	500	30	22	1,500	1.1	65, 68[1]	360[2]	10	91
		391		44.5	22.6						
Cuttlefish (*Sepia*)		509	200	63	22	9,200	1.2	62[3]	330–360[2]	10	88–91
		223		46							
Lobster (*Homarus*)	Leg nerve	228	75	61	22	2,300	1.3	62[2]			
Crab (*Carcinus*)	Leg nerve	217	30	90	22	7,700	1.1	82[4]	300[2]	10	86
		265	40	55	22	3,360	1.2				
Crab (*Portunas*)	Leg muscle	123	180	69	22	120	42	72			
Frog	Sciatic nerve	457		110	87						
	Node	478	8			8–20	3–7	71[5]	115[6]	2.5	97
	Internode	478	15			100,000	0.005				
		457	15			160,000	0.003				
	M. sartorius	120	100	250	87	4,000	6–8	92[7]	139[7]	2.5	94
		345	80	330	87	5,000	4.1	89[8]	153[8]	2.5	104
	M. adducter magnus	265	75	176	87	1,500	6.0				
	M. ext. longus IV	265	40	255	87	4,300	4.4				
Toad	Motoneuron	3				270	18	40–50			
Cat	Motoneuron	51	70		51	500	8	69			89
		154				1,000	1–1.5				
Calf, Sheep	Heart (Purkinje)	59	75	154	51	1,200	11	94	140[10]	2.7	105
Goat	Heart (Purkinje)	510	75	105	51	1,900	12	94[9]			

References: [1](70,158); [2](219); [3](226,509); [4](217,246); [5](246,491); [6](414,426); [7](2); [8](92); [9](96); [10](73, for ox heart A–V bundle, corrected for extracellular space of 15% as in mammalian ventricle, *cf*. 365,370).

* From 512.

potassium concentration—a result of theoretical importance since the maximum possible is 58 mV if potassium concentrations are solely responsible for the potential.

Studies with single fibers have also revealed the great rapidity of potassium depolarization. A technique particularly designed for rapid application of solutions to single nodes of Ranvier reveals completion of depolarization in one second (458).

In skeletal and heart muscle fibers microelectrode measurements reveal generally high membrane potentials, most often between 80 and 90 mV (Tables 1 and 2). Kuffler and Williams (288, 289) have called attention to the need to distinguish two types of muscle fibers, present in various proportions in different striated muscles, that have distinctive electrical, contractile (and probably ionic)

characteristics (30, 31). The "slow" tonus muscles, which are present in substantial numbers in M. rectus abdominis and in M. extensor longus IV, have significantly lower resting potentials (60 mV) than those of the "fast" twitch muscles (90 to 95 mV), which are in the majority in the skeletal muscles usually employed.

Exceptionally low values have been obtained for some heart fibers—from the cat auricle and frog ventricle—but since high values are also reported for these, technical factors, such as the nature of the solutions used, may be involved. Until electrodes are systematically checked for excessive diffusion potentials, (see Section B 1 above), high values are not necessarily more acceptable; for the time being, however, they are considered the more reliable.

We may note in Table 2 the low values of E_m found for smooth muscle; additional low values for other smooth muscles are given in (27) and (514).

The E-log $[K]_o$ curve of skeletal and heart muscle also resembles that of nerve (2, 28, 30, 162, 191, 239, 512); a complication in such determinations is that special precautions are necessary to assure a stationary state at the time of measurement (48) because KCl diffuses into muscle (18). Measurements under

TABLE 2

Comparison of in situ (i.s.) and in vitro (i.v.) potassium concentrations and electrical potentials in skeletal, heart, and smooth muscle

Reference	Animal	Fiber	$[K]_i$	$[K]_o$	E_K	E_m
			mM	*mM*	*mV*	*mV*
169	Rabbit (i.s.)	Skel. muscle	144	4.7	91	
500	Cat (i.s.)	Skel. muscle		4.8[1]		80
500	Guinea pig (i.s.)	Skel. muscle				85
169	Rabbit (i.s.)	Ventricle	107	4.7	83	
73	Ox (i.s.)	Ventricle	140	(12.5)*		
208, 365	Rat (i.s.)	Ventricle	140	4.5[2]	91	
208, 365	Rat (i.v., 6 hrs)	Ventricle	112	5-5.6	79-82	
57, 230, 499	Dog (i.s.)	Ventricle		4.4[3]		80-90
369, 370	Cat (i.s.)	Ventricle	151	4.8	91	
230, 499	Cat (i.s.)	Ventricle		4.8		81
396	Frog (i.s.)	Ventricle	137	3.6	92	
499	Frog (i.s.)	Ventricle		3.6		64
396	Frog (i.v.)	Ventricle	140	6	79	
284	Frog (i.v.)	Ventricle	62	2	87	
506	Frog (i.v.)	Ventricle	(62?)	1.9	(88)	85
523	Frog (i.v.)	Ventricle	(100?)	4	(81)	65
73	Ox (i.s.)	Rt. auricle	90	(12.5)*	(78)	
73	Ox (i.s.)	Lft. auricle	130	(12.5)*	(88)	
230	Cat (i.s.)	Auricle		4.8[1]		85
28	Cat (i.v.)	Auricle		5.6		60
72	Rat (i.s.)	Small intestine	193	4.5	100	
26	Guinea pig (i.v.)	Taenia coli		4.7		50
520	Rabbit (i.v.)	Uterus	98	2.7-4.7	81-95	45

* From shot animals; the value 4.8 for cattle (453) is probably more appropriate.
References: [1](369,370); [2](72); [3](453)

such conditions give a final slope for amphibian skeletal muscle approaching the theoretical 58 mV maximum—52 (2) and 57 (48) mV per ten-fold K_o^+ concentration change. In mammalian Purkinje fibers the slope is 45 mV (512), and only 40 mV in the cat auricular fibers with a low E_m (28).

2) Interactions. In whole crab nerve calcium depletion of the medium lowers the maximum injury potential which can be attained with low external potassium; this limiting effect, evident also with higher concentrations of calcium, can be counteracted at least in part by agitation of the solution in contact with the nerve, but only as long as agitation is continued (434)—a result suggesting that the potassium leakage, to be expected at low external potassium levels (137, 272, 409, 415), prevents the potassium level immediately surrounding the fibers from falling to as low a value as in the surrounding medium, thereby limiting the rise in potential as extracellular potassium is decreased. This has been observed to be the case even with single fibers (158). Hence attempts at theoretical deductions based on E_m changes at low $[K]_o$ should be preceded by an evaluation of the magnitude of the effect of such leakage on the measurements. Moreover, a depolarization induced by a given experimental condition must be checked for the involvement of increased potassium leakage and potassium accumulation around the fibers [see Section B 1 b 1)].

The effectiveness of intermediate concentrations of external potassium in causing depolarization can be reduced by high concentrations of multivalent ions (Ba > Sr > Ca) in crab nerve (189), in sheathed frog nerve (216), and in muscle (148, 153, 214), and enhanced by a decrease in the calcium concentration of the medium surrounding desheathed frog nerve bundles (468). In muscle eight-fold higher calcium maintains a 10 mV higher potential but does not prevent potassium depolarization (251). In the case of sheathed nerve the possibility that reduced sheath permeability is a factor requires examination.

Calcium has been classed as a "stabilizer" (19, 398, 402, 404) since, in common with this group of compounds, it produces little change in membrane potential or a slight hyperpolarization when added in excess (*e.g.*, 19, 61, 314, 406, 461, 469), yet markedly decreases *changes* in E_m brought about by potassium and by other experimental conditions (discussed below). In keeping with this, other stabilizers duplicate the action of calcium on potassium depolarization. This was observed many years ago by Höber (214) and again recently (153, 403, 410) in muscle. Straub (468) has now shown for medullated fibers that procaine and cocaine are effective in concentrations of 1–3 mM (0.03–0.08%); additional important observations by Straub are (a) partial reversal of potassium depolarization by procaine when applied after potassium has acted, (b) that the rapidity of the protective action of local anesthetics against potassium is as great as the potassium depolarization itself (equivalent to the diffusion time of 10 to 30 sec in the nerve bundles), and (c) the wearing off of procaine protection, as judged by the gradual increase of successive potassium depolarizations in the continued presence of procaine. He points out that observation (a) rules out the possibility of increased resistance to potassium diffusion by an external barrier around the membrane and that (b) indicates the anesthetics act at the fiber surface. Straub

also notes that procaine effectiveness against potassium is greater at pH 7 than at 9, and suggests that the cation form of the anesthetic may be involved.

These local anesthetics as well as antihistaminics have been shown earlier to reduce potassium depolarization in muscle (403, 410). Attempts at that time to demonstrate effects by local anesthetics on potassium depolarization in sheathed nerve were unsuccessful (405, 412), presumably because of diffusion limitations (468) as noted more recently for the action of pH change on rheobase (58, 348).

Physostigmine (eserine) (1–10 mM) and neostigmine (Prostigmin) (10 mM) also reduce potassium depolarizability in small frog nerve bundles (467). Tetraethylammonium at high concentrations acts similarly in spinal roots (317). Epinephrine is effective in muscle (352).

Anodal current, that is, current directed inward across the membrane, increases the membrane potential and exerts effects like those of stabilizers. In keeping with this, sheathed sciatics, depolarized in relatively strong KCl solutions, are repolarized under the anode (297, 310, 311), an effect observed even with isotonic KCl in contact with single fibers (459).

In frog spinal roots, KCl depolarization is slower in Ringer made twice hypertonic with NaCl than in Ringer made equally hypertonic with choline chloride or diethanol-dimethylammonium chloride (317). Considerably more hypertonicity with sucrose than with electrolytes is required to reduce the rate of depolarization of frog roots by potassium, but less suffices on the peripheral trunks (317). Desheathed, small nerve bundles also are depolarized more slowly by potassium in Ringer hypertonic with sucrose, and more rapidly in hypotonic Ringer; these effects develop gradually to a maximum (460).

The action of hypertonic solutions may reflect in part reduced rates of diffusion of potassium to the physiological membranes. Thus, after depolarization of roots in Ringer by potassium, excess NaCl does not reduce the depolarization (317). This negative result is surprising since the higher conductivity of the extracellular space should have reduced the measured depolarization; however, since excess NaCl will depolarize (317, 444), this may have balanced the conductivity effect. Monnier and his colleagues (339) have observed that inadequacy of the osmotic pressure of Ringer widens the gap at the node of Ranvier, where the predominant effect of potassium occurs, and that the addition of oncotic agents narrows it; such changes with hypertonic solutions could slow the action of applied potassium. Also, water removal from the sheathed nerve trunk (402) with hypertonic solutions substantially exceeds that from desheathed nerve (416) and from single fibers (209); this may be expected from the low diffusibility of polar substances through the sheath (417, 423), which would cause water removal from the interstitial space and hence tighter packing of the fibers than in the absence of the sheath. This may account for the need for stronger sucrose solutions at roots to slow potassium depolarization.

Depolarization by potassium may not only be depressed, it may be augmented as well. This occurs when the calcium content of the medium is reduced (461), when veratridine is present (469) or when the sodium level of the medium is reduced by replacement with choline (322).

b. Other small (hydrated) ions. 1) Rubidium, cesium, ammonium. In keeping with the older literature on intact nerve (215), rubidium depolarizes desheathed myelinated nerve less effectively than potassium (131). The same was reported for European crab leg nerve (61), but the reverse has been described for American spider crab preparations (516). Nerve and muscle are even less affected by

cesium (215), an observation of considerable theoretical importance, as is the finding for muscle that depolarization by NH_4^+ also is weak (163) (see Section VII).

2) Hydrogen and carbon dioxide. Medullated nerve fibers. The gas, CO_2, is included under ions because it probably acts at least in part by virtue of the H^+ it produces in solution. Its effects on membrane potential and excitability are the same as those of hydrogen ions except more marked (58, 348, 471). The greater effectiveness usually is attributed to better entry into the axoplasm, but a more marked uptake by the membrane itself should not be overlooked as a possibility. The exceptionally high lipid activity of CO_2 hydration products has been noted with emulsions (397).

Two obvious effects are produced by an elevation in H^+ and CO_2: (a) An initial rapid hyperpolarization (310, 402, 471), which is depressed in the absence of extracellular sodium (471), and (b) a slow secondary depolarization (93, 310, 402, 468), which in the case of CO_2 is suppressed by improved buffering (402). CO_2 hyperpolarization is accelerated and overshoots in low calcium but is slowed in elevated calcium (402); it is depressed or actually changed to a depolarization in blocking concentrations of cocaine and pyribenzamine (413).

H^+ acts as a stabilizer as well. Thus, in preparations in which a hyperpolarization effect is not apparent, it reduces K^+ depolarization as rapidly as the potassium action itself (93). It also reduces the depolarization of nerve bundles caused by the lowering of extracellular calcium (468).

Other fibers. CO_2 lowers the potential of crab nerve, an effect depressed by improved buffering (400). In muscle, too, a depolarizing action has been reported (*e.g.*, 305, 337). Excitability studies with arthropod nerves strongly suggest that depolarization results when membrane potential is high, and hyperpolarization when potential is low (14, 15, 296). This is in keeping with the hyperpolarizing effect of CO_2 in medullated fibers, where E_m is low, and its depolarizing action in muscle and crab nerve, which have higher values of E_m (Tables 1 and 2). However, E_m is probably secondary to another more basic factor in the polarity of the CO_2 effects, for pyribenzamine, which has a negligible effect on the membrane potential of medullated nerve (69, 413), nevertheless reverses the CO_2 effect (413).

c. Sodium. Reduction of the sodium content of the medium, for example by replacement with choline cation, augments the potential of small medullated nerve bundles (468, 469), of single myelinated fibers (206, 221, 247), and of muscle (344), an effect which is well maintained (459).

Excess sodium chloride (469), choline chloride (206), and sucrose (402), added to make the medium hypertonic, cause depolarization. Lowered sodium in spinal roots (322) and intact nerve trunks (425) also causes a depolarization, but since this represents a secondary decline (425), it is more likely due to an accumulation of potassium in the interstitial spaces which, by its depolarization effect, obscures the hyperpolarization by low sodium. This is to be expected from the leakage of potassium which occurs at low extracellular sodium levels (422).

As in the case of potassium, potential changes associated with changes in the sodium content of the medium can be depressed or enhanced. Thus, in small nerve bundles, veratridine (469) and reduction of the calcium content of the medium (461) augment the depolarizing action of sodium. Procaine and cocaine depress the hyperpolarizing effect of low sodium and the depolarizing action of excess sodium in the same preparations (468).

d. Lithium. In crab nerve this ion has little effect (61), but in spinal roots and small nerve bundles its replacement of sodium causes a hyperpolarization, which is augmented by lower temperatures (316) and by veratridine (469). The augmentation at low temperature may be due to the reduction of a secondary depolarization which occurs at higher temperatures (316).

e. Calcium. In general, elevation of the calcium level of the medium above normal produces either no change or a slight elevation in potential; a decrease in calcium usually lowers E_m or E_i. In whole medullated nerve, an increase in Ca^{++} to 17 mM causes only a hyperpolarization; at higher concentrations irreversible changes accompany a secondary depolarization (314). In small medullated nerve bundles calcium removal causes a depolarization of 8 to 10 mV (461, 469), but a preliminary report on single nodes of Ranvier indicates no potential change (206). Similarly, in giant axons calcium removal causes a small (5%) depolarization (424) or none (21), also noted in Purkinje fibers of the heart (499, 511). All agree, however, on a small or negligible hyperpolarization in excess calcium (206, 405, 469, 511).

Skeletal muscle potentials are more sensitive to calcium, an appreciable increase occurring in an excess of the divalent cation (249, 251) and a marked decrease in its absence (250, 332). In the former studies, E_m in Ringer was about 10 mV lower than found in the careful investigations of (2) (Table 1).

The absence of sodium in the medium can prevent the depolarization of medullated nerve bundles in low calcium, although at elevated potassium levels depolarization will occur even in the absence of external sodium (461); as already noted, at lower pH levels depolarization in low calcium is depressed (468). Depolarization by low calcium is prevented in squid axons by 0.9 mM cocaine and higher (424), and reduced in nerve bundles by as little as 0.05 mM procaine, an effect augmented at higher pH presumably because the effective form is the free base (468).

The initial depolarization of small medullated nerve bundles in low calcium solutions is slow compared to potassium depolarization, but successive alternation of Ringer and low calcium solutions speeds up the low calcium effect presumably by depleting a calcium reserve which delays calcium deprivation; repolarization upon return to normal calcium levels is as rapid as upon removal of excess potassium (461). Lüttgau (324) has also noted that the excitability characteristics of single nodes of Ranvier change progressively in citrate solutions over 15 to 20 min.

f. Anions. Early studies established that the bioelectrical effects of extracellular monovalent anions are negligible in vertebrate nerve as judged from the lack of changes in E_m in substitution experiments; crab nerve, however, shows

minor effects on replacement of the anions similar to muscle (516). The small increases in potential noted in muscle vary inversely with the degree of ion hydration (Table 3) and hence as SCN > NO_3 > I > Br > Cl (215, Chapt. 17). Höber (214) showed very early that this hyperpolarization is reduced by stabilizers (see Section VIII B6).

More recent research attempting to judge the role of anions has employed sulfate and phosphate as substitution anions; unfortunately, these anions may be expected to bind calcium since the solubility products are low. Consequently, reports of depolarization obtained with them in small nerve bundles and muscle (204, 469) must be regarded tentatively as the consequence of a lowering of the calcium in the medium. The lack of an effect by NO_3 replacement in small vertebrate nerve bundles (469) corroborates the older data for such tissue.

When the chloride ion is removed with sodium, *e.g.*, by substitution with glucose or sucrose, the polarization changes are not greatly different from those resulting from removal of sodium alone, *e.g.*, by replacement with choline chloride. This is indicated by studies with giant axons (221); in muscle, the hyperpolarization may be reduced (343, 344) indicating that chloride does contribute somewhat to E_m. However, in small bundles of medullated fibers a much larger hyperpolarization occurs and then gradually subsides (459, 469), as though chloride were originally depolarizing the membrane. In view of the substantial pH changes which can occur at interfaces when the electrolyte content of the bathing solution is very low, this may underlie the hyperpolarization, linked perhaps to permeability changes or to phase boundary effects. The high oxygen consumption of muscle in pure sucrose, which is greatly reduced by small quantities of many different ions, is consistent with this view, as is the early demonstration that small quantities of KCl greatly reduce the electrical effect of pure sucrose (133). The transitory nature of the hyperpolarization also is in keeping with these possibilities. Moreover, Tasaki (personal communication) reports that a large anion like glutamate can replace chloride with no obvious bioelectrical effect in vertebrate fibers.

Microinjection studies are discussed in Section III D 3 a.

2. Drug effects. a. Stabilizers. Agents categorized as stabilizers were originally defined as those which block the nerve or muscle impulse with small or negligible change of the resting potential (13, 33). The concept of "stabilization" was broadened as much as fifteen years ago with the recognition that it is associated with the reduction or prevention of *any* change in membrane potential (398, 403, 404). Thus, calcium, cocaine and procaine were shown to reduce anoxic depolarization of nerve, and the latter two to decrease potassium depolarization of muscle. Actually, as early as 1907 Höber (214) called attention to the reduced effectiveness of "negativizing" (KCl, RbCl, *etc.*) and "positivizing" salts (NaSCN, NaI, *etc.*) on muscle in the presence of narcotics (chloroform, phenyl carbamate, ethyl carbamate), which themselves do not affect membrane potential. This was extended by Okamata (352) to procaine and other drugs. We have seen in the preceding section that this concept of stabilization is indeed generally applicable to E_m changes by ions including CO_2. It will soon be discussed in

relation to depolarization by anoxia and "labilizers," and later with respect to more basic effects (Sections III D, IV, V). Let us consider first the direct effect of stabilizers on the resting potential.

A description of the effect of stabilizers on E_m must take careful account of the concentrations employed. Thus in vertebrate nerve, various carbamates (urethanes) (65), higher members of the alcohol series (13, 360), methylfluoracetate (17), and iodoacetamide (316), which hyperpolarize at lower, blocking concentrations, or antihistaminics, *e.g.*, Benadryl (diphenhydramine), Pyribenzamine (tripelennamine), Histadyl (methapyriline), and related but non-antihistaminic agents, *e.g.*, methadone (Amidon) (69, 413), which produce no detectable potential change at low, blocking concentrations, nevertheless all depolarize at higher concentrations (65, 69, 361). This effect with concentration has also been noted with ions, for example calcium and lithium, as already mentioned (314, 316). The depolarization frequently is only partly reversible (65, 69). It is not due to leakage and interstitial accumulation of potassium for it is accelerated by improved penetration of drugs in the absence of the peripheral sheath (69).

The frequent disagreements as to whether stabilizers do or do not produce a hyperpolarization in medullated nerve (*e.g.*, 13, 33, 413, 468, 477, 491a, 526) therefore are attributable (a) to too small a change in membrane potential to be detected by the technique employed or (b) to the use of excessive concentrations, at which the depolarization masks the hyperpolarization. The negligible potential change so often seen may well reflect a nice balance between two opposing processes—one hyperpolarizing, the other depolarizing—which are individually apparent with other drugs at appropriate concentrations or, as will be shown, under certain experimental conditions. The presence of two such processes is certainly indicated by the aliphatic alcohols; thus, the lower members only depolarize, the higher members hyperpolarize at low concentration and depolarize at higher concentrations, and an intermediate alcohol has no effect on membrane potential at low concentration (292, 360, 361).

Other drugs which act as stabilizers for a variety of depolarizing conditions in frog and rabbit nerve are (a) carbonic anhydrase inhibitors, including sulfanilamide, thiophene-2-sulfonamide (402), and Diamox (acetazoleamide) (349), (b) physostigmine sulfate (526), and (c) quinoline (67). (a) and (b) are of interest since their stabilizing action is probably quite independent of their "specific" enzymatic inhibitory properties. Thus, carbonic anhydrase is not present in detectable quantity in vertebrate nerve (349) and anoxic depolarization also is affected (402). A small depolarization accompanies physostigmine sulfate application (309, 526); however, the possibility of removal of ionized calcium through complex formation with the sulfate requires examination since the calcium sulfate solubility product was approached. In invertebrate fibers quinoline causes repetitive firing (179) rather than block, an effect which may be related to an important difference in the response of muscle and invertebrate nerve fibers to the stabilizers.

Muscle and invertebrate (especially crab) nerve are rather similar to each

other and differ from vertebrate nerve in that there is either no potential change or only a decrease upon application of stabilizers, especially at higher concentrations, never a hyperpolarization. This has been described for cocaine applied to squid axons (405) and to crab leg nerve (406) and for a variety of stabilizers (local anesthetics, antihistaminics), including CO_2, in skeletal muscle (151, 214, 410, 488) and in heart fibers (254, 507, 511).

This difference is probably related to the higher values of E_m which were noted to be characteristic of muscle and crab nerve fibers (Tables 1 and 2). In keeping with this are (a) the hyperpolarization of muscle from 60-day starved animals by 1.1 mM procaine, which normally causes a depolarization (251), (b) the change of the CO_2 hyperpolarization of frog nerve to a depolarization by a hyperpolarizing local anesthetic such as cocaine (413), (c) the indirect observations on arthropod nerves, already mentioned, that suggest that the polarization response to CO_2 depends similarly on membrane potential (14, 15, 296), and (d) the alteration of the direction of the polarization changes induced by procaine in medullated nerve as a result of changing the initial level of polarization electrically (470). We have already noted, however, that a higher initial membrane potential does not accompany the reversal of CO_2 action in frog nerve by pyribenzamine (413); moreover, most stabilizers have only a small effect on E_m. Hence, we may conclude that while elevation of E_m induces changes comparable to those brought about by stabilizers, the membrane potential changes which accompany stabilizer action are usually only incidental to and reflect other more basic changes, changes which will soon be related to the permeability characteristics of the cell membranes. The stabilizing action of electrical hyperpolarization against a variety of depolarizing conditions has been described in a number of studies on nerve and muscle (147, 148, 149, 151, 153, 297, 310, 459), as has that of CO_2 (291, 293, 310, 338, 348, 402). In the case of the latter, a stabilizing effect independent of the increase in E_m is likely in view of the action of H^+ in depressing potassium depolarization [Section III C 1 b 2)].

Low temperature and low extracellular sodium concentrations produce effects similar to the stabilizers. Thus, the former prevents or counteracts depolarization of spinal roots by veratrine and by anoxia and the secondary depolarization following the initial hyperpolarization produced by CO_2, iodoacetamide (316) amyl carbamate (66), and replacement of sodium by lithium (316); as a result, the hyperpolarization phases, when present, are augmented. The effect on potassium and rubidium depolarization is slight, however (316). Low sodium reduces or prevents the polarization changes produced by veratridine (469), low calcium (461), and CO_2 (471) in medullated nerve, and by anoxia in vertebrate (322) and invertebrate (407) nerve; unlike the stabilizers, however, it accentuates potassium depolarization (317).

b. "Labilizers." A term is required to refer to the action of experimental agents which is the reverse of that of the stabilizers, namely, the accentuation of electrical changes. "Unstabilizer" has been employed (404, 413), but "labilizer" seems preferable and will be used henceforth with suitable modifications to conform to different parts of speech.

It has long been recognized that removal of calcium from the medium, especially when supplemented by calcium precipitants or complexing agents, renders nerve and muscle hyperirritable (*e.g.*, 19, 324, 338). Recently attention has been called to other effects by low calcium which are the reverse of stabilizers, *viz.*, accentuation of anoxic depolarization (402, 403, 413), of CO_2 hyperpolarization (348, 413), and of potassium and of sodium depolarization (461). It has already been pointed out that depolarization may or may not occur, in contrast with the hyperpolarization which may or may not occur with stabilizers. Obviously, experiments which may change the calcium content of the medium (by addition of Ca-complexing agents, such as $SO_4^=$, HCO_3^-, $PO_4^=$, of organic phosphates like adenosine triphosphate, or by changes in pH of solutions containing such complexing agents) must be carefully controlled.

The spontaneous repetitive activity which occurs in the complete absence of extracellular calcium no doubt contributes to accentuation of anoxic depolarization (125); however, the latter and the other effects of low calcium are still apparent when calcium concentration is reduced to only half (413), at which concentration no repetitive activity occurs (21). Moreover, veratrine, now to be discussed, acts like low calcium although it is known not to cause spontaneous activity (*e.g.*, 405, 406, 413).

Alkaloids derived from veratrine, and probably other veratrum derivatives, but not all—*e.g.*, veratramine (281), which resembles quinine and quinidine instead—exhibit the properties of labilizers. Thus, in veratrine concentrations too low to affect appreciably the membrane potential, frog nerve (413) and crab nerve (406) depolarize more rapidly in the absence of oxygen; also, in CO_2, veratrine makes frog nerve hyperpolarize with an overshoot, as does lowered calcium (413). Depolarization by potassium is enhanced by veratridine (469), an effect which may account for the increased sensitivity of rectus abdominis muscle to potassium in the presence of related drugs (175). At higher concentrations, veratrine depolarizes whole vertebrate nerve (310, 413), invertebrate nerve (188), and striated muscle (153) and veratridine small medullated nerve bundles (469), an effect prevented or reversed by stabilizers such as procaine, cocaine (153, 413), calcium (153, 469), barium (188), CO_2 (310), low temperature (316), by removal of sodium from the medium (469), and by electrical hyperpolarization (153, 310).

It has been concluded from the stoichiometric relation of the antagonism between calcium and veratrine (and other compounds) on repetitive activity that calcium is displaced from the cell surface (*e.g.*, 179, 513). However, veratrine still markedly accentuates anoxic depolarization of medullated fibers after long periods of exposure to strong solutions of a calcium precipitant such as phosphate; on the other hand, the alteration of CO_2 hyperpolarization by veratrine disappears (413). The effect of veratrine on CO_2 action therefore appears to be dependent on the presence of calcium in the medium which maintains a calcification of the membrane surface that is reduced by veratrine, while that on anoxic depolarization involves an additional factor.

It should be noted that the agents discussed as labilizers have a demonstrable effect on the changes in membrane potential produced in the resting cell by a

variety of experimental agents. Labilizers are to be distinguished from compounds which cause repetitive activity without involving the properties of the resting cell [*e.g.*, insecticides such as DDT (1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)-ethane); see 406, 413], a phenomenon which will be discussed in Part II.

3. Metabolic factors. a. Inhibition. 1) Nerve. Under moist chamber conditions vertebrate and invertebrate nerves undergo depolarization with deprivation of oxygen, interference with oxygen utilization, or through action of glycolytic inhibitors (*e.g.*, 310, 406, 431, 525). Glucose utilization under anaerobic conditions initially delays anoxic depolarization in frog nerve, an effect prevented by iodoacetate, which itself accentuates anoxic decline (402, 406, 413); prolonged anoxia in glucose eventually leads to faster depolarization (310). Inhibition by iodoacetate alone is delayed by pyruvate and lactate; that by fluoride by pyruvate only. Pyruvate and lactate are ineffective as substrates under anaerobic conditions (402). Cat spinal roots require glucose even in oxygen to maintain the resting potential (322).

In nerve, metabolic inhibitors and various substrates have the same effects on metabolic intermediaries and oxygen consumption as in other systems (112, 164, 165, 166, 236, 390). These effects appear to be related to the bioelectrical data from the general standpoint that the membrane potential is at least partly dependent on continuous energy turnover, a view in accord with the parallelism between heat production and membrane potential during anoxia and return to oxygen (126). In keeping with this, Wright (524) finds for nerves from a wide range of animal species that sensitivity of the polarization level to oxygen lack is proportional to the resting level of oxygen consumption, a phenomenon also observed with change in temperature. A similar conclusion may be drawn for calcium precipitants and for excess calcium, which augment or depress oxygen consumption (19, 21). Less sensitive preparations may make greater use of anaerobic processes—as will be pointed out for muscle—or may be less dependent on metabolic turnover because of mechanisms which reduce the leakiness of the fibers to ions, a matter discussed later.

It must be emphasized that the studies which have just been described were conducted under moist chamber conditions, with an epineurium present in the case of vertebrate nerves. Since potassium leaks from the fibers during inhibition (see Section IV B 4), obviously the change in E_m is at least partly dependent on potassium accumulation in the interstitial spaces during such experiments. However, a comparison of the E_m changes with well washed and unwashed fibers offers a means of separating the potassium effect from any other which may underlie changes in E_m with anoxia. Recent studies with desheathed toad nerve reveal E_m changes with anoxia half as great as in the moist chamber (425).

Development of a slight depolarization in dinitrophenol has been seen in small, washed vertebrate nerve bundles (93, 469); this effect is obtained only at lower pH (*e.g.*, pH 6), apparently because penetration of the inhibitor requires the lower pH (93), an effect also noted for invertebrate fibers (34). In keeping with the role of potassium accumulation discussed above, intact trunks of C fibers depolarize more in 0.1 mM dinitrophenol, cyanide, iodoacetate, and

azide than small, sheath-free bundles composed of these fibers; also, the same observers note that 15 μM ouabain causes depolarization of whole trunks but not of small bundles of C fibers (366).

While a depolarization of vertebrate nerve fibers independent of potassium accumulation in the extracellular space may occur with inhibition, available evidence indicates this does not occur in giant invertebrate axons. Thus, washing of squid axons during anoxia completely restores functional activity over long periods of time (412). Direct measurements of E_m in Sepia giant fibers, during exposure to strongly inhibitory concentrations of cyanide, dinitrophenol, and azide, revealed no significant change (224, 225).

Stabilizers mimic the action of glucose in slowing anoxic depolarization, but differ from it in exerting many other effects (*e.g.*, conduction block, lowered excitability, modification of CO_2 action on membrane potential) as well as in exerting protection against an inhibitor such as iodoacetate rather than being blocked by it (413, 414). Stabilizing agents and experimental conditions which delay anoxic depolarization under moist chamber conditions are barium (314), calcium (310, 398), magnesium (308), low external sodium (308, 322, 407), low temperature (316), physostigmine (308, 526), carbonic anhydrase inhibitors like sulfanilamide and thiophene-2-sulfonamide (402), local anesthetics and antihistaminics (403, 413), yohimbine (413), and CO_2 (291, 293, 310). With washed desheathed nerve cocaine and low sodium nearly obliterate anoxic depolarization (425).

Labilizers (low calcium, calcium-complexing agents, veratrum alkaloids) superficially resemble the glycolytic inhibitors in accelerating anoxic depolarization, but again differ from the inhibitors in inducing repetitive firing, in modifying CO_2 action on E_m, and in not impairing recovery following anoxia (405, 406, 411, 413, 431). Moreover, the antagonism of stabilizers to depolarization by higher concentrations of labilizers can be complete whereas the antagonism to depolarization by metabolic inhibition is only partial (413). Consequently the mechanism of action of stabilizers and labilizers is unlikely to be that of substrates and inhibitors, a conclusion repeatedly pointed to in many studies (33, 241). Rather the mechanism must be one which is common to other functional aspects of excitable cells, an electrochemical one to be discussed shortly. In keeping with this view, no good evidence is at hand for metabolic influences by these agents related to their effects. Thus, at pharmacologically active concentrations stabilizers generally lack a demonstrable effect on respiration (thermal measurements would be more crucial) (33, 439) while labilizers either augment it slightly (39, 387) or have no influence but accelerate it at high concentration (364).

2) *Muscle*. In frog skeletal muscle anoxia and cyanide poisoning cause only a slow, small fall in E_m; iodoacetate substantially accentuates the decline. Here, too, flow of solution past the impaled surface fibers is necessary to prevent accentuation of depolarization, probably because of potassium accumulation (306). The depolarization of muscle by metabolic inhibitors is slowed by a lowering of temperature (306), as in nerve.

Ischemia of mammalian muscle leads to immediate depolarization (500), but potassium accumulation in the extracellular spaces may have been involved under these conditions.

The depolarization of heart muscle is delayed during anoxia (496, 507, 508).

b. Recovery from inhibition. In both vertebrate and invertebrate nerve, under moist chamber conditions, return to oxygen after anoxia causes a larger increase in potential than the decline during anaerobiosis (310, 431, 434). This overshooting is larger in mammalian than in amphibian nerve (322). It is depressed by low sodium (322), by inhibitors (431) and by conditions which reduce the anoxic decline (413); conversely, increased anoxic decline augments overshooting except when inhibitors hasten anaerobic depolarization (413). The possibility of accounting for the overshooting in terms of potassium depletion in the extracellular spaces has been pointed out (411). According to this, the secondary decline in potential following the maximum repolarization would be due to entry of potassium into the extracellular spaces from the external medium or to secondary leakage from the fibers; reduction of the potassium level of the medium does reduce the secondary fall in potential during recovery (322). Repolarization of skeletal muscle following inhibition by cold (92, 466) and in papillary muscle of the heart following anoxia (496) has been observed but is not as striking as in nerve.

D. *Hypothetical considerations*

1. The cell as a 3-phase system. The approach most likely to prove substantial is that expressed in the simplest terms consistent with cellular structure and composition and with the observed phenomena. The cell cannot be divorced from its environment, with which it forms a 3-phase system—an intracellular and extracellular phase and the intervening membrane.

a. Extracellular phase. This is best known with respect to ionic composition, particularly under *in vitro* conditions with single cells. In whole tissues, especially *in situ*, the complexities (Donnan equilibria, binding, *etc.*) increase; these have been summarized by Manery (329) and noted more recently for mammalian (71, 283) and amphibian (427, 428) nerve and for heart (208, 370). Such difficulties contribute to the uncertainty of estimating the ionic content of the protoplasm; the error for potassium, which is predominant in the cells, is much less, of course, than for sodium and chloride, which are present there in small amounts although in high concentrations extracellularly. Potassium concentrations in plasma, which are somewhat larger than in the extracellular spaces *in situ* (329), and those in various bathing media which have been employed *in vitro*, are listed in Tables 1 and 2. Sodium and chloride data are given in detail in the references in this and the immediately following section.

b. Intracellular phase. The "effective" concentrations of the free ions in the protoplasm—more properly known as the thermodynamic activities of the ions —must be known with considerable exactness to determine the validity of electrochemical models proposed to account for the resting potential. This involves (a) distinguishing the ions in the extracellular spaces and in the sheath-

ing elements, perhaps in the membrane itself, from those in the protoplasm, and (b) determining the thermodynamic activity of the ions in the protoplasm.

With respect to (a), the situation is simplest for giant axons, where the protoplasm can be extruded from the fibers (*e.g.*, 10, 374, 464, 465), but this is by no means a panacea. Thus few have taken the precaution of pretreating the sheaths with low electrolyte solutions to minimize contamination, particularly by sodium and chloride, during extrusion. Moreover, in many early measurements the ends of the axons, where substantial interchange of sodium and potassium as well as gain of chloride occur (414, 429), contributed appreciably to the results. And, finally, it must be remembered that the large squid fibers have many branches, which must be cut, through which some exchange can occur; this can be kept small by leaving sufficiently long branches, although better still, work carried on with cuttlefish (*Sepia*) fibers, which lack branches, eliminates this particular problem.

In more complex systems, such as whole nerve and muscle, the peculiarities of the extracellular spaces with respect to volume, ion content, binding, *etc.*, must be defined with maximum precision by the use of compounds that remain exclusively extracellular and by a study of the kinetics of entry and exit of these compounds and of the ions of interest. Until kinetics data are available, simple analyses cannot be considered an adequate test of current hypotheses (*e.g.*, 437, 438). Available evidence for the binding of sodium, calcium, and chloride in the extracellular spaces, especially by connective tissue (201, 205, 283, 330), certainly dictates caution in interpretations based solely on direct analytical results.

Uncertainty also arises from the inhomogeneity of tissue with respect to the cells present. Thus, skeletal muscles differ in the proportions of their "fast" and "slow" fibers (288) and nerve trunks with respect to their A, B, and C fibers; hence, if one fiber type should have a high sodium content, this would ordinarily be averaged for all the fibers. Information is needed on electrolyte distribution in tissues composed predominantly of the fibers whose presence hitherto has been neglected in the commonly studied preparations—*e.g.*, abdominal muscle for slow fibers, autonomic nerves for small axons. One systematic comparison of sodium and potassium in various parts of the heart (*e.g.*, Table 2, p. 72) reveals interesting differences (73); although the significance of the findings is limited by the absence of data on the extracellular spaces, the presence of real intracellular differences is quite likely. Obviously, the application of analytical data from one part of the heart to another is certain to involve some error.

A final element of uncertainty in the determination of the ions actually contained in the protoplasm is the inhomogeneity of individual cells—the mitochondria, many nuclei, and perhaps striations in muscle and the myelin sheaths in nerve. Myelin appears to be part of the extracellular space (427, 428). The organelles (nuclei, mitochondria) must be considered suspect as a possible basis for the dual kinetics—a faster and slower component—of potassium entry and exit in muscle. This kinetics has been interpreted as the consequence of a free and bound fraction of potassium (197, 198, 205). However, the membranes of mitochondria and nuclei may enclose potassium at essentially the same concentration as in the protoplasm but function as additional barriers. A high sensitivity of such membranes to temperature could account for the accentuation of the 2-stage kinetics at low temperature (197, 198, 200, 334).

The technical difficulties which have been outlined by no means obscure the basically different distribution of ions inside and outside cells which has been so long known. Data for potassium, based on the best available corrections and given in Tables 1, p. 71 and 2, p. 72, are seen to be consistently high for the cell interior. Sodium and chloride concentrations are usually low in the cells. Further details, especially with respect to sodium and chloride, will be found in the references in the text and in the tables and in 48, 200, 329, 512.

The concentration figures for potassium in Tables 1 and 2, and most estimates of intracellular concentrations of sodium and chloride, are based on the assumption that the ions are completely dissociated in the water of the protoplasm as they are in ordinary aqueous solutions. However, chloride binding by blood protein is known and its occurrence within cells is likely (*e.g.*, 276). Multivalent cations like calcium have long been known to be bound by protein and lipid; consequently one is not surprised at its absence as the free ion in squid protoplasm (37), at its indiffusibility when injected (227), at its anomalous transport in the electric field because of binding to a protoplasmic anion in mammalian nerve (452), nor at other available evidence of binding within the cell (171, 201, 490).

On the other hand, the case for binding of sodium and potassium may be considered questionable. It is certainly not easily demonstrable as for calcium and chloride. Hill's early data on the vapor pressure of muscle (210) and recent findings on diffusibility and mobility of potassium within giant axons and muscle (199, 223, 227) and on the emergence of radioactive sodium injected into giant fibers, in contrast to the behavior of calcium (237), point to negligible binding of the monovalent cations. In Table 1 the specific resistance of protoplasm can be seen to range from twice to three times that of the medium. Although this seems high, it must be remembered that organic anions, especially in vertebrates, are not likely to be free to migrate, hence intracellular resistance will be at least twice that of the medium; since cellular volume is generally taken on a geometrical rather than on an aqueous basis, this will further increase the value; and, finally, the protoplasmic viscosity is likely to exceed that of water, which would further raise protoplasmic resistance.

Available measurements therefore are consistent with the existence of the monovalent cations largely in unbound form. The complete or nearly complete exchangeability of intracellular ions for radioisotopes—observed for potassium in invertebrate nerve (272), for chloride, sodium, and potassium in amphibian nerve (425–427), and for potassium in some amphibian (269) and mammalian (35, 64) muscle—is in keeping with this. However, it by no means constitutes final evidence of free ionization because binding is not usually the poorly reversible process (276) seen in the case of potassium in *Escherichia coli* (62, 367) or of calcium generally.

In addition to high potassium and low sodium and chloride concentrations, axoplasm and myoplasm are characterized by a substantial deficit of intracellular inorganic anions relative to the cations (*e.g.*, 10, 135, 137, 138, 329, 462, 465). Among invertebrate nerves much of the anion deficit is made up of organic anions, chiefly aspartate in lobster and crab fibers, isethionate (2-OH-ethanesulfonate) and aspartate in squid (277, 303, 440, 441); in muscle the organic phosphates represent a major fraction of the anion deficit (18, 304); and in vertebrate nerve the anion deficit is met largely by structural components (441).

Since the organic anions composing the deficit are largely indiffusible, the conditions for a Donnan equilibrium are present and diffusible cations will greatly exceed diffusible anions, in keeping with the situation as described for

skeletal muscle (18, 48). A major difference from the simple Donnan equilibrium lies in the predominance of potassium over sodium within the cell (brought about by work processes to be described later). Because of this there is a continuous leak of potassium outward, and of sodium inward, which gives rise to a membrane potential not quite equal to the equilibrium value for potassium (see Section 3 below). The situation is only slightly modified in systems such as vertebrate nerve, where anion penetrability may be low.

Muscle is outstanding in the lability of its diffusible anions. Important shifts in potassium may be expected to take place which depend on the rise and fall of the organic phosphates; this may not involve extracellular sodium, but rather exchange with hydrogen ions or diffusion with an anion (*e.g.*, lactate or phosphate) (304, 306). The organic anions of invertebrates also may function as a labile pool of indiffusible ions (277, 303). In small cells, *e.g.*, smooth muscle, shifts of this kind could bring about rapid concentration changes by virtue of the high surface to volume ratio. This may be expected to contribute to observed electrical changes and will be discussed later.

c. The membrane. The delicacy of this phase apparently requires one or more relatively rigid structures surrounding it which occasionally are mistaken for the membrane—in muscle the sarcolemma, which is substantially thicker than the estimates of the physiological membrane (7), and in nerve the myelin layer or layers, Schwann cells, and connective tissue sheaths (11, 90, 91, 168, 356, 388, 527). Investigations of the giant axons of cephalopods show that much equipment and disturbance can be tolerated in the axoplasm for hours, but functional derangement immediately follows scraping of the inner surface.

Electrical measurements with direct and alternating current have demonstrated that the physiologically active surface of excitable cells is a relatively high resistance to current flow, of 10 to 10,000 ohms for 1 cm^2, shunted by a capacitance, ranging from 1 to 40 microfarads/cm^2 (Table 1, p. 71), which represents the ion-impermeable portion of the membrane.

That the resistance is an attribute chiefly of the physiological membrane rather than of the supporting structures is demonstrated by its large decline with excitation in nerve and muscle (43, 120, 264, 393, 480, 481). The capacitance allows high-frequency currents to bypass the membrane resistance, and hence it necessarily is in parallel with the resistance (44); yet it undergoes no appreciable change with excitation (43), with temperature (88, 344, 478, 482), nor with hydrostatic pressure (454), and consequently it is a relatively inert component which, as will soon be seen, probably represents the major structural expanse of the membrane.

The absolute magnitudes of resistance and capacitance of the membrane were early shown to provide estimates of membrane thickness. In the case of a membrane predominantly composed of large fatty molecules (dielectric constant 3), or of smaller molecules, for example guaiacol (dielectric constant 12) 1 microfarad/cm^2 represents a thickness of 30 to 150 Å ($0.3–1.5 \times 10^{-6}$ cm or 0.003–0.015 μ); similar values have been obtained for a variety of other cells by this and other techniques (76, Chapt. 15; 215, Chapt. 15).

The myelin lamellae which surround each internode of myelinated fibers in

"jelly-roll" fashion (originally assumed to be concentric), while not part of the active membrane which occurs only at the node of Ranvier, nevertheless each represents a double cellular membrane derived from the investing Schwann cell (167, 368). The relation of these membranes to physiological membranes is further emphasized by their similar dimensions (140–142), by their comparable resistance, capacitance, and dielectric constant (219), by their similar increase in resistance in local anesthetics (477, 478), and by their depolarization in KCl (246, 477). It is of interest that X-ray and polarized light studies (141–144, 388) assign a structure to the 85.5 Å half-section of a lamella that is essentially the same as inferred for the cell membrane from early physiological studies, *viz.*, a double-layer of lipid molecules, radially (or normally) oriented, perhaps bounded at each aqueous surface by a layer of protein (76). If we accept 100 Å as the approximate thickness of the physiological membrane, it is still large on the ionic scale, being about 50 potassium ions thick (see Table 3, p. 135). The inner and outer faces of the membrane may be expected to differ in their properties, if not composition, by virtue of the different surface-active components in the intracellular and extracellular phases. That they really are different in myelin is indicated by low-angle X-ray spectra, which show that the two membrane layers forming each lamella actually constitute a distinctive unit of 171 Å thick (141, 389), as might be expected from the observations indicating that each myelin lamella represents 2 Schwann cell membranes deposited with the originally outer surfaces in contact, the inner surfaces directed outward. Other data, of a physiological nature, also suggest this difference and will be mentioned later.

In addition to inhomogeneity in a direction perpendicular to the surface, we may note the possibility of inhomogeneity laterally. It has already been pointed out that excitation (in the squid axon) occurs with a negligible change in capacitance, so that the ion-impermeable surface may be regarded as essentially constant. It is interesting to compute from resting and excited membrane resistances the minimum proportion of the surface area which might be occupied by channels containing electrolyte at, say, the concentration of the medium, to see whether this would be small relative to the total. For squid axon membrane, containing sea water with a specific resistance of 20 ohm cm, the channel area would be 2×10^{-6}% of the total at rest and 8×10^{-5}% when excited; for the node of Ranvier, which has a much lower resistance (Table 1, p. 71), the corresponding proportions would still be 10^{-3}% and 4×10^{-2}%, all more than small enough to account for constancy of the capacitance.

If the channel resistance at rest is greater than given by the specific resistance of the surrounding media, more of the surface area will be proportionately occupied by the channels; this resistance can be at least 1000 times higher without involving more than 1% of the physiological membrane at the node of Ranvier, and substantially higher still for squid axon. The resistance change of activity could now involve a decrease of resistance of such pre-existing channels rather than the increase in the number of low resistance channels implied by the previous calculation.

The distance between such hypothetical channels may now be estimated

roughly. If we take the average channel diameter as 10 Å—about double that of the smaller hydrated ions (Table 3, p. 135)—and its specific resistance as comparable to that of the medium, it would be separated from its neighbors by 100,000 Å in squid axon and 3000 Å at the node of Ranvier. Channels with 1000 times this resistance would still be separated by 2000 Å and 100 Å, respectively. These are still large dimensions on a molecular scale. As we shall see later, channel diameters, at least at rest, are probably closer to 3 Å. Similar calculations are described by Tobias (490a).

Whether such channels represent pores, faults, or intermolecular spaces in an otherwise relatively homogeneous membrane, or further specialized regions, will be considered later when more data are at our disposal. Here we may note that the channels and interchannel regions may be expected to show a variability consistent with the semi-rigid, semi-fluid characteristics of cell membranes. The rigidity is reflected, for example, by the resistance of myelin to manipulation or injection (91, 356) and by the low surface tension in cells where this is measurable (76). Fluidity is indicated by the reversible deformability of myelin by manipulation (91) and by dehydration (141), as well as by the "snapping in" of oil drops in desheathed egg cells (38, 278). Hence, the lateral molecular forces in the membrane may be looked upon to be such as to create a "smectic mixed fluid-crystalline" structure, as described by Schmitt and his collaborators (388) and in keeping with theoretical considerations (76). Such a structure would allow entry of relatively large molecules by virtue of their solubility, *i.e.*, their ability to displace laterally the membrane molecules by intermolecular attractions, as observed by Skou (445, 448) on the uptake of local anesthetics by whole nerve and by myelin films. This need not involve entry into the channels through which the ions move. Such channels are probably best looked upon as having a certain lability with respect to size and perhaps charge as well—a lability dependent on the balance between thermal (kinetic, vibrational) and attractive and dispersive intermolecular forces; the latter in turn are susceptible to the surface-active components present in the aqueous phase bathing the membrane. A generalized approach to the thermal characteristics of penetration which would follow from some of these properties has been described and utilized (76, 114, 357), but cannot be discussed here. We should note, however, that this view of the membrane, which will be developed in greater detail in Section VII, provides for penetration of large molecules by virtue of their solubility in the region between channels, and passage of ions through small channels; this is consistent with many of the permeability characteristics described by classical physiology (76, 215).

At this point in the presentation no commitment is necessary concerning the detailed mechanism of penetration of ions into and through the membrane. Instead, we may lump the one or more steps involved by expressing the ability of ions to penetrate in terms of their transference numbers, *i.e.*, their relative ability to carry electric current (96, 275, 451), or in terms of their permeability coefficients, *i.e.*, the net effect of their relative uptake at the interfaces of the membrane (the distribution coefficients) and their relative mobilities in the membrane (177, 221). Permeability is the more familiar concept in biology and

closer to the measurements actually made in living systems, and consequently it will be employed in this review.

2. Implication of the membrane. a. Ion effects. In addition to providing magnitudes of depolarization which are close to the absolute values, modern techniques were pointed out in Section C above to have reduced diffusion limitations to the point that one may state unequivocally that the electrical effects of ions applied to the surface are extremely rapid and in keeping with surface action. The same conclusion must be drawn from earlier observations. Thus, the depolarization of myelinated nerve by potassium occurs without entry of potassium into the fibers when concentrations are not too high (426); statements to the effect that potassium entry into vertebrate nerve fibers is required for depolarization (129, 310) have been shown to be erroneous (426). In invertebrate nerve and in muscle, although potassium (and chloride) entry accompanies depolarization (3, 18, 399), depolarization is completed much sooner (2, 403, 434), again as may be expected from a surface effect. Moreover, in these preparations potassium entry may occur without appreciable change in internal potassium concentration (due to the water uptake by the fibers when potassium replaces sodium in the medium); nevertheless, the depolarization obtained is about the same or larger than when the potassium concentration of the fibers increases (2, 434).

b. Drugs. By virtue of the alteration of the effectiveness of cations as depolarizing agents by labilizers and stabilizers, the latter also may be considered to act at the cell surface. This is further verified by the rapidity of action of local anesthetics (468), of hydrogen ions (93), and of calcium ions (323) which is comparable to that of potassium. Labilizers, on the other hand, generally act more slowly—*e.g.*, calcium precipitants and low calcium (209, 324, 461) and veratridine (467). Stämpfli and Nishie point out in the case of lowered calcium that the rate of depolarization is slow with the first deprivation of calcium, but repolarization upon restoration of calcium, and depolarization with succeeding depletions of calcium, are much faster and comparable to rates with potassium (461); consequently, a surface effect still is the likely situation. The initial delay is attributable to a cellular reserve of ionic calcium which must be removed; the amount of this reserve is probably small compared to the total fiber calcium, which is largely bound (227, 452, 490). The slower action of veratridine, which persists for succeeding applications (469), is less easily explained. It may be due to a slow displacement of calcium from the membrane surface (see Section VII C 2).

c. Metabolism. Since the contribution of metabolism to membrane potential may be small or negligible when precautions are taken to prevent changes chiefly of the potassium ion concentration in the extracellular spaces, its role appears to be a secondary one related to maintenance of ion distribution. The effects of extracellular sodium and cocaine on the depolarization of washed, desheathed vertebrate nerve during anoxia implicate some alteration of the membrane in this preparation during metabolic inhibition.

With these considerations and the preceding facts before us, a simplified model will be presented to integrate this material and to provide a basis for understanding (a) additional facts which will be brought forward and (b) other phenomena characteristic of excitable cells which will be discussed in Part II.

3. A quantitative formulation of membrane potential. The difference in potential across the cell membrane must arise from asymmetry in the system. The asymmetry of the ion concentrations on both sides of the membrane is most obvious. We have seen, too, that cations differ from each other in their relative effectiveness in decreasing E_m. That K^+ applied to the outside does so more effectively than Na^+ means that K^+ can enter the membrane more readily to discharge the negativity of the protoplasm (or to discharge the positivity of the extracellular phase). However, the effectiveness of this discharge will depend, too, on separation from anions. If Cl^- could enter the membrane as readily as K^+ the effectiveness of the latter would be nullified.

Obviously, the concepts involved are simply an extension of those which have given us the rigorous Planck equation and the approximate Henderson equation of classical electrochemistry for predicting diffusion potentials in homogenous solutions (328). These equations have proven useful for analyzing the origin of potentials in model membranes and some natural membranes from the standpoint of modified diffusion potentials (352, 486, 516). But even these become somewhat involved when applied to the living membrane. In view of our ignorance of the detailed characteristics of this membrane, a still simpler formulation is preferable as a working basis pending accumulation of data which will permit a more realistic model to be constructed. A particularly useful and surprisingly effective equation in this respect is that derived by Goldman (177, 221).

It is obtained on the basis that in the membrane, which is considered homogeneous, the electric field is uniform, the ions move by virtue of differences in concentration and in electrical potential essentially as in free solution, and that in the interfaces in contact with the intracellular and extracellular phases ionic concentrations are governed by distribution coefficients, *i.e.*, are directly proportional to the concentrations in the adjoining aqueous solution; also, that the ion permeabilities, which are proportional to the mobilities and distribution coefficients of the ions, are unaffected by the ions or membrane potential and that there are no interactions between ions moving in opposite directions or between different ion species. We have already had occasion to note membrane inhomogeneity; the other assumptions will later be seen to be oversimplifications. Nevertheless, it may be regarded as a semiquantitative approach for relating E_m to membrane characteristics and to ion concentrations and, as we shall see, especially for evaluating *changes* in E_m in terms of these parameters.

If we regard the potassium, sodium, and chloride ions as most important because of their high concentrations and because of appreciable permeability to them, we may write that, at 18° C,

$$E_m \text{ (in mV)} = 58 \log \frac{P_K[K]_i + P_{Na}[Na]_i + P_{Cl}[Cl]_o}{P_K[K]_o + P_{Na}[Na]_o + P_{Cl}[Cl]_i} \qquad \text{(I)}$$

Equation (I) summarizes several general principles: (a) The addition of cations to the outside solution (in the denominator as subscript o) will decrease E_m, while addition of anions (in the numerator as phase o) will increase E_m. The reverse electrical change occurs if the concentration is decreased or if the addition is made inside (subscript i). (b) The effectiveness of this change in potential will depend on the amount of the ion added relative to that originally present

and the magnitude of its permeability coefficient relative to the permeability coefficient[2] and concentrations of other ions of the same charge on the same side of the membrane, and relative to oppositely charged ions on the other side of the membrane. (c) Everything else being equal, an increase in permeability to a cation will make the phase with the lower concentration of the same ion positive. When the permeability to any one ion greatly exceeds that of the others, the terms containing the other ion concentrations become negligible. We then have the familiar equilibrium potential, which is the largest obtainable for that ionic species. Thus, in the case of potassium, it would be

$$E_K = 58 \log \frac{[K]_i}{[K]_o} \tag{II}$$

The effect of a change in permeability on E_m will depend on whether the ion ratio is greater or less than the equilibrium potential. Thus, an increase in P will cause E_m to move in the direction of the equilibrium potential. In cells in the steady state, $[K]_i/[K]_o$ generally exceeds that to be expected from E_m (Section IV B 1), a consequence of work done in transporting potassium; hence, an increase in P_K generally will raise E_m. Under ideal conditions, if chloride moves solely as a free ion through the membrane, it may be expected to attain the ratio fixed by E_m, and hence a change in P_{Cl} alone would have no effect on E_m. However, the chloride ratio usually is actually somewhat smaller than the theoretical, possibly because chloride may also enter as an "ion pair" (Section V C 1 b); in this case an increase in P_{Cl} will depolarize.

In Section VI, particularly, it will be shown that, by suitable alteration of E_m or of specific ion ratios, one may readily determine from the modified change of E_m to a physiological or pharmacological agent how ion permeabilities are affected by the agent. The principle involved is that of altering the equilibrium potential relative to E_m.

The coefficient 58, which is 2.3 RT/F in mV, will change with absolute temperature, T. Thus, it is 59 at 25° C, 61 at mammalian temperatures. Careful measurements, carried out under conditions with negligible secondary effects, such as changes in intracellular and extracellular concentrations, show indeed that E_m is but little dependent on T in giant nerve (222), skeletal muscle (307), and heart (497, 523) fibers. Because of the possibility of such secondary effects, as well as of changes in ion permeabilities, to be expected on theoretical grounds and from the "stabilizer" action of lower temperatures, the relation of E_m or E_K to a physical process on the basis of its dependence on temperature cannot be pressed.

a. Applications to membrane potentials. It can be shown that equation (I) satisfactorily describes the E_m-log $[K]_o$ curve discussed in Section C 1 a above. Thus, when K_o^+ is completely removed, a maximum potential is attained governed by $[Na]_o$ and $[Cl]_i$ (and by the numerator, of course, which is considered as essentially unchanged during the course of observation). As $[K]_o$ is gradually increased from low values, the sodium and chloride terms in the denominator are relatively so large that the $[K]_o$ increments reduce the potential only to a small extent; but as the K_o term increases relative to the others in the denominator, it begins to dominate and finally reduces E_m as the logarithm of the K_o concentration. Deviations will arise in part from leakage of potassium from the fibers when

[2] Hereafter, the term "permeability" will usually be used in place of "permeability coefficient", as has been customary for some time. Thus, "potassium permeability" will refer to the membrane permeability to the potassium ion.

the potassium content of the medium is small (due to inadequate active transport, discussed later), so that the potassium level immediately at the fiber surface is not as low as in the medium (434) even in single giant fibers (158) or for superficial muscle fibers (305–307). Of course, allowance must also be made for changes in the potassium content (K_i) of the fibers themselves if these are appreciable, as they may be for small invertebrate fibers or for muscle, when KCl penetrates (48, 399). When potassium equilibrium is complete at higher potassium concentrations, or when $SO_4^=$ is used in place of chloride to prevent K entry, the final slope of the E_m-log $[K]_o$ for muscle approaches the theoretical maximum of the 58 mV coefficient (2, 48, 438).

Changes in $[K]_i$ of muscle, induced by tonicity changes or by penetration of excess potassium from the medium, also modify the equilibrium potentials very much as expected (2).

By far the greater number of experiments on ions and potentials has been carried out from the outside rather than the inside. Negative effects have been reported for injections of potassium and chloride (and other ions) in giant squid fibers (183, 186), skeletal muscle (117), and motoneurons (52). In the last instance, entry of chloride with potassium by leakage from micropipettes left E_m unchanged, whereas ionophoretic injection of chloride caused an anticipated depolarization once the injection current was stopped. This suggests that the outward diffusion of KCl, when both ions are introduced, sets up a hyperpolarizing diffusion potential since potassium diffuses more rapidly than chloride, which nullifies the depolarization to be expected from an increase in $[Cl]_i$ alone. The negative results with injections of KCl in muscle may be similar in nature. However, the significance of these and other injections for E_m determinations cannot be pressed until answers are available for the following important questions: (a) To what extent do microelectrode diffusion potentials, which would be particularly marked when the pipette-electrodes are unselected for low charge and filled with other than KCl, obscure expected changes in E_m because of a change in the tip diffusion potential? (b) Since in the squid axon experiments large volumes frequently were injected and the axon sheath is fairly inextensible (401), how much ion loss occurred by filtration through the membrane and by leakage through cut branches? (c) Since in the muscle and motoneuron experiments hypertonic solutions were involved, to what extent did water uptake reduce effective ion concentrations? Osmotic experiments are less complicated, which may explain the success obtained with them in muscle. Unfortunately, squid, crab, and myelinated fibers are poor osmometers (189, 209, 213, 401), so that such studies are not readily extended to them.

Adrian (2) noted that equation (I) seemed inapplicable to the effects of low $[K]_o$ on E_m in frog muscle. However, this is true only if the 3 M KCl microelectrodes employed are assumed to be free of a diffusion tip potential. This was pointed out in Section III B 1 b 1) as unlikely, and actually more likely to be in the neighborhood of 5 mV for amphibian muscle. The addition of a diffusion potential term to Adrian's calculations shows that if it is at least 4 mV there is agreement with the Goldman equation!

According to equation (I) the effectiveness of extracellular potassium as a depolarizing agent stems from a greater permeability to potassium ions than to sodium. From the change in membrane potential with potassium at lower potassium concentration levels P_K/P_{Na} has been estimated in squid giant axon (221) as 1/0.04, in muscle (2, 204, 248) as 1/0.03, and in myelinated fibers (425) as 1/0.06. The exact value assigned depends somewhat on the value taken for P_{Cl}/P_K; for giant axon and muscle 0.4 and 0.2 (221, 248) appear to be satisfac-

tory, whereas for medullated fibers it may be taken as close to zero (425). However, a careful check on these values of P_{Cl} based on a systematic study of E_m-$[Cl]_o$ curves is not available.[3] See Section VIII B6.

The markedly smaller depolarizing action of sodium is in keeping with these figures. We may note, too, that the reduced effectiveness of potassium in elevated sodium and the increased effectiveness in low $[Na]_o$ (317) follow from the increase and decrease of the $P_{Na}[Na]_o$ term in equation (I).

The depolarizing action of rubidium, similar to but usually less than potassium, is to be expected from a similar permeability to this ion. Direct measurements of rubidium entry in muscle, and more so for cesium, show a somewhat slower rate (49, 315) in keeping with their weaker depolarizing action (215); this has been puzzling since the hydrated ion diameter, usually assumed to govern penetrability and depolarizability, is smaller for rubidium and especially cesium (Table 3, p. 135) and should favor them. This is discussed in Section VII.

Consider, now, the effect of a change in potassium permeability. Because the $[K]i/[K]_o$ ratio usually exceeds that expected from E_m (*i.e.*, $E_K > E_m$), a decrease in P_K alone will cause a depolarization, an increase a hyperpolarization. The effect of an increment in $[K]_o$ will be reduced or increased depending on whether P_K is greater or less. If P_K is sufficiently large (or all other permeabilities sufficiently small) we are left with the equilibrium potential, E_K, which, from equation (II), is independent of P_K changes unless it is greatly reduced or unless the other permeabilities are greatly increased, when equation (I) must be applied.

A change in the same direction in sodium permeability will have the opposite effect on membrane potential from that of P_K because of the opposite orientation of the sodium ion gradient; hence an increase in sodium permeability depolarizes, a decrease hyperpolarizes. Moreover, it is apparent from our equation (I) that as the sodium in the medium becomes smaller, depolarization due to an increase in sodium permeability may be reduced, prevented, and even reversed. And, of course, the sensitivity of membrane potential to changes in the sodium content of the medium is directly related to the sodium permeability.

The last two paragraphs summarize many of the facts reported (Section III C) if stabilizers are recognized as agents or conditions which decrease permeability, labilizers as those which increase permeability to sodium and potassium.

Thus, the reduction by local anesthetics, calcium, *etc.* of the E_m changes induced by increments or depletions of sodium and potassium in the medium indicate that the permeabilities to *both* ion species are reduced. Similarly, the augmentation by veratrine alkaloids, low calcium, *etc.* of the polarization changes

[3] Mullins reports that anion substitutions in the medium leave E_m of the squid axon unaltered (personal communication), as in frog nerve, and in keeping with the little difference in electrical response whether only Na^+ is removed, by replacement with choline$^+$, or both Na^+ and Cl^- are removed, by replacement with sucrose. Hence, P_{Cl} may be much smaller than estimated, a definite possibility in the light of evidence that potassium leakage from the axon at low $[K]_o$ can restrict the rise in E_m with decreasing $[K]_o$ (158), a restriction which was previously attributed to the $P_{Cl}[Cl]_o$ term in equation (I).

induced by alterations in the concentrations of these ions indicates that permeability to both ion species ordinarily is increased. The usually weak effect of stabilizers and labilizers on resting potential is ascribable in part, therefore, to the fact that both sodium and potassium permeabilities are changed, since they have opposite effects on the potential, but also to the small magnitude of the permeability change (*cf.* Section 3 b 2) below). There is no *a priori* basis to ascribe an identical intensity to the two permeability changes; in fact, the different directions of the modification in resting potential—in some cases with concentration, in others with position in a homologous series, and in still others with the tissue studied—point to P_{Na} and P_K being altered to different degrees.

Additional features of the relative effectiveness of changes in these permeabilities in altering the potential are worth keeping in mind. In cells with membrane potentials which are high and hence close to the potassium equilibrium values, P_K must be substantially larger than P_{Na}; hence, stabilizers will exert little effect through P_{Na}, which is already small, and little effect through P_K until the action is strong enough to lower P_K to that of P_{Na}, which may involve drug concentrations which not only lower potential via P_K but cause secondary effects (enzyme inhibition, surface disruption) as well. In keeping with this, it has been shown in Section III C that muscle and crab fibers, which have high membrane potentials, when a stabilizer is added, undergo either no electrical change or only a depolarization, which may not be reversible especially at higher drug concentrations. Conversely, in preparations like medullated fibers, where E_m is substantially below the equilibrium value for potassium, P_{Na} is presumably higher and therefore closer to P_K; the condition is therefore favorable for hyperpolarization through a decrease in P_{Na}, as observed. An explanation is also provided for the reduction and even reversal of the CO_2 effect on nerve membrane potential by stabilizers: In keeping with Straub's demonstration that the usual hyperpolarization by CO_2 is dependent on external sodium, CO_2 may be considered to reduce sodium permeability; however, when other stabilizers reduce P_{Na} first, the change of P_{Na} in CO_2 must be smaller or contribute less to the electrical change, and the decrease in P_K, which may also be expected to occur with prior stabilization, will be accentuated by CO_2 to the extent of causing depolarization. (It will be recalled that the lowering of pH reduced potassium depolarizability and hence P_K.) The opposite effects of CO_2 action observed in insect nerve depending on the type or condition of the fibers are readily explained on the same basis. Thus, the approach presented accounts for the frequent correlation of the direction of stabilizer changes with membrane potential. It can also account for those cases in which stabilizers cause altered effects without a prior change in E_m; in these cases both P_{Na} and P_K would have been reduced so that their opposite electrical effects canceled. Additional stabilization (*e.g.*, in CO_2) would still cause depolarization since only P_K could be affected further.

The similarity of low extracellular sodium to stabilizers is understood from the standpoint that both lower the sodium content and rate of penetration of sodium ions in the membrane; or in terms of equation (I), both have the same

effect on the P_{Na} $[Na]_o$ term. This readily accounts for the rapid and complete reversibility of depolarizations in veratrine, veratridine, and low calcium by calcium, local anesthetics, and removal of extracellular sodium.

The role of temperature must be left for discussion in Section VII.

Anions have not been given much attention because of the limited amount of available information—a consequence of the lack or probable lack of striking changes in their distribution under many experimental conditions (414, 424, 429). The values of P_{Cl} assigned on the basis of E_m-log $[K]_o$ curves remain to be verified by other measurements. In view of the probable relation of anion effects on E_m to chloride penetrability (Section IV B 2), Höber's old observation that stabilizers reduce the hyperpolarization resulting from anion substitution can be related to the prior, similar effect by the stabilizers. The observation that rapid depolarization in muscle by excess KCl, which must depend on K^+ entry exceeding Cl^-, is reduced by stabilizers (410), indicates that permeability to potassium is reduced more than to chloride (403); if chloride permeability were depressed more, of course, stabilizers would augment KCl depolarization.

In summary, therefore, the electrical data which have been discussed—largely qualitative but to some extent quantitative—conform surprisingly well to an equation which attributes the membrane potential to diffusion by the monovalent ions present in *high* concentration in the system; changes in this potential, by agents at *low* concentration, are explainable as the consequence of alterations in those properties of the membrane which govern the penetrability to these ions.

b. Applications to membrane conductance. Formulations such as equation (I), coupled with a specific procedure such as changing ion concentration for evaluating permeabilties, serve to put the concept of permeability on a concrete basis. They compel recognition not only that the permeabilities to various ion species differ, but that they may be altered to different degrees, the direction of the electrical change being a criterion of the permeability most affected; verification of these permeability changes has been seen to be obtainable through study of the electrical effectiveness of individual ions. But, more important for establishment of the principles of membrane behavior, the assumptions involved in the derivation of an equation such as (I) also permit predictions concerning two additional parameters susceptible to measurement: Membrane resistance and the rate of movement of ions. The latter will be discussed shortly.

Since conductance is directly related to permeability, rather than inversely as in the case of resistance, it is more convenient for the present purpose. In electrolyte systems it provides a measure of the rate of transfer of charge by both positive and negative ions for a given applied potential difference, and is governed by the concentrations and mobilities of the ions present in the membrane. The ionic mobility is a property of the membrane alone; however, membrane concentration depends both on the characteristics of the membrane and on the concentrations of the ions in the aqueous phases bounding the membrane.

The total conductance of the membrane, G_m, is the sum of the contributions

by each ion species, which may individually be designated G_K, G_{Na}, *etc.* Changes in permeability to a given ion will therefore affect the total conductance only in proportion to its initial or final magnitude relative to the total.

Several different methods have served to measure membrane resistance. The most direct involves the application of Ohm's law and the use preferably of two electrodes within the fiber, one to pass small direct or alternating current, the other to measure the potential difference resulting from the R_mI drop, where I is the membrane current (*e.g.*, 81, 83, 120, 123). In cylindrical systems the absolute magnitude of R_m is obtainable with sufficiently long electrodes and with a guard ring system, or when microelectrodes are used, by correction for the network characteristics of the fiber; such refinements are unnecessary when the investigator is interested only in the relative changes of R_m. Another technique utilizes the magnitude of the "electrotonic potential" attained at an electrode lateral to one but outside the two current electrodes; this increases at constant currents with R_m. These procedures may also utilize the rate of rise (or fall) of potential with passage (or cessation) of small constant currents. The time taken to rise to within 1/e of the final potential or to fall to 1/e of the initial value, gives a measure of the time constant (R_m C_m) of the membrane; since C_m is known to be constant in the few cases in which it has been measured, an estimate of R_m may be obtained (217, 218, 228). Still other methods involve impedance measurements with external transverse or longitudinal electrodes (44, 45, 160, 392). Tasaki (477, 478) describes additional special techniques applicable to the node and internode of single myelinated fibers; of particular importance for evaluation of the nodal membrane is a comparison of the internode and the nodal region so that the inescapable contribution of the myelin in the nodal area can be corrected for [see Section III B 1 b 2)].

1) Ions and conductance. In crab fibers (218, 220), squid (41, 46, 392) and Sepia axons (509), and the single node of Ranvier (327), elevation of extracellular potassium raises the membrane conductance; where this effect has been followed as a function of concentration (218, 220, 509) the factor by which conductance increases is at least equal to, and usually greater than, the factor by which concentration is raised. In a few experiments, a comparison of the relative effectiveness of various cations in chloride solution, all of which act on the crab nerve membrane in a matter of seconds, led Hodgkin to the following tentative sequence: Rb > K > Cs > Na = Li; the proportions required for the same conductance change, relative to potassium, were roughly as follows: 0.8:1.0:-2.2:40, 40 respectively (218). Ringer made hypertonic with NaCl raises the conductance of the region containing the node of Ranvier (326), but since the myelin on either side of the node is necessarily involved in these measurements, and the internode itself increases in conductance with salinity (477, 478), the actual effect on the nodal membrane is uncertain. On the whole, these data on monovalent ions are consistent with the expectation that cation entry into the membrane determines the extent of depolarization. The relative conductance effects of the cations merit more precise study to determine whether potassium and rubidium really differ with respect to their depolarizing effectiveness.

In skeletal muscle, too, G_m is reported to increase with $[K]_o$ (248).

Early variable results with transverse impedance measurements of muscle (188) may have been due to the use of solutions in which potassium replaced sodium; these have already been pointed out to cause geometrical changes in addition to potassium uptake by the fibers—alterations known to affect such measurements seriously. Tamasige (475), who

also employed solutions with potassium substituted for sodium, noted no appreciable increase in G_m until $[K]_o$ attained a contracture level (ca. 15 mM). Below this concentration the volume changes certainly could not have been great; but the negligible effects after exposure to such low K^+ concentrations may well have been the consequence of potassium leakage into the extracellular space during the measurements, which were made in a moist chamber (474).

Anions such as NO_3^- and Br^-, which increase the polarization of muscle, decrease its conductance (356a). This is the reverse of the effect to be expected if the hyperpolarization were due to greater penetrability by these anions than by Cl^-. It will be shown in Section IV B 2 that the conductance data correctly indicate the ability of the anions to penetrate. The significance of the electrical effects are discussed in Sections V C 1 b, VII, and VIII B 6.

The addition or removal of divalent ions exerts effects in keeping with postulated permeability changes. Thus, reduction of the calcium content of the medium increases the membrane conductance of crab (220) and squid (41, 46, 157) nerve fibers. In the latter, evidence of an increase in sodium and potassium conductance has recently been presented (157). The nodal region of single myelinated fibers does not change in conductance with an increase in calcium from 1.8 to 5 mM, but complete substitution of calcium by 5 mM barium causes a substantial decrease (325), presumably of G_K in accord with its depolarizing action at low concentration (314). In muscle, addition of calcium (250, 475) and of magnesium (475) is associated with a decrease in conductance. The effectiveness of magnesium on conductance is surprising in the light of its inability to prevent the depolarization by low calcium (332), but since the resistance and potential measurements were on nerves from cold and warm blooded forms, species or temperature differences may be involved. The large decrease in muscle resistance reported in isotonic $CaCl_2$ (and $BaCl_2$) (188) is probably related to the irreversible (toxic) depolarization (membrane rupture?) of nerve at high concentration (314). Kopac and Chambers (278) noted the increased brittleness of Arbacia membranes in elevated $[Ca]_o$.

2) Drugs and conductance. Such data are meager, although supplementary information is to be found in work done with transmitters (Section VI).

Local anesthetics such as sodium Pentothal (Thiopental) and procaine, at about conduction-blocking concentrations (0.2 and 1%, respectively) do not appreciably affect the conductance of single medullated fibers (394). Also, in the case of cocaine, a significant decrease in conductance becomes apparent only above the blocking concentration (478). Such findings are consistent with an effect at low concentrations predominantly on sodium permeability, which does not contribute appreciably to G_m but raises E_m, and at higher concentrations on G_K, which is a major part of G_m. The previously described polarization changes with ion concentration apparently are more sensitive for detection of the small changes in P_K and P_{Na}. Bufotenine, which does not block conduction but reduces potassium depolarization (Tasaki, personal communication), appreciably reduces membrane conductance (478); this suggests a selective decrease of P_K.

In muscle, lower concentrations of iso-amyl carbamate (which correspond to those which hyperpolarize nerve) decrease membrane conductance, and higher, toxic concentrations (which cause depolarization of nerve) increase the conductance (188). Chloralose, paraldehyde and pentobarbital are also reported to reduce G_m, whereas ethyl carbamate has little effect except at high concentrations, at which level it increases conductance (488); presumably the former have a marked effect on P_K (and P_{Na}?), whereas the latter acts predominantly on P_{Na} until toxic levels are reached. These studies again indicate that resting permeability changes are small.

IV. ION DISTRIBUTION AND RESTING POTENTIAL

The concept that membrane potentials reflect ion penetrability and that drugs may act through their effects on permeability is by no means new. But in recent years it has been consigned to limbo by those engaged in the search for explanations in terms of enzymatic processes only. The loss of interest is understandable in part as a reaction to the period when many phenomena were loosely ascribed to "permeability" with little if any experimental facts besides electrical or even less direct measurements to support such statements. Moreover, reports had appeared (61, 134) that the well-known changes of membrane potential with metabolic inhibition were unaccompanied by alterations in the ion contents of the cells—observations thought to render unlikely any explanation of bioelectrical phenomena solely in electrochemical terms.

We have seen that refinements in electrical measurements and in our concept of membrane potentials have led to a gratifying correlation of much data, particularly those related to ion and drug effects. But these views obtain their greatest strength from a more direct analysis of the relation of ion movement to the bioelectrical phenomena. Not only have these studies succeeded in showing that the effects of metabolic inhibition are in keeping with strictly electrochemical processes, but a number of clues to more detailed mechanism have been uncovered.

We turn, then, from the predominantly electrical to the chemical aspect of cellular electrochemistry.

A. Cautionary comments

This section will present data obtained by standard analytical methods. Such data provide not only absolute values for the ionic contents of the tissues but a means of following gross or *net* changes under a variety of experimental conditions. It is important to keep in mind that these changes are a consequence of a modification in *either* the rate of entry or rate of exit of a given ion species, or both. Thus, a decrease in intracellular potassium content may result from an increased exit of potassium and a reduced rate of entry. By the use of radioisotopes the unidirectional rates of movement (fluxes) can actually be followed. These will be discussed in Section V.

It should be recognized that net entry or exit of an ion by no means necessarily signifies that the permeability to it has changed. The gain or loss may also be the

consequence of increased permeability to other ions, which now can exchange or diffuse with it. A case in point would be the effect of an increase in sodium permeability. In this instance, sodium would exchange more readily with potassium, which therefore would emerge more rapidly; we know from equation (I) that an increase in sodium permeability must lower the membrane potential, which therefore serves as one means to identify this type of potassium loss. We may note, too, that the increased exit of potassium may be looked upon as a consequence of the depolarization, since the rate of outward movement (efflux or outflux) of K^+ is normally retarded by the membrane potential. Had the increase in potassium movement been due to increased potassium permeability, equation (I) tells us that the membrane potential would have been raised.

There is no need to expand on the limitations in evaluating intracellular ionic concentrations described in Section III D 1 b. As in the case of bioelectrical potentials, the uncertainties involved in estimates of absolute values render them less favorable for testing theoretical relationships than *changes* in concentration which, with certain precautions, are more certainly attributable to cellular behavior.

B. *Results in relation to electrical findings*

1. Potassium concentration. Despite the uncertainties inherent in the absolute values of concentration and potential, it is remarkable that practically no exception exists with regard to one aspect, *viz.*, that the resting potential, E_m, does not exceed and frequently is less than the equilibrium value for potassium, E_K. It will be recalled from Section III D 3 that E_K is the maximum obtainable potential from the potassium concentrations when the system is in equilibrium and when the permeability to potassium greatly exceeds that to other ions.

Hodgkin (219) pointed out this upper limit on the basis of data available for a number of cells in 1951. This principle obtains with more recent data, on the same and other cells, given in Tables 1, p. 71 and 2, p. 72. Unfortunately, the comparison is usually not a strict one since the conditions for electrical measurements are seldom the same as for the analytical determinations. Where this has been done carefully (2) the principle holds. Only Shaw *et al.* (438) report values for E_m in excess of E_K—for tropical toad muscle—but since this could have been due to unselected electrodes with additive diffusion potentials (2), verification must be awaited.

2. Ion penetration. In keeping with the electrical evidence for anion as well as cation permeability of muscle, KCl added to the medium bathing muscle penetrates and attains a new equilibrium (18). The many conditions under which this has been observed, and objections which have been raised, are fully discussed by Conway (48). Moreover, the relative rates of penetration of K^+, Rb^+, and Cs^+ (49, 315) are in complete accord with those to be expected from the relative effectiveness of the cations on E_m.

Br^- and NO_3^- salts of K^+ penetrate muscle more slowly than the Cl^- salt (49). This is in the reverse order to be expected from the increased hyperpolarization in Br^- and NO_3^-, but in keeping with resistance measurements noted in Section

III D 3 b 1). A remarkable feature of the resistance and diffusion data is their quantitative agreement. Thus, G_m in chloride, bromide, and nitrate Ringer is as 1.0:0.67:0.5, which are exactly the ratios of penetrability to KCl, KBr, and KNO_3, whether the potassium content of the Ringer solution is normal or high. One must conclude that the conductance and salt penetrability are governed by the content of the potassium salts in the membrane which in turn is governed by the anions according to their unhydrated diameters (Table 3, p. 135; see Section VII). The possible significance of the electrical effects of the anions in muscle is discussed later (Sections V C 1 b, VII, and VIII B 6).

In keeping with the electrical evidence for anion impermeability of vertebrate nerve, potassium replacement of sodium in the medium, or in excess as KCl, does not lead to potassium entry into the fibers except at very high potassium concentrations (426). Previous indications to the contrary were due either to damaging swelling at high concentrations (137) or to a miscalculation (129; see p. 429 in reference 426).

The resemblance of crab nerve fibers to muscle extends to their high penetrability to chloride, as would be expected from their electrical sensitivity to anions. Thus, unlike vertebrate nerve, but as in skeletal muscle, KCl diffuses into crab fibers readily (399).

As may be expected from the electrical evidence for increased cation permeability, a reduction in the calcium content of the medium causes an increased loss of potassium from smooth muscle (301), from skeletal muscle (135), and from vertebrate nerve (12, 134). In such studies it is essential to prevent spontaneous repetitive activity which arises and causes interchange of sodium and potassium. This may be done with low concentrations of stabilizers which do not otherwise affect the functional activity (413); in the presence of low concentrations of the stabilizers, potassium loss into Ca- and K-free Ringer is substantially reduced, but still greater than in normal Ringer (12).

The action of CO_2 merits careful study. It has two distinct effects—formation of intracellular anions (HCO_3^-), which are poorly diffusible, and of H^+; if the external medium is well buffered, an exchange H_i^+–K_o^+ takes place with an uptake of potassium in nerve and muscle (136). The reverse has been described for amphibian (4, 135, 136) and mammalian muscle (371) when external pH is allowed to fall. These processes may be expected to cause E_m to vary with $[K]_i$; on the other hand, the changes in permeability to be expected from changes in H_o^+ and which remain to be verified by more direct measurements, should exert a modifying influence on this. On the basis of the available data, it may be suggested tentatively that the initial rise of potential in vertebrate nerve in CO_2 is due to the depressed sodium permeability indicated by the electrical measurements, and the secondary decline either to the outward movement of potassium or to a slower decline in P_K. In muscle only the latter are apparent.

3. Drugs and ion penetration. a. Stabilizers. Observations with muscle first called attention to a demonstrable reduction of ion penetrability by stabilizers (403). These utilized the swelling of muscle in Ringer solution in which part of the sodium is replaced by potassium; Boyle and Conway (18) had shown that

this swelling was in proportion to the entry of KCl, to which muscle is permeable. A typical stabilizer, cocaine, was shown to reduce the rate of entry of KCl, while the response to hypotonic solution was left unchanged or actually slightly improved; the reduced depolarization in KCl also indicated a greater reduction in permeability to potassium. This approach was successfully extended to other local anesthetics and to antihistaminics (410); only yohimbine failed to alter KCl penetration, which suggests its stabilizer effects are exclusively due to a reduction of sodium permeability, in keeping with its similarity to low external sodium on the active aspects of nerve and muscle functioning (435) (see Part II).

Another procedure for demonstrating ion permeability effects is that utilizing the leakage of potassium in potassium-free solution which takes place because of reduced active uptake of potassium (414). Thus, cocaine slows such potassium leakage in vertebrate nerve (414) and in guinea pig auricles (234); quinidine and physostigmine also exert this effect in the latter (233, 235).

It was noted earlier that while physostigmine sulfate exhibits properties of a stabilizer, nevertheless minor labilizer action might be expected by virtue of the complexing of ionized calcium by the sulfate. The possibility that the slight depolarization and potassium loss of frog nerve in 20 mM concentration of the drug (526) is due to this action, perhaps with spontaneous activity, merits investigation.

In the light of the general use of phosphate, sulfate, and other calcium-precipitating anions as substitutes for chloride, attention should be called to the marked and *reversible* reduction in KCl penetrability and depolarizability as well as in the response of muscle to hypotonic solutions, which were observed as a result of treatment with phosphate and citrate but not oxalate (410). This is suggestive of a surface precipitation or complexing action involving calcium with a clogging of channels which may be peculiar to muscle. It would be of interest to determine whether the reduced loss of potassium in potassium-free solution with elevated extracellular phosphate (135) is due to this phenomenon rather than to a contribution by the phosphate to the intracellular pool of indiffusible organic anions, such as creatine phosphate, adenosine triphosphate, and phosphorylated intermediaries.

b. Labilizers. Probably the most complete correlation of permeability, membrane potential, and ion distribution exists for veratrine and one of its components, veratridine. We have seen that in the case of these alkaloids the evidence for increased permeability to sodium consists in the strong dependence on the external sodium concentration of the depolarization produced by these drugs. But even before this electrical finding it was shown that depolarizing concentrations of the alkaloids cause sodium to enter in exchange for intracellular potassium, and that this interchange is prevented by the two conditions which are now known to prevent the depolarization of washed nerves—low external sodium and local anesthetics (411, 413, 414). It has been pointed out already that depolarization is consistent with an increase in sodium permeability, the depolarization and greater escape of potassium being attributable to this, rather than to an increase in potassium permeability, which could cause only a

hyperpolarization. Moreover, the action of the local anesthetic is fully in keeping with the evidence for its action as a depressant of sodium permeability. Since recovery of potential is far more rapid in low sodium and in stabilizers than restoration of the intracellular ions, the electrical shifts are attributable more directly to permeability changes than to the ion concentration changes (469).

Two additional aspects of these studies may be noted: (a) Following loss of substantial potassium in veratrine, the addition of cocaine in the continued presence of veratrine not only prevents further loss of potassium but enables the system to reabsorb this cation for a considerable period thereafter (411, 414); presumably, reduction of the leak to sodium enables the metabolic processes to function effectively to restore potassium (and to extrude sodium?). Cocaine has been shown to reduce leakiness to ions without appreciably affecting active transport (Section V C 4 b). (b) The block of conduction associated with depolarization, as with anoxia or veratrine, may be expected to be reversed or prevented temporarily by stabilizers by virtue of a repolarization or sustained polarization, as actually found (148, 403); a simple basis is therefore provided to account for antagonism of two blocking agents under certain conditions.

Observations on limb muscle with a variety of other depolarizing agents, particularly tribromoethanol (Avertin) and caffein, demonstrate a similar correlation with potassium leakage as in the case of veratrine; likewise, prevention of depolarization (and contracture) by stabilizers [*e.g.*, procaine, 3-dimethylamino-1,2-dimethylpropyl-p-aminobenzoate (Tutocaine), tetracaine (Pontocaine)] is accompanied by potassium retention (194). Sodium movements were not followed.

4. Metabolism. a. Inhibition. 1) Nerve. A "break-through" in the approach to the role of metabolism resulted from the recognition that ion shifts much smaller than originally expected had to be sought to account for the bioelectrical changes associated with metabolic inhibition (402, 434). This came with the realization that electrical studies had been carried out almost invariably under moist chamber conditions, where small losses of potassium from the fibers would have caused a substantial increase of $[K]_o$ in the interstitial spaces—a view supported by a few earlier comments in the literature to the effect that washing of inhibited vertebrate nerve could delay or partly reverse the effects of inhibition (6, 125, 127) and by more recent experiments in which washing of invertebrate nerve achieved even more rapid and complete recovery despite continued inhibition (411, 412).

Indeed, the order of magnitude of this potassium leakage was successfully predicted in our laboratory from the electrical measurements (*cf.* 402, 434 and 409, 414). Such leakage was corroborated first for invertebrate nerve (407, 409) and shortly after for vertebrate nerve (128, 139, 408). Also, in keeping with their slowing of anoxic depolarization in nerve (94), glucose, cocaine (407–409, 411, 414), and low temperature (*cf.* 139 and 414) were subsequently found to slow potassium liberation during anaerobiosis. Iodoacetate, veratrine (408, 411, 413), and high temperature, as exemplified by results with mammalian nerve (505),

accelerated potassium leakage during anoxia, again in accord with their effects on anoxic depolarization. In mammalian brain slices, anoxia, glucose and a glycolytic inhibitor (fluoride) affect potassium leakage as in nerve (94); glutamate may also contribute to retention of potassium (75).

In general, the loss of potassium was paralleled roughly by corresponding entry of sodium (411, 414). These studies therefore led to the conclusions (a) that cessation of aerobic metabolism reveals an ionic leak to sodium and potassium which normally is present (and is responsible for the diffusion potentials already discussed) but that this leak normally is obscured by the metabolically linked, aerobic transport of potassium and sodium against their gradients, (b) that under anaerobic conditions glycolysis can supply energy, but not rapidly enough in nerve even when glucose is supplied, to achieve adequate metabolic transport, and (c) that the stabilizers and labilizers reduce or enhance the anoxic interchange because of their effect on the permeability (leakiness) to the cations (414), a conclusion reached before the findings in Section 3 above supporting this were obtained. It was also noted (414) that one could not rule out from these studies (a) a small change in permeability during metabolic inhibition and (b) a direct contribution by metabolism to membrane potential over and above that due to processes implicit in equation (I), but that radioisotope studies, soon to be described, could provide answers to these questions.

The recent electrical measurements (425) demonstrating that metabolic inhibition (anoxia) of washed, desheathed toad nerves causes a depolarization which is prevented by cocaine and low external sodium (much as in the case of veratrine and veratridine depolarization) indicate that an increase in sodium permeability actually does occur during inhibition in vertebrate fibers. But the electrical indifference of washed, cocainized vertebrate nerve during the early stages of inhibition (425), as in the case of untreated cephalopod fibers (224, 433, 434), despite the cessation of active Na-K interchange, is strongly indicative (a) of the absence of a direct contribution by active transport to E_m prior to inhibition and (b) of the presence of the same ionic leak, evident during inhibition, also prior to the inhibition.

2) *Muscle.* In muscle fibers, too, metabolic inhibition depolarizes and leads to Na-K interchange; as might be expected from the well-developed glycolytic system in this tissue, anaerobiosis alone is relatively ineffective in amphibian muscle and requires inhibition of glycolysis as well to demonstrate marked effects (77, 306, 442). Rat diaphragm at 38° C, on the other hand, loses potassium with only partial anoxia (64) and guinea pig atria lose potassium in dinitrophenol (234).

The decline of intracellular potassium and of membrane potential in muscle may be dependent not only on the failure of active Na-K interchange but on the breakdown of indiffusible organic phosphates to diffusible components as well; changes in organic phosphate, potassium content, and in membrane potential can be delayed by suitable substrates (lactate and pyruvate in the case of iodoacetate inhibition) (306) and by adrenaline (304, 352). Such correlations may be anticipated if, as already pointed out, the potassium content depends

in part on a Donnan equilibrium in which labile organic phosphates serve as the requisite indiffusible, negatively charged intracellular ions. Such shifts would be distinguished by the absence of corresponding sodium movement. The improved retention of potassium by rat diaphragm when bicarbonate is in the medium (63) should also be examined from this standpoint in the light of the "sparking" effect of bicarbonate on pyruvate oxidation (181); the possibility of a direct permeability effect as in the case of phosphate (Section IV B 3 a) also requires exploration.

3) Low temperature. Low temperature, which has been discussed as an agent reducing permeability, also interferes with metabolic transport in muscle and in the nerves of invertebrates, of toads, and of mammals (304, 336, 419, 436, 442). In keeping with its antagonistic effect with respect to passive sodium and potassium movement, low temperature is less effective than inhibitors (iodoacetate with cyanide) acting at normal temperatures in causing the decline of ionic gradients (442).

b. Recovery. 1) Nerve and muscle. Restoration of potassium, following depletion brought about in various ways, has been studied in nerve and muscle. Such studies provide direct information concerning the metabolically linked transport processes referred to as "active." In crab and frog nerve it has been observed following cessation of repetitive activity, but not in cephalopod fibers (20, 412, 419); in crab and sheathed and desheathed frog nerve on cessation of anoxia (139, 411); in toad nerve upon return to Ringer following exposure to NaCl (427); in skeletal muscle usually following exposure to a combination of low temperature and low extracellular potassium (78, 92, 315, 436, 443, 463, 464, 466); and in mammalian heart muscle immediately following removal from the animal (365). Following anoxia and repetitive activity of vertebrate nerve, sodium extrusion accompanies the potassium uptake (20, 425); no extrusion of sodium follows NaCl treatment (427). In heart muscle potassium uptake is also more marked than sodium extrusion (365). This active transfer is depressed in nerve by low temperature (442), by dinitrophenol (423), and by azide concentrations which affect resting respiration (20); and in muscle by oxygen lack (78), by insufficient intracellular sodium (463, 464), and by an insufficient excess of potassium or an inadequate depletion of sodium in the bathing medium (48). When equal quantities of K^+ and Rb^+, or K^+ and C_s^+ are presented to K-depleted muscles, the relative uptake is $Rb^+ > K^+ > C_s^+$ (315).

2) Cardiac glycosides and allied drugs. Studies of recovery also have proven useful in demonstrating the interference of cardiac drugs with active transport. Thus, ouabain and strophanthidin, but not the cardiac inactive drug dihydrostrophanthidin, at 10^{-7} to 10^{-6} M, prevent the metabolically dependent K_o^+–Na_i^+ interchange in skeletal (257) and heart (365) muscle. Such experiments also provide further evidence that active transport does not contribute directly to the potential, for microelectrode measurements of E_m showed no significant difference whether active transport was present or not.

Similar experiments on ionic movement have been carried out on red cells, which have been known for many years to gain sodium and lose potassium in the cold, and to reverse the

transfer of these ions by a process requiring metabolism (see 173,200,492 for references). Schatzmann (384) first called attention to the prevention of energy-dependent Na^+-K^+ exchange in human erythrocytes by strophanthin, digitoxin, strophanthidin, and digitoxigenin. This has been carried substantially further by Kahn and collaborators (259–261), who showed that a wide variety of compounds—certain simple lactones (*e.g.*, α-butyrolactone; β-propiolactone, α,β-angelicalactone), adenine, higher concentrations of adenosine (*e.g.*, 40 mM), and others—in addition to numerous cardiac glycosides and aglycones, block active transport; adenosine at lower concentrations (between 0.3 and 15 mM) and inosine above 0.3 mM accelerate the metabolic transport. No correlation was found between $PO_4^{\equiv}$ and K^+ transport; 8 compounds which inhibit K^+ uptake leave radiophosphate uptake unaltered, while 2 which increase K^+ uptake decrease P^{32} entry (259). It is also pointed out that diisopropylphysostigmine, at a concentration fifty times that needed to inhibit cholinesterases, has no effect on active cation transport (260), in keeping with findings with physostigmine, diisopropylfluorophosphate, and neostigmine (484). These and other studies to be described provide evidence that the predominant effect of cardiac drugs resembles inhibition of metabolism. Whether all the above compounds act in this fashion or by another mechanism requires an analysis of their metabolic effects. It is discussed in Section V C 5.

C. *Summary and preliminary conclusions*

1. Recapitulation. The facts presented up to this point are consistent with the view that the resting potential is an electrochemical process in the cell membrane set up in response to the ionic and other components of the protoplasm and of the extracellular fluid which bathe it. The absolute potential is governed by the aqueous concentrations of the ions and by the relative membrane "permeabilities," a term which encompasses not only the ionic mobilities but the distribution coefficients (governed by charge effects and other adsorbability or solubility factors) as well. Membrane permeability is highly labile, that to different ions being susceptible to change to different degrees, although the change usually is in the same direction. Drugs and experimental conditions are conveniently classified as either "stabilizers" or "labilizers." Stabilizers reduce or prevent an increase in permeability, labilizers increase or enhance an increase in permeability. Metabolism contributes to the potential only indirectly: (a) through prior work, which is responsible for the ionic gradients and for the stable structure and selectivity of the membrane; (b) through continuous energy turnover, which maintains ionic gradients by supporting a labile reservoir of indiffusible anions and by extruding or excluding sodium and restoring potassium at a rate equal to the normal leakage, or which restores ionic gradients following a period of uncompensated leakage; and (c) in some systems, such as vertebrate nerve, by continuous energy turnover to accentuate further the selective properties of the membrane.

2. Possible mechanisms for experimental effects. These studies focus attention on several mechanisms whereby an ion, drug, hormone or other experimental variable may act: (a) Metabolic stimulation or inhibition; (b) decoupling of the enzymatic reactions from ions normally transported; (c) entry into the membrane as an ion, thereby setting up a membrane potential, as in the case of Na^+ and K^+; (d) reversible change in the ion permeability of the membrane; and (e) irreversible disruption of the basic structure upon which these mechanisms depend. The last is readily discarded and should be since it is trivial; but we have

seen that its occurrence may be overlooked when a search for positive results leads to toxic concentrations which are unrecognized as such. Similarly, the search for a metabolic effect has frequently led to concentrations well above those which produce other effects; in such cases it may be suspected that lower concentrations act on a physical mechanism, either by setting up a membrane potential or by altering the membrane permeability characteristics.

Obviously, caution is necessary before the action of an agent can be ascribed only to one mechanism. The use of the lowest feasible concentration is helpful, but it by no means guarantees only one effect, any more than the use of a known metabolic inhibitor assures action only through metabolic inhibition. A proper evaluation of action requires that as many criteria as possible be available for the selection of one mechanism and for the rejection of others.

3. Criteria for a physical effect by experimental agents or conditions. Those discussed so far are:

(a) A rapid change in E_m induced by the agent or condition when the rate of diffusion is not limiting.

(b) A rapid alteration in the response of E_m to physiological ions by the experimental condition when the latter is not diffusion-limited. Simply a change in the effectiveness of ions or of other agents known to depend or act on permeability, even if not rapid, is also an indication of a physical (permeability) effect in an experiment.

(c) Alteration by experimental agents of the rate of depolarization of tissues in a moist chamber or of the ionic interchange during metabolic inhibition. It is important in such experiments that the possibility that the agent modifies the degree of inhibition be minimized. Ideally, prior complete inhibition is desirable of the metabolic pathways that may be involved, and this should be achieved with inhibitors that lack permeability effects themselves. In frog nerve, anoxia combined with iodoacetate poisoning reduces heat production to zero (126). This suggests complete inhibition and has been so utilized; but the possibility remains of a balance between endothermic and exothermic reactions, or of the stimulation of other pathways by experimental substances. Moreover, inhibitors lacking permeability effects are certainly to be desired, but those currently available have not been carefully studied from this standpoint. In aerobic systems, displacement of oxygen with an inert gas is least likely to be complicated by permeability effects; the presence of a glycolytic inhibitor assures more complete inhibition, but iodoacetate and iodoacetamide may behave as stabilizers as well (316). Obviously, modification of the effects of metabolic inhibition must be carefully considered in the light of other criteria.

(d) Alteration by the experimental condition of the rate of depolarization in a moist chamber during metabolic inhibition without interference with the return of potential upon removal of inhibition.

(e) Change in membrane conductance when a permeability change induced by an experimental agent involves an ion species contributing appreciably to G_m.

(f) Modification by an agent of the ionic interchange occurring in the absence of extracellular potassium.

(g) Ionic shifts, brought about experimentally, which are in keeping with accompanying electrical effects as predicted by hypothesis [*e.g.*, equation (I)].

(h) Alteration by experimental agents of the depolarization phase of spike production which in Part II will be shown to serve as an index of the ability of excitable fibers to produce an increase in P_{Na}.

4. Criteria for a metabolic effect by experimental agents or conditions. These are:

(a) A slow change in E_m, brought about by the agent under test, which is more marked under moist chamber conditions than when the fibers are washed, especially if this occurs with known metabolic agents acting at expected concentrations.

(b) Alteration of the rate of depolarization of tissues in a moist chamber during metabolic inhibition by agents known to act metabolically at the concentrations employed, especially when they also affect recovery of potential following inhibition; these effects are prevented or reduced by washing.

(c) Sodium and potassium shifts occur under the experimental conditions with no change in membrane potential in well washed fibers.

(d) Spike production is little affected by the agent until secondary changes develop (Part II).

Until the nature of the coupling between ion transfer and metabolism is clarified, one must bear in mind at least two possible means whereby active transport could be hampered: (a) Interference with the metabolic reactions which provide the necessary energy or (b) interference with physical interactions between the ions and metabolites (such as in ion pair or complex formation; see Section VII C 4 and 5) which are necessary to achieve transfer. The latter is equivalent to decoupling metabolism from a final step which is itself not necessarily enzymatic thereby leaving the biochemical reactions free-running, much as phosphorylations may be decoupled from respiratory processes or the wheels of a car disengaged from a running motor. The presence of such decoupling would be reflected by a cessation of active transport without or before a change in metabolism. The time aspect is important since the failure of active transport necessarily leads to a change in the intracellular ionic composition at a rate governed by the surface to volume ratio of the cells and by their permeability. Such a change in ionic composition can be anticipated to lead secondarily to altered metabolism because of the well-known dependence of the activity of many cellular enzymes on the sodium and potassium content of the milieu (see 173 for references). The finding that cardiac glycosides depress active transport in red cells without appreciably affecting glycolysis (384) and in muscle without altering heat production (201a) is suggestive of simple decoupling.

V. UNIDIRECTIONAL ION FLUX AND RESTING POTENTIAL

A. General

The criteria just given for the action of physiological and pharmacological agents by way of metabolic transport are substantially fewer than for physicochemical effects. This situation has improved much in recent years through the

use of radioisotopes. Because unstable (radioactive) ions exhibit chemical behavior indistinguishable from that of stable ones, they may be added as a contaminant to predominantly stable ions and thereafter the investigator is in a position to measure the rate of entry (the *influx*) or the rate of exit (the *efflux* or *outflux*) individually for a given ionic species. Since the ionic content of a cell is constant when the opposing fluxes are equal, and changes when one increases or decreases relative to the other, the observer can better assess the cause of such changes. Six years ago attention was called to the possibility of distinguishing permeability from active transport as the mechanism responsible for increased potassium retention; thus, everything else being equal, if permeability were reduced, the rate of potassium influx and outflux would be decreased, but if active transport were responsible, the influx would be increased (414).

B. Cautionary comments

The uncertainties inherent in estimating the distribution of the familiar stable ions within tissues and single fibers, described in Section III D 1 b, apply equally well to radioisotope studies. But flux measurements include several additional sources of error.

The estimate of outflux depends on a determination of a percentage change in the contained radioactivity and on the "effective" ion concentration, *i.e.*, the thermodynamic activity, within the cell. If the estimate of the ionic activity is incorrect—as in the case of ion binding or of misinterpretation as to the correct location of the given ion species—the computed efflux will be too high. This can be checked by determining the influx and the actual ionic content of the cell. The influx is obtained with little error from the initial rate of radioisotope entry and the ionic concentration of the medium, but only if measurements are made prior to appreciable uptake of the isotope if sequestering of ions, as suggested in Section III D 1 b, occurs in the cells. Corrections for diffusion limitations and for the rise in intracellular radioactivity are too well recognized to require comment. The net change in ionic content over a given period of time, especially if small, substracted from the influx over the same interval, should equal the outflux during that time. This test should be applied when incompleteness of isotope exchange suggests ion binding; as pointed out in Section III D 1 b, limited exchange may be the consequence of trapping of the ion within intracellular structures. In any case, information on the intracellular content of ions is generally a desirable adjunct to flux determinations.

In the light of the discussion in Sections IV A and IV B 4, a change in cation (or anion) flux may reflect not only a change in permeability but (a) a change in active transport, (b) a change in membrane potential, and (c) a change in the cations available for exchange or in the anions available to accompany the cations. As we shall see later, fluxes may also depend on still other forms of penetration which are subject to modification. Ideally, therefore, an interpretation of the action of a drug or other experimental agent should be based on a knowledge of E_m and of the fluxes of all major ions in the system. Thus, if for example the membrane potential falls, it becomes necessary to assess whether this underlies

an increase in the outflux, O, or a decrease in the influx, I, of potassium should these occur. An approximate check on this is possible by use of the following equations, which apply if the original assumptions (Section III D 3) are reasonably correct and *with the additional assumption that active transport is not taking place:*

$$O_K = [K]_i P_K (E_m F/RT)(e^{E_m F/RT} - 1)^{-1} = [K]_i P_K (E_m/25)(e^{E_m/25} - 1)^{-1} \quad \text{(III)}$$

$$I_K = [K]_o P_K (E_m/25)(1 - e^{-E_m/25})^{-1} \quad \text{(IV)}$$

The value for RT/F given in equations (II) and (III) is for 18° C and is in mV.

The ratio of the fluxes reduces to a convenient form:

$$\frac{I_K}{O_K} = \frac{[K]_o}{[K]_i} e^{E_m/25} = e^{(E_m - E_K)/25} \quad \text{(V)}$$

This shows that outflux exceeds influx when E_m is less than E_K, as it usually is; when the fluxes are equal the familiar equilibrium equation follows, and E_m then becomes E_K.

Ussing (502) derived equation (V) from more general considerations than those underlying equations (III) and (IV), one of the requirements still being independent diffusion of ions; he pointed out that conformance to equation (V) by potassium and other ions might be taken as evidence of passive diffusion and this has been so utilized for measurements carried out in cephalopod axons, myelinated nerve, and muscle.

However, the application of such equations to metabolizing cells requires caution until the role of active transport is clearly defined and until the equations can be shown to be a correct description of the behavior of the living cell membrane (*e.g.*, 503).

Implicit in the discussion of recovery of normal ion distribution was a definition of active transport, *viz.*, ion transfer dependent on energy turnover. This is an "operational" definition, *i.e.*, one susceptible of measurement by a specific procedure, for example, as the loss in ionic transfer as a result of complete metabolic inhibition. Ideally, this measurement is valid if indeed complete inhibition is possible while no other changes take place, such as in the ion permeabilities, ion concentration gradients, and membrane potential. Fortunately, in some systems subjected to inhibitors, permeabilities are sufficiently low and well sustained (in amphibian nerve) and the volume to surface area sufficiently large (in giant axons) that intracellular concentrations do not change rapidly and the membrane potentials and structure are stable (225, 226, 414, 423).

The study of metabolically inhibited cells therefore offers a means of determining whether the equations we have been utilizing, and hence the underlying assumptions, completely describe the properties of the cell membrane at least under these particular conditions. From the changes which occur during the transition from the uninhibited to the inhibited state, a reliable reconstruction of the situation in the normal cell becomes a practical possibility. This is considered the best available means at present for distinguishing passive transfer—that governed solely by electrical and concentration gradients—from active transport—ideally described as transfer against or in excess of electrochemical

gradients—and for determining the factors which govern both types of ion movement.

C. Results

1. Metabolic inhibition and passive transfer. As already pointed out, an examination of the characteristics of the fluxes in inhibited fibers provides a preliminary test of the applicability of membrane hypotheses to the cell membranes without the complication of active transport.

a. Potassium. In the case of inhibited (anoxia plus iodoacetate, as usual), cocainized toad nerve fibers, the residual fluxes and the concentrations of potassium inserted into equation (V) provide an estimate of membrane potential which agrees with that obtained directly on frog fibers and estimated for toad axons (423). From this it may be concluded that potassium is moving as the free ion in the membranes of this preparation. Since cocainization prevents the secondary changes in membrane potential and in potassium outflux associated with inhibition, the flux data may be regarded as approximating those normally present in the uninhibited nerve, and hence the implication is that passive potassium fluxes are behaving similarly during intact metabolism except for the superposition of potassium influx through active transport. Moreover, the agreement of the potassium data with the membrane potentials is in keeping with the preliminary conclusion (Section IV C) that E_m is determined largely by passive diffusion of potassium.

The outstanding analysis of passive potassium flux in relation to K_o and E_m is that of Hodgkin and Keynes on Sepia giant axons (226). This study confirmed the passive nature of potassium flux in inhibited (chiefly dinitrophenol-treated) fibers by demonstrating that at different equilibrium values of $[K]_o$ (*i.e.*, when $I_K = O_K$), E_m varied according to the calculated Nernst equilibrium potential, E_K (Section III D 3). But two corrections to the simple model which has until now been employed were discovered to be required for Sepia axons. First of all, P_K was not a constant independent of membrane potential; rather, it decreased with hyperpolarization and increased with depolarization, in keeping with "rectification," as found in squid giant axons (42), which greatly exceeds that to be expected from the simple theory (176, 485). Secondly, the increased transfer of potassium in one direction interfered with the flux of potassium in the opposite direction, an effect which could be accounted for by postulating very narrow channels through which K^+ passes; this was shown to be reflected by two changes in the basic equations, *viz.*, the flux ratio equation (V) became

$$\frac{I_K}{O_K} = \left(\frac{[K]_o}{[K]_i}\right)^n e^{nE_m/25} = e^{n(E_m-E_K)/25} \qquad \text{(VI)}$$

and the simpler definition of conductance at 18° C at equilibrium (219):

$$G_K = FI_K/25 = FO_K/25 \qquad \text{(VII)}$$

became

$$G'_K = nG_K, \qquad \text{(VIII)}$$

where n is greater than one. In other words, the flux ratio changed more rapidly with E_m than it should according to simple theory, and conductance was higher than would be estimated from the fluxes at equilibrium.

Hodgkin and Keynes point out that the last result provides at least a partial explanation of previous low estimates of potassium conductance from flux measurements [from equation (VII)] compared to electrical estimates of this parameter in *Sepia* axons as well as in amphibian muscle.

The general applicability of these findings to other biological systems remains to be determined. The simpler result obtained with frog nerve may reflect a genuine difference or less precision in the available data. In general, rectification appears to be much less striking in crab fibers (217), in myelinated nerve (395), mammalian motoneurons (51), and in muscle (83, 248, 266). In slow muscle fibers rectification appears to be as marked as in giant axons (30). In fast muscle fibers the situation is obviously complicated, for a reversal of the normal rectification curve (*i.e.*, G_m decreases under the cathode, increases under the anode) is the consequence of an elevation of extracellular potassium, especially in sulfate solutions (266, 267), or of removal of phosphate buffer (248). This raises the possibility of a second, opposing effect which obscures a more marked rectification in these systems. One such effect, in terms of "ion pairs" (see below), might be a decrease in the number of dissociated ions (which are partly the consequence of disruption of ion pairs in the intense electric field of the membrane) with depolarization. In any case, further study of rectification in other systems is certainly to be desired.

Another indication of the applicability of these findings is excessively large changes in potassium flux with $[K]_o$. Influx should be studied in the absence of active transport, in which case I_K should increase at least in proportion to $[K]_o$ or more. In toad nerve, under these conditions, we find in 4x and 20x $[K]_o$ that I_K is approximately 4.5-fold and 19-fold larger (425). Corresponding figures derived from *Sepia* data (226) are 26- and 90-fold. Obviously, permeability changes and ionic interaction are more important factors in the giant axon than in amphibian nerve. Potassium outflux, on the other hand, is less likely to be affected by active transport, and hence it may be studied in either inhibited or in uninhibited preparations and compared with the predictions of simple theory on the effect of $[K]_o$ and the associated depolarization. Thus, the Goldman equation predicts an increase roughly inversely as the relative magnitude of E_m, and hence somewhat less than the logarithm of the relative increment in $[K]_o$. For amphibian fibers this can be calculated to be 50% for a 4-fold rise in $[K]_o$, and almost 100% with a 20-fold increase in $[K]_o$. We actually find increments of 100% and 650% for toad fibers, compared to 350% and 600% indicated by Sepia data. The similarity between vertebrate and invertebrate fibers is therefore better with respect to O_K than I_K.

In metabolizing muscle, increments in extracellular potassium produce about the same or somewhat less than proportionately greater enhancement of I_K and O_K (197, 198, 202, 269, 333). With respect to outflux, then, but not to influx, muscle also appears to show behavior resembling that of giant axons.

On the whole, giant axons appear to be more extreme in the deviations of fluxes from the simple hypothesis, although as careful a study remains to be carried out in other systems. In any case, we may note that the major effect of these findings is (a) that an increase in P_K will be depressed secondarily if E_m increases simultaneously (as when P_{Na} is initially large); (b) that an increase in P_{Na}, which causes depolarization, will tend to increase G_m because P_K will increase as well; (c) fluxes will be modified correspondingly, and conductances will measure higher than expected from flux measurements. While these conclusions are of special importance in rapid transitory phenomena, discussed in

Part II, they may be considered as refinements which do not alter the largely semi-quantitative data and relationships which are the subject of this review.

b. Sodium and the concept of ion pairs. With respect to sodium the flux situation is very different from that of potassium. Thus, the fluxes of sodium during inhibition of vertebrate nerve bear no obvious relation to E_m; the fluxes in either direction approach the relative magnitudes of $[Na]_i$ and $[Na]_o$ (423). This suggests that sodium movement is *not* predominantly as the free ion, which would be affected by E_m. A simple interpretation is that sodium moves chiefly as an ion pair (*e.g.*, with Cl^-), for preliminary measurements of flux by radioisotope techniques show that the emergence of Na_i^+ in sucrose is identical with that of Cl_i^- (433).

The data for inhibited cephalopod axons are given as $I_{Na} = 15 \times 10^{-12}$, $O_{Na} = 1.5 \times 10^{-12}$ moles/cm^2 sec, approximately (224, 225, 270); hence, the fluxes are as the respective sodium concentrations, again suggesting an independence of the electric field. Here, too, sodium passes through the membrane largely in undissociated form, again most easily understood from the standpoint of ion pairs.

It will be pointed out that at least some active sodium transport, like sodium movement in the absence of such transport, is determined largely by sodium concentration, rather than by a combination of electrical and concentration gradients, and that ionic exchange can be ruled out. The concept of "ion pairs", *i.e.*, association with oppositely charged ions by virtue of electrical attraction and/or other forces in a region of low effective dielectric constant, could explain such observations. It is consistent with indications (Section VII) that the ions may penetrate the membrane in the unhydrated or weakly hydrated state. The theoretical and experimental aspects of ion pair formation in low dielectric solvents have been extensively developed in recent years (161, 279, 280, 328).

The following are some important conclusions pertinent to ion pairs:

(a) Below a critical dielectric constant, depending on the effective radii (including solvation when present) and on polarizability of the ions, ion association occurs.

(b) This association is greater the smaller the dielectric constant, the smaller the effective radii, and the greater the polarizability of the components in the system.

(c) The greater the ion concentrations the greater the association, the quantitative relationships being given by association constants, of which there may be several if multiple ion pairs form.

The behavior of alkali picrates in nitrobenzene appears particularly pertinent to nerve. Thus, the association constant for sodium, because of its smaller radius (unhydrated), is 25 times that for potassium (280). Hence, sodium could exist predominantly as ion pairs at membrane concentrations that leave potassium unassociated, the situation suggested by the data for nerve.

It, therefore, appears reasonable that within the membrane Na^+ will tend to associate with available anions, organic and inorganic, *e.g.*, Cl^-, more than K^+ will. Moreover, since ion pair formation depends on polarizability, such association will increase with larger anions (Table 3, p. 135). Ion pair formation by sodium may account for the following additional phenomena:

(a) The faster swelling of muscle in proportion to the polarizability (ionic

radius) of anions used to replace the chloride of Ringer (40); this would follow from faster sodium entry with the anions because of a greater effective concentration of ion pairs in the membrane.

(b) Increase or decrease of NaCl in muscle fibers roughly in proportion to $[NaCl]_o$ (138, 438, 442a).

(c) The frequency with which $[Cl]_i$ is in excess of that to be expected from E_m.

(d) The high chloride fluxes in cephalopod and vertebrate nerve (428, 429) despite the small P_{Cl} suggested by the negligible effect on E_m of replacement of Cl_o^- with much larger anions and by the failure of KCl to enter at moderate concentrations (426); in muscle, too, the flux rates appear very high (302) although KCl entry is substantially slower (18, 410).

(e) The tendency of chloride to enter with sodium during membrane depolarization; the steep electrical gradient across the membrane should normally tend to prevent ion pair formation just as high electrical gradients increase dissociation of weak electrolytes or raise activity coefficients (dissociation field effect).

Further study of sodium and especially anion fluxes obviously is necessary before the applicability of the concept of ion pairs to the above phenomena can be considered established. The same physical characteristics of the ions may equally well be responsible for interactions with membrane channels rather than between the ions alone, with similar final effects. For example, in certain anion-selective artificial membranes unidirectional anion fluxes substantially exceed those to be expected from membrane conductance (Gottlieb, personal communication), a result which would be characteristic of ion fluxes due to ion pair formation; transfer accomplished via anions strongly adsorbed along channel walls might be responsible for this observation. But net movements of both sodium and chloride such as observed in nerve and muscle would require a similar process for sodium as well.

The hyperpolarization of muscle by anions which penetrate more slowly and reduce membrane conductance poses an interesting problem also not solved with the Goldman equation [*i.e.*, equation (I)]. We have seen that the rate of penetration of potassium salts into muscle fibers parallels the effect of the anions on conductance. The question may be raised whether the membrane conductance is governed primarily by the net uptake of potassium with the anion within the membrane, and the polarization change by alteration of the rate of efflux of intracellular chloride (see Section VIII B 6). Information on the behavior of membrane potential as a function of $[K]_o$ in media with different anion might clarify the mechanisms involved. Details are also needed on cation and anion fluxes with different anions composing the medium. Edwards *et al.* (108a) report negligible changes in the sodium and potassium fluxes of muscle; however, since active transport, which tends to obscure changes in passive fluxes (422, 423), can be reduced or eliminated in muscle with ouabain (108), such studies with anions might be profitably repeated in the presence of cardiac glycosides.

2. Metabolic inhibition and active transport. a. Nerve. The most complete data currently available for excitable cells are those on unstimulated sciatic nerves of the toad (423, 430) and on giant axons from Sepia and Loligo which had been previously stimulated to accentuate active transport (225). In both preparations a striking effect of metabolic inhibition is the decline of potassium influx, I_K; the outflux, O_K, is little changed or increases slightly in the cephalopod fibers (see also 429), and clearly increases about 50% in the vertebrate nerve.

The behavior with respect to sodium differs in the two species. In the single giant axons, O_{Na} falls proportionately more than I_K during inhibition, with little change in I_{Na}, whereas in the amphibian fibers O_{Na} undergoes no detectable change, as has been also reported for amphibian muscle (274, 519), and I_{Na} increases appreciably.

Thus, the coupled active transport of potassium inward and of sodium outward is quite evident for the invertebrate fibers; the loss of potassium with inhibition is clearly due to a failure of its inward transport, and the gain of sodium to the decline in its outward transport. Such coupling is not quite as obvious in the sciatic nerve; here the loss of potassium is due to *both* an increased outward leak and a reduced inward transport, whereas the gain in sodium is due only to increased entry.

It was pointed out earlier that some depolarization occurs in washed sciatic nerves, probably because of an increase in P_{Na}, which is absent in invertebrate fibers. This requires the answer to 2 questions:

(a) Would this depolarization explain the increased O_K and the decreased I_K? By use of equations (III) and (IV), the applicability of which has been pointed out, it can be shown from the magnitude of the change in E_m that the increase in outflux is exactly accounted for, but the decrease in influx is much too great to be so explained (425). Moreover, the question of depolarization as a factor in the reduced I_K during inhibition is easily eliminated by having 0.1% cocaine in the medium during inhibition. Not only is depolarization then negligible, but the increase in potassium efflux is prevented, in keeping with the previous conclusion that an increase in P_{Na} now fails to occur, and potassium influx still declines, even somewhat more than before because of the reduced P_K (422, 423, 429). The same conclusion may be drawn from experiments in which $[Na]_o$ is low; again depolarization and increased O_K are reduced or prevented, owing to absence of an appreciable increase in K_i^+—Na_o^+ exchange, but I_K still declines with inhibition (422, 423, 430).

(b) Could the increase in P_{Na} have led to an increase in passive sodium outflux which, by nearly equaling the loss of active outward sodium transport, masked the latter during inhibition? Quantitative considerations have seemed in keeping with this (423). That active transport of intracellular sodium outward is possible is shown by an actual rise in sodium outflux during the heightened metabolism immediately following recovery from inhibition—an acceleration which is blocked by dinitrophenol and which is dependent on extracellular potassium, in accord with Na_i^+—K_o^+ exchange.

However, the critical experiment of inhibition in the presence of cocaine, which prevents the increase in P_{Na} and in potassium exit, fails again to reveal a change in sodium outflux (433), although it should have dropped by ⅓ or ½ if the original interpretation in terms of greater passive leak had been correct.

We must conclude from the available facts that two types of active Na—K exchange are possible and both may be present. They are shown schematically in Figure 1. That exemplified by stimulated invertebrate nerve and recovering vertebrate nerve is the more familiar type, A, which may be designated Na_i—K_o exchange and involves intracellular sodium. The other, exhibited by unstimulated nerve, type B, will be called Na_o—K_o exchange; in this case, as soon as extracellular sodium enters the membrane it is carried out again in exchange for K_o, and hence work is done not to accelerate exit of intracellular sodium but rather to slow the entry of extracellular sodium. When this work process fails, extracellular sodium continues to move in and exchanges with intracellular potassium.

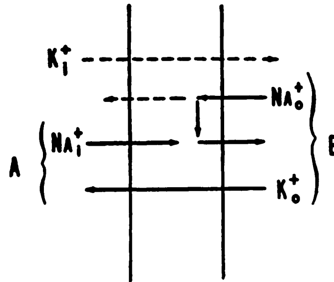

FIG. 1. A. Conventional active transport, as observed in previously stimulated cephalopod axons and in previously inhibited vertebrate nerve, which involves sodium extrusion and potassium uptake. B. Another form of active transport seen in vertebrate nerve which effects sodium exclusion and potassium uptake.

as shown by the broken arrows, giving the net effect of increased leak to sodium, with depolarization and increased potassium exit. Thus, the latter phenomena can no longer be considered fortuitous concomitants of the failure of active transport but rather a natural consequence of the mechanism involved. Thus, the increase in O_K and I_{Na} with inhibition are really equivalent to an increase in P_{Na}, although it is not a genuine increase inasmuch as I_K and O_{Na} will not be altered except insofar as they are changed by the drop in E_m. Put another way, we can speak of work done by active transport to reduce the inward permeability to sodium, or better still as sodium *exclusion* in contrast to *extrusion*.

b. Muscle. The same transport mechanisms are indicated by studies on muscle. The potassium fluxes in rat diaphragm change as in frog nerve during anoxia at normal temperature (37°C), *i.e.*, outflux increases, influx decreases (35); in the same preparation low temperature reduces only the influx (35), as in the squid axon, but since low temperature reduces all fluxes generally (35, 108, 196, 197, 333), the rise in outflux which might have occurred with inhibition by low temperature may well have been obscured by a general decrease in permeability. In rat *ext. dig. longus* muscle, inhibition by dinitrophenol, azide or cyanide reduces potassium influx to half (334).

The resemblance of frog muscle to frog nerve extends to the indifference of its sodium outflux to inhibition of phosphorylations and of respiration and glycolysis (274, 519). However, Edwards and Harris (108) have succeeded in demonstrating that half of the sodium outflux is linked to potassium influx by confirming that it is dependent on $[K]_o$, as shown previously and by others for muscle (269, 335, 473) and for giant fibers (224, 225), and by the additional important finding that ouabain, which blocks active transport (see section on cardiac glycosides below), has the same effect as the absence of extracellular potassium. Preliminary experiments of this nature in resting frog nerve have revealed no such effects by removal of extracellular potassium (423, 433) nor by a cardiac glycoside (433) although, as already mentioned, the potassium effect is quite apparent after $[Na]_i$ is raised by prior inhibition (423, 430).

Hence, although data on potassium fluxes are needed for frog muscle and on sodium fluxes for mammalian muscle, as well as on membrane potentials in both

systems, the indications clearly point to similar active transport mechanisms as elaborated in nerve. The same can be said for heart muscle, as pointed out below in the section on cardiac glycosides.

c. *Additional important findings.* Certain additional aspects of these and other studies are of considerable importance from the standpoint of the nature of the ion transport mechanism:

(a) The demonstrations noted above that influx of potassium in nerve and muscle is dependent on metabolism implies a transport mechanism located in the cell membrane.

(b) The decline of sodium outflux with lowered extracellular potassium in nerve and muscle is rapid, as it is with ouabain in muscle. This is in contrast with the appreciable time required for metabolic inhibition to be effective in giant axons. The rapidity again is indicative of a surface effect and one intimately associated with the transport process. It is of interest that a lowering of temperature also acts with great rapidity in depressing O_{Na} in the giant axon.

(c) Only part of the metabolically dependent sodium outflux of giant axons is depressed by the absence of potassium from the medium. This suggests that other ions may participate in sodium transfer. In the case of the giant axons an intracellular anion is implicated, for the remaining sodium outflux which is susceptible to inhibitors is not reduced by removal of all additional cations and anions from the surrounding medium. Moreover, since sodium outflux in the giant axon is unaffected by hyperpolarization (225), the sodium and this anion must be combined as an uncharged complex or as an ion pair (Section 1 b above).

(d) While the metabolically dependent outflux of sodium is frequently in excess of the active potassium influx, as in the case of the cephalopod axons (and in red cells, summarized by reference 359), the reverse also may occur. Thus, net potassium uptake in Ringer by toad sciatic nerve, following a decline of ionic gradients in NaCl, occurs without sodium extrusion (427).

(e) Net loss of potassium may occur by other than exchange with extracellular sodium during inhibition, one possibility being diffusion with an intracellular anion such as may appear during inhibition (*e.g.*, lactate), while less diffusible bicarbonate is lost as CO_2 because of acid production, or by exchange with another extracellular cation. Thus, in frog nerve the net loss of potassium is independent of $[Na]_o$ at the beginning of anoxia (413) and the net escape of potassium in azide exceeds the net gain in sodium (20); in rat diaphragm O_K rises during anoxia before I_K falls (35), accompanied by a large production of lactic acid.

(f) Following or accompanying the depletion of cellular potassium through bicarbonate loss, lactic acid production may itself contribute to potassium retention depending on the amount of lactate retained in the steady state and on the rate of H_i^+—K_o^+ exchange in a manner similar to the CO_2 experiments of Fenn and Cobb (136). In accord with this, prevention of lactic acid production during anoxia with iodoacetate reduces potassium influx to half in mammalian muscle (35). It was pointed out earlier for anoxic frog nerve that H_i^+—K_o^+ exchange resulting from lactic acid production accounts quantitatively for the different rates of net potassium loss under a variety of experimental conditions (414).

(g) Potassium influx depends on $[Na]_o$ in frog nerve under aerobic conditions, but not in the absence of active transport (422), as would be expected from the Na_o—K_o, sodium exclusion transport.

d. General transport mechanisms. These results therefore call attention to active transport brought about by

(a) K_o—Na_i exchange, the familiar sodium *extrusion* principle;

(b) K_o—Na_o exchange, a new sodium *exclusion* principle revealed by vertebrate nerve studies;

(c) Na diffusion outward, without its charge exposed, by complex or ion pair formation with another diffusible component; and

(d) Formation of indiffusible or slowly diffusible intracellular anions (organic phosphates, bicarbonate, isethionate, aspartate, glutamate, and other organic anions) with H_i^+—K_o^+ exchange.

The last will function selectively for potassium because of the relative permeabilities of the membrane, but only during a period of change, for sodium must eventually leak in and replace K_i^+ unless mechanism (a), (b), or (c) is functioning.

The flux changes corresponding to the cessation of these mechanisms are, respectively, (a) a decline in sodium outflux and in potassium influx, or in sodium outflux upon removal of K_o, (b) a decline in potassium influx with a rise in potassium outflux and sodium influx, (c) a decline only in sodium outflux whether or not cations are present in the medium, and (d) augmented potassium outflux without increased sodium influx.

e. Active transport and membrane potential. The fact that active transport mechanisms are predominantly ion exchanges or involve combined transfer of cation and anion makes quite understandable the absence of a change in E_m by inhibition of active transport in giant axons (225), in muscle (257), and in washed, cocainized, vertebrate nerve (425).

The clear-cut studies of Ussing on frog skin, which could demonstrate the active transport only of sodium and its correlation with membrane potential under most conditions, led to the conclusion that the transfer of sodium was itself the source of potential and other ion movement purely passive (252, 501, 502, 504). This view has had a strong influence in nerve and muscle physiology, for many have assumed that the outward transfer of sodium generates the membrane potential, which in turn is responsible for the potassium gradient. Early measurements, particularly on giant axons, appeared to conform to this approach since potassium data obeyed equation (V) (222, 268, 273); but a more careful analysis, involving the removal of active transport, has required a rejection of single ion transport in Sepia and Loligo axons (224, 225). Actually, the fact that in many fibers in the stationary state the potassium concentration exceeds that possible from the membrane potential is clear evidence for active potassium transport (Section IV B 1). This has been known to be true of red cells for some time (501).

It is important to note that Ussing and his associates have rejected unidirectional transport as the source of membrane potential for frog skin as well. It appears that a high permeability to sodium and a low permeability to potassium at an outer layer of the skin obscured active Na^+—K^+ interchange occurring at the inner surface, which is selectively permeable

to potassium. Consequently, the middle region of the skin is depleted of sodium and enriched with potassium and thereby gives rise to a diffusion potential which is the sum of that due to sodium at the outer layer and that due to potassium at the inner layer (253). At least two steps in potential have actually been noted with a microelectrode traversing frog skin normal to the surface (237). Huf's findings with regard to ion distribution and potential in frog skin (240) are in harmony with the revised view.

Consequently, explanations of bioelectrical potentials and their changes in terms of the active transport of only one ion species should be recognized as having no strong experimental basis at the present time.

3. The components of flux. By way of summary, we may note here the several components of flux which have been discussed and others which are encountered:

(a) Movement as the free ion, *e.g.*, through pores, susceptible to the electric field and aqueous concentrations, and without chemical interaction with the membrane or with other ions; this includes exchange with the same or other ion species of the same charge from the opposite side of the membrane or diffusion accompanied by an ion of opposite charge from the same side of the membrane; it contributes to the conductance of the membrane. This would be described by equations (I) to (V) (Section III D 3 and V B) and underlies the entry of potassium salts in muscle and the potassium ion in vertebrate nerve.

(b) Movement as a free ion with interaction with the same ion species moving in the opposite direction. A change of flux alters the opposite flux in the reverse direction, equation (V) is altered as though the entire right side were raised by a power greater than one, and the membrane conductance is greater by the same factor than expected from the individual fluxes, as in equations (VI) and (VIII) (this Section, C 1 a).

(c) Exchange diffusion, whereby a charged site is occupied alternately by ions from one side, then from the other side of the membrane; in this situation thermal energy accomplishes ionic transfer in both directions but no net or gross movement of ions occurs despite an apparent electrochemical gradient, and hence no work is actually performed (302, 473). Membrane potential will have no effect on such fluxes, and lowering the ion level below saturation on one side of the membrane will reduce the exchange flux in the opposite direction by an equal amount. The half of sodium outflux in muscle which is unaffected by $[K]_o$ appears to be of this nature (473).

(d) Ion pair diffusion, whereby oppositely charged ions, by virtue of the low effective dielectric constant of the membrane and possibly other forces, are undissociated and diffuse independent of the electric field and simply as a function of concentration from the side on which they are formed. Passive and possibly active sodium movement seem to be of this nature.

(e) Active transport, whereby ions are moved against electrochemical gradients by a mechanism which appears to involve ionic exchange and ion pair diffusion. This is discussed in greater detail in Section VII.

Electrical effects are obtainable only with the movement of free ions and, conversely, only the movement of the free ions can be affected by membrane potential. It follows, then, that electrical measurements are likely to be a better index of free ion permeability than flux determinations; from the results with

anions, we have seen this may be misleading unless all the ion fluxes involved are carefully determined.

4. Stabilizers. a. Calcium. The effect of this ion on the flux of monovalent ions apparently has been studied directly only on radiopotassium emergence from rat diaphragm (180), where it almost instantaneously raises E_m and greatly reduces O_K. This is qualitatively in keeping with a decrease in P_{Na}. The effect on O_K was larger than expected from the simplified theory from the change in E_m, but this may reflect an additional decrease in P_K, either because of the dependence of P_K on E_m, as described for Sepia, or because of a more direct effect of Ca_o^{++} on P_K. Addition of potassium to the medium in the presence of the excess calcium elevates potassium outflux again, but the depolarization must be substantially greater than the initial hyperpolarization before the original potassium outflux is restored (180). This indicates a direct effect of calcium on P_K as well, in keeping with previously discussed findings.

That excess calcium brings about a decrease in P_{Na} and P_K, and its reduction causes an increase in both permeabilities, has been verified by indirect measurements with the "voltage clamp" technique applied to squid giant axons (157). This study reinforces the well-known similarity of the effects of hyperpolarization to those of excess calcium, and of depolarization to calcium deficiency, which has frequently led to the proposal that changes in permeability with E_m and with experimental agents are a consequence of modifications in the calcification of the fiber surface (*e.g.*, 19, 179, 405, 406). Karreman (263) has provided a mathematical model which has interesting possibilities for adaptation of this concept to current knowledge of excitable cells.

The most promising direct evidence for surface calcification is the recent report by Niedergerke and Harris (346) of a reversible enhancement of Ca^{45} uptake by frog vertricle strips when $[Na]_o$ is lowered, and a slowing of Ca^{45} outflux in low $[Na]_o$. The rapidity of the effects suggests surface action. The increase in E_m to be expected from the decrease in $[Na]_o$ or $[K]_o$ cannot be ruled out as a contributing factor to increased Ca^{45} entry, but this is generally small and the rapid reversibility of the Ca^{45} uptake in low $[Na]_o$ is more in keeping with a displacement effect suggested by the findings of Niedergerke and Lüttgau (347).

Nevertheless, the concept of calcification may not suffice in all cases. Thus, Stämpfli (459) finds that repolarization of myelinated fibers in isotonic KCl under the anode, presumably through a decrease in P_K, occurs in the absence of Ca_o^{++}. It will be recalled that veratrine, which acts like low $[Ca]_o$, nevertheless still exerts its characteristic effect on the anoxic depolarization of frog nerve in calcium-free solutions containing calcium precipitants; and local anesthetics exhibit their characteristic effects on membrane depolarizability in the absence of Ca_o^{++}. The possibility remains, of course, that intracellular calcium may play a part, but the demonstration of this would be very difficult. It will be recalled that the slow development of low calcium effects in single fibers and its rapid reversibility suggest a calcium reserve available in the cell despite its bound nature.

b. Cocaine. Direct measurements of the effect of stabilizers on sodium and po-

tassium flux have thus far appeared only from our laboratory; they have been limited to cocaine on toad sciatic nerve (422, 423) and on squid giant axon (429, 432). Because of their potential usefulness in explaining the detailed mechanism of stabilizer action, as well as the considerable promise exhibited by such agents as a tool for studying the nature of the passage of ions through the membrane, the results obtained are carefully examined here.

In an effort to resolve effects via active transport as distinguished from passive ionic movement, the action of this alkaloid has been observed on metabolically inhibited and uninhibited nerve. In the former preparation cocaine obliterates the increase in potassium outflux which normally occurs with inhibition and reduces I_K proportionately to the same extent (423). This effect is in keeping with a reduction in P_K. However, since we have already seen that increased O_K during inhibition is dependent on $[Na]_o$ and on the depolarization which occurs, the effect of cocaine on outflux appears more properly attributable to a suppression of the increased sodium influx previously discussed. Cocaine may therefore affect only the influx components of sodium and potassium. Apparently in keeping with this, the alkaloid does not affect sodium outflux in either inhibited or uninhibited fibers (433).

In uninhibited toad and squid fibers cocaine causes an appreciable decrease in potassium outflux, but in the former it is largely transitory; depression of potassium influx, on the other hand, is well sustained (422, 423, 425, 429). The simplest explanation that can be offered is in keeping with the above indications that cocaine acts only on an outer layer of the membrane. This would reduce both sodium and potassium influx, but potassium outflux would be delayed only initially, when cocaine is first applied. Such delay would occur while outflowing potassium ions build up in the membrane at the inner boundary of this layer to a concentration which reestablishes the outflux through this layer to that in the inner part of the membrane. The final outflux would be only slightly less than before because $[K]_i$ is so large that the change in concentration gradient due to the increment in potassium concentration in the outer layer of the membrane could not affect the outflux much.

The absolute decrease in influx of potassium in cocaine is about the same as or perhaps only slightly greater in uninhibited nerves in the presence of cocaine than in inhibited preparations despite the three-fold greater total potassium influx in the former (422). Since the additional influx in metabolically active nerves is primarily due to active transport, the conclusions appear justified (a) that cocaine affects the passive I_K in the same way whether active transport is present or not and (b) cocaine does not appreciably affect potassium transferred by active transport, presumably because its route and form are different from that of passive transfer.

On the basis of these data three tentative proposals may be made:

(a) Cocaine depresses free ion movement but not other types of transfer, *e.g.*, ion pairs, whether of the "active" or "passive" variety. Thus, its failure to act on sodium outflux and on active potassium influx would be in keeping with other evidence for transfer as ion pairs.

(b) While experimental agents may act on the membrane as a whole, thereby altering ionic movements in both directions in accord with the Goldman equation, the present data suggest the additional possibility of effects limited to the outermost layer of the membrane, so that influxes are appreciably affected while outfluxes are not. From this standpoint, which is equivalent to postulating a phase boundary potential at the outermost interface, reduction of either potassium or sodium penetrability in this region alone will augment E_m, since movement of these cations into the membrane from the medium is in a direction which

will depolarize. This would explain why potassium must be present for frog fibers to depolarize when calcium is removed from a medium containing no sodium (459). If this layer is sufficiently thin relative to the rest of the membrane, its alterations may not be reflected by conductance changes.

(c) If our picture of active Na_o-K_o transport is correct, this layer on which cocaine and possibly calcium act underlies the region of the fiber surface where sodium first enters but is carried out again, for cocaine does not interfere with active exclusion yet prevents Na_o^+ from entering the membrane when active transport is blocked.

This would place at least one enzyme system related to sodium exclusion at the outermost surface of the cell. The presence of enzymes at the cell surface is strongly supported by the demonstrated high concentration of cocarboxylase in the sheath of giant axons (341) and by the susceptibility of cellular reactions to inhibitors and to activating agents which do not penetrate, for example in yeast (8, 375, 379, 380), in intestine (381), and in rat diaphragm (377). The finding with respect to cocarboxylase is especially suggestive in the light of the report that pyruvate oxidation (decarboxylation?) was the reaction most effective in the metabolic support of E_i (431), now recognized to be the consequence of active transport.

It is obvious that only a beginning has been made in relating stabilizer action to ionic flux, and that much remains to be done with this and other stabilizers, with other drugs, and with other biological systems. The technique of comparison of drug action in the presence or absence of active transport is promising in its ability to distinguish free ion flux from passive transfer of other types and from transport due to metabolism.

Another form of flux measurement—the indirect voltage-clamp technique already mentioned in connection with calcium—applied to squid axons has recently demonstrated that procaine and cocaine both reduce the increase in sodium entry and potassium exit brought about by depolarization (432).

5. Cardiac glycosides and allied drugs. Only in recent years has the indirect demonstration by Guttman and Cattel (190) that ouabain (g-strophanthin) and digitoxin augment potassium leakage from muscle been exploited. Attention was called in Section IV B 4 b 2) to the finding—in skeletal and heart muscle and in red cells—that these and related drugs block reabsorption of potassium and extrusion of sodium during recovery from inhibition or activity. It has also been demonstrated that these agents act like metabolic inhibitors in causing a net loss of intracellular potassium and a net uptake of sodium in heart muscle (192, 396) and a decline in $[K]_i$ of red cells (258).

The decline of $[K]_i$ in potassium-free solution must occur with little active potassium uptake, hence the accelerated decline in guinea pig auricles exposed to ouabain at a high concentration, 0.01 M (234), is more probably due to other factors, *e.g.*, to increased cellular permeability (to sodium, potassium, *etc.*) or to formation of diffusible intracellular anions. The same conclusion appears applicable to a comparable study in skeletal muscle, where the effect of 10^{-5} M K-strophanthin was weaker but the same (385).

Studies of the net changes in ions do not eliminate other possibilities (*e.g.*, increased permeability, as with veratrine), which cause ionic interchange despite

normal active transport or which prevent active transport from achieving recovery. But measurements of unidirectional fluxes do rule out permeability except as a secondary factor. Thus, in frog ventricle ouabain abolishes the slow (active) components of potassium influx and sodium outflux without affecting O_K (396); in frog skeletal muscle it reduces sodium outflux to the same extent as removal of K_o (108). In human erythrocytes not only does digoxin reduce I_K (172, 258) and O_{Na} (172), but the reverse fluxes are also reduced; therefore, passive penetrability may be affected in these cells (172). The lack of an appreciable change in resting potential (145, 472, 522), except at high drug concentrations (56), also indicates little alteration in the ionic permeability of heart cells.

The similarity of the effects to those of metabolic inhibition raises the question whether the drugs act by impairment of metabolism. This possibility appears supported by the partial shift of glucose metabolism in dog heart slices from aerobic glycolysis to CO_2 production in low concentrations of ouabain (518). However, here one is faced with the problem as to whether alteration in the spontaneous physiological activity or in the ionic content of the cells, brought about by the glycoside, secondarily modifies metabolism. Such studies should be repeated under conditions which prevent spontaneous activity (*e.g.*, with a low concentration of local anesthetic) and should be compared with the metabolism of the same tissue with a similar internal milieu brought about by other means (*e.g.*, anoxia). In the red cell, the uptake of sodium still is rapid enough to pose a problem, although anaerobic acid production, which is the major source of energy in these cells, appears to be unchanged (384). More recently, the rate of active transport in glucose-starved red cells has been described to parallel the level of adenosine triphosphate (ATP); strophanthidin slows the decline of ATP, with little effect on ATP generative processes, so that a change in ATP utilization appears to be involved, but to an extent far too small to account energetically for the decrease in transport (100). In skeletal muscle heat production is unaltered by strophanthin although $[Na]_i$-$[K]_o$ transport is blocked (201a).

The available data do not rule out the possibility that cardiac glycosides act to inhibit a metabolic process intimately related to ionic transport. On the other hand, their effect on heart contractility (*e.g.*, 192, 365, 396, 517) and on membrane phenomena, such as the action potential (*e.g.*, 512, 522), is in some respects so different from that of ordinary inhibitors (495, 496, 508) as to suggest another mechanism, that of interference with the actual coupling process between sodium, potassium, perhaps calcium movement and metabolism. The technique of examining glycoside action by comparing fluxes with and without inhibition as a function of membrane potential and ion concentrations should prove helpful in further resolving the mechanism of action.

VI. APPLICATIONS TO TRANSMITTER-SENSITIVE MEMBRANES

A. Introduction

Until now we have dealt with membranes that are largely unaffected by physiological concentrations of neurohumoral agents (also referred to as transmitters) such as are liberated by axonal terminations at a variety of junctions.

This may reflect a lack of reactivity rather than inaccessibility, for such neurohumoral agents when injected into giant axons or muscle cells are without the effects they exert on post-junctional cells or at the endplates (117, 182). However, close microinjections, effective from the outside, are also ineffective immediately underneath the endplate (82, 87).

The neurohumoral agent has been positively identified as acetylcholine at neuromuscular junctions, such as in fast and slow fibers of skeletal muscle (*e.g.*, 1) and of course in the sino-auricular fibers of the heart, and at autonomic ganglia, such as the familiar superior cervical ganglion. In these cases careful study has been possible of the nature of the interaction of acetylcholine, released by stimulation or applied directly, with the effector cell membrane. Transmitter action in electric organs appears to be quite similar, as might be expected from the origin of these structures from muscle. Inasmuch as these systems have been thoroughly reviewed recently (185), and detailed information on their ionic characteristics is still largely lacking, they will not be discussed in detail here.

At other junctions—those of invertebrate muscles, motoneurons, and invertebrate stretch receptors—the transmitter is as yet not definitely identified, but the application of suitable techniques permits evaluation of the mechanism whereby the transmitters alter the post-junctional membrane.

This membrane may be a distinct entity (but not as distinct histologically at myoneural junctions as once believed, *cf.* 1, 368a), and occupies more or less of the cellular surface. An important concept, most fully developed and discussed by Grundfest (184, 184a, 185), is that the post-junctional myoneural and synaptic membranes or endplates are electrically inexcitable and incapable of setting off the typical regenerative all-or-none action potential unless they are adjacent to electrically excitable membranes. His studies of the modified muscle cells which function as electric organs in fish have shown that two types of cells are distinguishable—those which possess only the endplate type of membrane and therefore respond electrically only to a transmitter such as acetylcholine, released by the pre-junctional axonal termination or applied directly (as in elasmobranchs) and those which have both this and the more familiar membrane capable of producing the all-or-none action potential in response to direct electrical stimuli (as in teleosts).

Similar differences exist among muscle fibers in both vertebrates and invertebrates. Thus, the slow fibers of vertebrates are completely inexcitable electrically and only they, not the fast (twitch) fibers, respond with a sustained depolarization to acetylcholine (Ach) and with a maintained contraction in Ach and KCl (31, 89, 118, 288, 289, 487). Indeed, Kuffler and Vaughan-Williams (289) state "that all pharmacological assays of acetylcholine on, for example, the frog rectus preparation have in fact been carried out upon the slow fiber system." The graded electrical changes in response to motor nerve stimulation appear uniformly over these cells by virtue of the many fibers and branches which innervate them; in this way a propagated action potential in the muscle fibers is unnecessary for complete contraction, and graded contraction is also possible. Such innervation, but with membrane potentials induced exclusively by transmitters of unknown nature (119), is extensive in insect muscle (80, 191), although some fibers give evidence of the presence of an excitable membrane as well (80). The crustacean muscle fiber resembles that of insects in having a widespread series of endplates, but a substantial electrically excitable membrane is also present (122, 123). And, of course, fast vertebrate muscle cells have a highly localized junction with properties which only in recent years have been recognized to be quite distinct from those of the more prominent, electrically excitable membrane surrounding and extending from it (1, 86, 119, 286). Whether this principle of distinct membranes applies to

individual sino-auricular fibers of the heart as well cannot be stated. But the absence of the electrically excitable membrane in certain cells provides preparations of great potential usefulness for the study of the reactions of transmitter-sensitive membranes without the complications of the electrically excitable regenerative system which have usually required involved techniques for separating their individual properties.

We shall be concerned in this section solely with the graded, non-regenerative bioelectrical manifestations of the interaction of transmitters and related substances with the sensitive membrane of post-junctional fibers known as junctional (endplate, post-synaptic) potentials, and the modification of this interaction by other agents. The vast literature which deals with transmitter action based on the final response of the effector cell—the nerve impulse or muscle contraction—to pre-junctional activity or drug action without specific attention to the several individual intermediate steps which may be affected (*cf.*, 1, 286) cannot be discussed here.

It will be shown that, by the criteria of potential, conductance, and ionic movement, in accord with the principles of cellular electrochemistry described in previous sections, the action of transmitters is ascribable to an increase in permeability to one or more ions, depending on the nature of the transmitter and of the cell or junctional membrane on which it acts; also, that interference with the action of the transmitter in many instances corresponds to the process we have called stabilization.

B. *Acetylcholine and sino-auricular fibers*

1. Membrane potential. Since in heart fibers the rate of beating modifies bioelectrical and contractile phenomena (493, 494), a proper evaluation of the effect of pharmacological agents on other than the rate requires that it be either kept constant by electrical stimulation or eliminated altogether. Some differences in reported results are attributable to failure to control the rate.

In the beating heart, the membrane potential fluctuates with each action potential, and hence the maximum polarization between impulses often is referred to as the resting potential. Strictly speaking, of course, the potential of quiescent heart cells is E_m, but its measurement is not always practicable. In pacemaker fibers of the frog, quiescence leads to a 10 mV higher value (245) whereas in dog auricles a *depolarization* occurs (99).

The effect of acetylcholine (Ach) and vagal stimulation is predominantly on pacemaker and auricular fibers (231, 245). The most prominent action is a hastening of repolarization at the end of the action potential which is believed to reflect an increase in P_K (29, 84, 85, 231, 245, 255, 507). This will be discussed in Part II. An increase in the resting and maximum membrane potential also is usually evident, particularly in fibers in which the potentials are lower to begin with (29, 84, 85, 231, 245, 255, 507). Carbachol (carbamylcholine) duplicates the action of Ach in cat and rat auricles (29, 507). The increase in E_m, as well as the steepening of the E_m-log $[K]_o$ curve, is that to be expected from an increase in P_K relative to P_{Na} (29) and is in keeping with equation (I) (Section III D 3). These effects may be anticipated to be more marked when E_m is low by virtue of a higher P_{Na}, as in active frog fibers.

Physostigmine (10^{-4} M), by preventing the splitting of Ach, enhances the effectiveness of the ester 100-fold (507). Atropine abolishes the action of carbachol, Ach, and vagal stimulation (29, 245).

2. Membrane conductance. The directly measured increase of the membrane conductance of pacemaker fibers by Ach is also in accord with an increase in potassium permeability (498).

3. Ion transfer. The first indication of the effect of Ach on ionic movement was the discovery of potassium loss from mammalian hearts during vagal stimulation (238). That this potassium was derived from the heart fibers was not clear until Lehnartz (300) showed that in the tortoise auricle Ach acts like vagal stimulation, and both are counteracted by atropine. The net loss of potassium in Ach has more recently been described for guinea pig auricles (234, 235), where it is also reduced by atropine (235).

Such losses, by themselves, do not reveal whether potassium is leaving because of increased diffusibility of intracellular anions (which enable potassium to escape with them), of increased sodium permeability, or of increased potassium permeability. The last is implicated by the hyperpolarization.

The increase in P_K by Ach is further established by the demonstration that both influx and outflux of potassium, in frog and tortoise sinus venosus fibers, are increased by low concentrations of the ester and by vagal stimulation, and these effects are abolished by atropine (203).

In accord with its action as a stabilizer, quinidine reduces net loss of potassium from guinea pig auricles in Ach (233).

4. Mechanism of antagonism. Since quinidine exhibits stabilizer properties in other respects, including reduction of potassium leakage from auricular fibers in the absence of extrinsic Ach, the mechanism of its action may be suspected to be that of preventing the increase of ion permeability directly rather than of competition for "sites" with the ester. In the case of atropine, which is so much more selective at "muscarinic sites," the possibility that it prevents Ach interaction with the membrane is more appealing. However, its similarity in structure to cocaine and its local anesthetic activity at high concentrations (178) certainly make the alternative a possibility as well. A comparison of the antagonisms as functions of concentration should be helpful in determining whether atropine does indeed differ from stabilizers.

5. Summary. Thus, the hyperpolarization, increased membrane conductance, and greater ionic fluxes produced by Ach are characteristic of auricular and pacemaker fibers and are in keeping with an increase in P_K.

C. Acetylcholine and skeletal muscle

1. Slow fibers. a. Membrane potential. This has been pointed out to average substantially less than in the fast, twitch fibers. Its change with inward and outward current may show considerable rectification; in other words, as in squid axons, increase in E_m reduces G_m (presumably at least G_K) and a decrease elevates it (30). As a result of the delay in the rise of G_m (delayed rectification) during passage of constant cathodal current, the membrane depolarization shows some return to a higher although still depressed level; on cessation of the current

E_m returns to a value temporarily higher than prior to the application of current, as would be expected from a delay in the decline of an elevated P_K (30). Insect muscle fibers, which resemble the slow vertebrate fibers, do not exhibit rectification until depolarization is appreciable (191). Thus the difference in susceptibility of membrane permeability to E_m described for electrically excitable membranes is apparent among transmitter-sensitive, electrically inexcitable membranes as well.

The reaction of the membrane potential to acetylcholine by direct application or through motor nerve stimulation is a depolarization; the temporary hyperpolarization following withdrawal of the ester or of indirect stimulation is identical with that obtained following an electrically induced depolarization, presumably for the same reason, *viz.*, a temporarily sustained elevated P_K (31, 288).

The magnitude of the indirect electrical response, referred to as the small-nerve junctional potential (s.j.p.), varies directly with the membrane potential, becomes zero when E_m is about 15 mV and reverses sign and becomes larger as E_m decreases further; this suggests that liberated acetylcholine increases P_{Na} as well as P_K, since as E_m is lowered the electrochemical gradient for net sodium entry would be reduced, for net potassium exit augmented, until they balance (at 15 mV) (31). The presence of a change in P_{Cl} cannot be determined from these data. A similar dependence on E_m has been described for junctional potentials in locust muscle, except that they disappear when E_m is zero (80).

Thus, at normal membrane potentials, the depolarization by the transmitter apparently is due predominantly to the entry of Na_o^+, which exchanges for K_i^+ (see below). A further electrical test would be the demonstration, as found with twitch fiber junctions (83, 118, 342, 343), that low $[Na]_o$ reduces the s.j.p. Moreover, in view of the similarity of Ach action at the junction to that of veratrine alkaloids, stabilizers might be expected to reduce the s.j.p., as they do the endplate potential of fast fibers (see below).

Since abdominal muscle in the frog is composed chiefly of slow fibers (289), the studies of Fleckenstein and associates (147, 151–153) on this tissue supplement those on the single fibers. Their work shows (a) that many junctionally active drugs (*e.g.*, nicotine, decamethonium, carbachol), as well as veratrine, depolarize and cause contracture and (b) that a wide variety of stabilizers (local anesthetics and antihistaminics, but calcium usually weakly or not at all) and anodal current prevent the depolarization and contracture. These results strongly suggest that the depolarization of the myoneural junction by the transmitter and related drugs is very similar to that of veratrine or veratridine on electrically excitable membranes, *viz.*, it is brought about by increased P_{Na} and P_K, but calcium is less effective as an antagonist.

b. Membrane conductance. A substantial increase in G_m occurs during the s.j.p. (31). Since this is associated with a depolarization, an increase in P_{Na} is implicated. The same has been described for endplate potentials in insect muscle (191).

2. Fast (twitch) fibers. a. Membrane potential. The transitory decline of the membrane potential in response to indirect stimulation is called the endplate

potential (e.p.p.). Since the endplate membrane is a minute portion of the total cellular surface, its study in the presence of the surrounding electrically excitable membrane has involved the application of a variety of ingenious techniques and analytical procedures (*e.g.*, 83, 120, 286, 343). This has included studies of the e.p.p. in the presence of the action potential, which arises when the e.p.p. attains a critical magnitude, and in the presence of derivatives of curare, particularly d-tubocurarine, which depress the effectiveness of Ach and thereby reduce the e.p.p. to magnitudes which fail to stimulate the muscle. Reviews of this and many other aspects of junctional activity not discussed here have recently appeared (86, 119).

The e.p.p. has a time course which indicates that the release of Ach and its disappearance are very rapid processes, the maximum depolarization being attained during its brief action (104, 120). The time course of repolarization is governed by the dissipation of charge in two ways: (a) discharge through the local membrane and (b) spread laterally in addition to discharge through the membrane in more remote regions. Consequently, conditions which change the resistance of the surrounding excitable membrane may affect the time course of the e.p.p. Conversely, these membrane characteristics may obscure changes to be expected from an alteration of the junctional membrane during the e.p.p.

The application of Ach and other "nicotinic" drugs to the entire muscle induces a depolarization in the vicinity of the junctions when Na^+, Li^+, or NH_4^+ is present (32, 89, 118, 487). The depolarization spreads laterally, gradually involving more and more of the adjacent electrically excitable membrane—a characteristic response of the latter to any type of depolarization, which disappears only slowly after the junctional membrane repolarizes (32).

When the transmitter is applied directly, its effectiveness will depend on the presence and activity of an enzyme in the post-junctional membrane (acetylcholinesterase in the case of acetylcholine); since we are not concerned in this section with the interaction with the enzyme, it is desirable to ascertain that a drug or ion does not modify the transmitter effect by hastening or slowing its breakdown. This may be done by checking the effect of the modifying agent on the enzyme directly (*e.g.*, 79), by using a compound duplicating the action of the transmitter but which is not split by the esterase (*e.g.*, carbachol instead of acetylcholine), or by inhibiting the enzyme completely with another drug. The last is complicated by the possibility of a secondary effect on transmitter action (Section c 3) below).

In studies where the transmitter is applied indirectly, *viz.*, by stimulation of the motor fibers, experimental effects on transmitter action must be distinguished from alteration in the rate of liberation of the transmitter; the latter is detected by analysis or bioassay of perfusion fluid passing through the junction or, when the transmitter is known, by comparison of the action of the transmitter applied directly with that obtained indirectly. The last procedure is subject to error since the direct application of the transmitter does not duplicate the conditions of natural liberation (*cf.*, 83, 121, 342, 343).

Unfortunately, a consideration of all these factors is not always found and this may be a source of confusion. For this reason many studies, especially those dealing with overall transmission, must be carefully evaluated. Consequently, as pointed out in Section A above, the studies to be described are limited largely to those dealing with the e.p.p. and which permit analysis of events solely in terms of interaction with the endplate, as distinct from the splitting or release of the transmitter.

Particularly at higher concentrations, the depolarization by Ach and "nico-

tinic" agents is not sustained (89, 118, 487). This repolarization is associated with reduced responsiveness to subsequent applications of these agents and with failure of transmission through the junction (32, 487); brief application of nicotine to the cat cervical ganglion leaves a persistent block although repolarization may be complete (106). Thesleff's conclusion that Ach and nicotinic substances at high concentrations contribute to junctional block by interfering with the action of the transmitter (487) therefore seems correct, although the action is probably to be attributed to a relatively non-specific action like that of stabilizers in preventing depolarization. Thus, Kuffler (285) and del Castillo and Katz (87) have shown that procaine also prevents Ach depolarization at the myoneural junction. This will be considered further in Section c immediately below.

That the e.p.p. or Ach depolarization is due to the influx of extracellular cations to which the endplate normally is poorly permeable is shown by the dependence of the electrical change on extracellular sodium (83, 118, 342, 343), lithium (118), or ammonium ion (163), all of which sustain the membrane potential. The possibility of an effect of $[Na]_o$ on e.p.p. by alteration of the Ach released by prejunctional fibers was ruled out by studies on cat cervical ganglia (243).

As already discussed for s.j.p., increase or decrease in E_m augments or depresses e.p.p., the reversal being at about the same level of membrane potential as for s.j.p. (81, 120); this is therefore evidence for a general increase in permeability as for the s.j.p.

Of particular importance from the standpoint of the locus of transmitter action is the observation by del Castillo and Katz (82) that ejection of Ach or carbachol (with or without neostigmine) directly under a junction causes no e.p.p., whereas its application in the same manner from the outside causes the usual depolarization. This suggests the reaction is with the outer surface of the endplate.

b. Membrane conductance. By a variety of direct and indirect methods the endplate conductance has been shown to increase substantially during the e.p.p. (81, 83, 120, 264). An elevation in P_{Na} is indicated by the increase in conductance to inward (Na^+) current with fibers in Ringer solution, and in P_K by the rise in conductance to inward (K^+) current with fibers in isotonic K_2SO_4 solution and to outward (K^+) current in either Ringer or a K_2SO_4 medium (83). Of course, anion permeability changes may also be involved.

c. Synergists and antagonists. 1) Multivalent cations. Calcium and magnesium, at lower concentrations, do not affect the sensitivity of endplates to Ach (79, 89). Isotonic solutions of these ions block the action of the transmitter (89, 163). Great care is necessary in studies with these ions; for example, measurements merely of myoneural transmission would not be able to distinguish the enhanced release of Ach in elevated calcium nor its depression by magnesium and the enhancement of cholinesterase activity by the latter (79, 89, 242, 243).

2) Epinephrine and norepinephrine. Stimulation of the sympathetic supply of frog muscle fibers enhances the e.p.p., as does the application of the amines (244). This may be comparable to the action of labilizers, *viz.*, an enhancement of

the increase in P_{Na}. The fact that the time course of the e.p.p. was unaltered cannot be used to reject this since the decay is determined largely by the cable properties of the surrounding muscle membrane rather than by the endplate. The possibility that cholinesterase may be inhibited by these amines also merits investigation; the absence of a change in time course may not have been an adequate indication of the absence of such an effect. The significance of these observations for the block of single impulses with facilitation of transmission of several successive impulses in mammalian sympathetic ganglia by the amines (321) remains to be evaluated; such block is more likely to be related to the hyperpolarization seen with skeletal and heart muscle (Section F below).

3) Blocking drugs. Attention has been called to the secondary blocking action of Ach and nicotinic drugs on electrogenesis associated with repolarization. This was compared with the effect of a stabilizer like procaine (Section a above). Hence, an agent may have a primary depolarizing effect as well as a secondary, less specific action which is duplicated by so many other different compounds classed as stabilizers. The ionophoretic experiments of del Castillo and Katz (87, 87b) most recently confirmed the stabilizing action of decamethonium (and procaine) against Ach and stable depolarizing esters such as carbachol and succinylcholine. The kinetics and weakness of the endplate depolarization induced by ionophoretic application of decamethonium alone (87a), indicate that with this agent, unlike most other nicotinic drugs, stabilization develops more rapidly than its depolarizing action.

With usual methods of application, physostigmine, and to a lesser extent neostigmine, also exhibit stabilizer action at high concentrations (105, 118), although their more familiar action is that of cholinesterase inhibitors. With brief ionophoretic applications, neostigmine exhibits only the curariform effect (87b).

The stabilizer action obviously is not a very specific effect, as was noted for electrically excitable membranes. It is therefore not surprising to find that, in addition to the familiar stabilizers already mentioned, other drugs block transmitter electrogenesis without inducing a depolarization. These are the same for skeletal junctions and sympathetic ganglia (358), and largely the same for eel electroplaques (184, 185), and include pentamethonium, hexamethonium, tetraethylammonium, and d-tubocurarine. It will be recalled that tetraethylammonium reduced the depolarizing effectiveness of potassium in spinal roots (317). It is of interest that in ganglia decamethonium lacks the depolarizing action found at muscle junctions, exhibiting only the stabilizer effect which appears as a concurrent phenomenon in muscle.

d. Mechanism of antagonism. The facts which have been presented indicate that one form of antagonism to transmitter electrogenic action is of a nonspecific, stabilizer type which is encountered with the transmitter itself, with other depolarizing drugs, and with non-depolarizing agents which have included cholinesterase inhibitors, local anesthetics, and antihistaminics. Whether this is a consequence of a non-specific interaction with the membrane which prevents Ach from reacting with the surface, or whether the Ach reaction is not affected but only the increase in ion permeability is, cannot be answered with complete

certainty. However, since stabilization has been observed in the previous sections under so many conditions involving no reaction with external agents, it seems more reasonable to suppose that the latter certainly occurs.

In view of the wide variety of agents which can act as antagonists to Ach, one is compelled to doubt that competition for the same specific "sites," as usually assumed, need necessarily take place. The similarity of procaine to d-tubocurarine in its antagonism to Ach when they are applied by ionophoresis, except for a faster disappearance of its effect (87), leads one to wonder whether even the curare derivative does not act in the non-specific, physical fashion characteristic of stabilizers. This is by no means inconsistent with formulations based on the assumption of competitive inhibition as applied to final effector response (3a); similar formulae are obtainable but with different assumptions concerning the nature of the interaction with the membrane. On the basis of the observations which have been treated in this review, it seems not unreasonable that the antagonism between Ach and at least procaine may reflect not displacement of each other but interactions with different sites on or in the membrane whereby they exert opposite effects on membrane permeability (see Section VII C).

The recent experiments of del Castillo and Katz carried out by ionophoretic application of curariform and endplate-depolarizing drugs do not provide evidence for the concept of competition for the same site, although del Castillo and Katz attempt to interpret certain aspects of the interactions at endplates in conventional competitive terms (87b). If true competition did exist between d-tubocurarine (DTC) and Ach, for example, the slow subsidence of DTC action which occurs following a brief ionophoretic application to the endplate should be hastened by successive Ach applications. No such effect is described nor was it sought; the statement is made that "it is very doubtful whether, in fact, the dissociation of the inhibitor-receptor complex is fast enough to allow an appreciable displacement to occur within the brief period of rise of the Ach potential" (87).

Here we may note at least two means whereby "competitive" antagonism may be distinguishable from less specific "stabilizer" action, *i.e.*, that which reduces the ion permeability change without interfering with Ach interaction with the membrane:

(a) The concept of competitive antagonism customarily implies a similarity of structure and/or charge in the agonist and the antagonist as the basis for mutual interaction for the same site. "Curariform" agents are considered to require a cationic charge (102, 483). Consequently, this is readily tested by altering the pH and using antagonists which are weak electrolytes. It will be recalled that the stabilizer effect of procaine on an increase in P_{Na}—by low calcium or veratridine—was determined by the free base, not the cation (468) (see also Section VII C 1 a).

(b) Evans and Schild (113) suggest that the rapidity of action and the dimensions of molecules such as the transmitters restrict their immediate effect on smooth muscle contraction to a surface reaction, which need not involve depolarization. If this is correct, the observation that in tetraethylammonium the transmitter causes contraction in insect muscle without the more usual de-

polarization (191) may be taken to indicate Ach interaction without the usual change in ionic permeability. A systematic study of this phenomenon in slow fibers of frog muscle with other Ach antagonists is desirable.

Del Castillo and Katz (87) note that d-tubocurarine does not directly affect either the resistance or the membrane potential at the junction. Hence, it interferes with the increase in permeabilities with little detectable effect on the permeability at rest. However, the endplate is so small a part of the total area at a junction that this is not surprising; measurements in slow fibers would be more critical.

e. Summary. We therefore emerge with the conclusion that transmitter action at myoneural junctions, and possibly at sympathetic ganglia as well, consists of an increase in sodium and potassium permeability. Chloride may also be involved but evidence for this is unavailable. The effect is therefore very similar to that of veratrine and veratridine, a similarity accentuated by the ability of stabilizers to antagonize it. Antagonism may not necessarily involve "competition" for sites.

D. Motoneuron junctions

By ingenious use of double-barreled combination micropipettes and microelectrodes, Fatt, Eccles and their collaborators have added substantially to our knowledge of the excitatory and inhibitory synapses as well as of the rest of the soma membrane (reviewed by 103). In addition to studying the effect of varying E_m on the synaptic potential generated by the pre-synaptic fibers, they have also altered the internal ionic milieu of the cell by using different salt solutions in their pipettes and (a) allowing these to leak into the soma or (b) controlling the ion species ejected with the direction of applied current flow.

1. Depolarizing (excitatory) synapse. As in the case of e.p.p. and s.j.p., the excitatory post-synaptic potential (e.p.s.p.) is a depolarization (53). Like them it decreases with E_m, and reverses in sign when E_m is sufficiently low, actually when E_m is reversed, for as at invertebrate junctions it disappears only when E_m is zero. This is in keeping with an increase in conductance equivalent to a non-selective increase in all permeabilities which shunts the rest of the soma membrane and thereby reduces E_m. The lack of an effect by changes in cations and anions in the motoneuron further suggests the disappearance of all ion selectivity during the e.p.s.p.

Some peculiarities noted by Coombs *et al.* (53), such as failure of the amplitude to increase beyond a maximum with hyperpolarization, a refractory period following the action potential which also is reflected by the e.p.s.p., and the absence of a shorting effect of e.p.s.p. on the action potential, suggest that the synaptic membrane bears a closer resemblance to the rest of the soma surface than the myoneural endplate and the inhibitory synapse now to be discussed.

2. Hyperpolarizing (inhibitory) synapse (52). The inhibitory post-synaptic potential (i.p.s.p.) is a hyperpolarization which increases when E_m is reduced, decreases to zero as E_m is increased to 13 mV above the normal resting potential,

and becomes a depolarization at higher values of E_m. This result is to be expected if, because of the inhibitory transmitter, (a) P_K increases, tending to give an equilibrium potential which is indirectly estimated to be 22.5 mV above the original resting level and (b) P_{Cl} also increases, thereby tending to keep E_m at the resting level of 66 mV; the result would be the intermediate value of E_m at which i.p.s.p. becomes zero.

This explanation receives strong support by the demonstration that increase of $[Cl^-]_i$ (or of intracellular Br^-, NO_3^-, and SCN^- as well), by simple leakage from micropipettes or by ionophoresis, changes the hyperpolarizing i.p.s.p. into a depolarizing one; this would follow from the outward diffusion of Cl^- or the other anions when anion permeability increases. Conversely, increase in $[K^+]_i$ raises i.p.s.p., a decrease lowers it; these effects would follow from an increase in P_K, since the outflow of K^+ hyperpolarizes.

Inhibitory extracts from brain and spinal cord have recently been found to contain considerable γ-aminobutyric acid (9). Purpura *et al.* (362, 362a), by application of this and other ω-amino acids to the cortex, obtain cortical potentials which are interpreted as exhibiting inactivation of depolarizing synapses by γ-aminobutyric and smaller ω-amino acids and inactivation of hyperpolarizing synapses by larger amino acids. Unicellular studies comparable to those described above are needed to establish that the mechanism of action of these agents in the cortex differs from that of the hyperpolarizing transmitter at motoneuron junctions.

E. Peripheral inhibitory junctions

1. Crustacean stretch receptor. This organ (115, 116), innervated by single inhibitory fibers, has inhibitory transmitter potentials which behave like those of motoneurons. Thus, as the membrane potential is changed (by stretch of the receptor), the inhibitory potentials are altered, being a depolarization at the higher values of E_m, disappearing with a certain decrease in membrane potential, and becoming a hyperpolarization with a further reduction in E_m (287). An increase of Cl^- (and Br^-) permeability is revealed by the change of the transmitter hyperpolarization, at lower values of E_m, to a depolarization by replacement of either Cl_o^- or Br_o^- with glutamate, I^-, and NO_3^- (107). The increase of P_{Cl} (or P_{Br}^-) causes Cl^- (or Br^-) to diffuse into the cell when E_m has been previously lowered, since the extracellular concentrations now exceed the equilibrium value for the lower E_m; glutamate, I^-, and NO_3^- apparently do not enter during transmitter action, and hence their replacement of Cl_o^- now causes chloride to leave during the increase in P_{Cl}, thereby producing a depolarization because of the outward flow of negative charge. An increase in P_K may not be involved, for elevated $[K]_o$, which should reduce the hyperpolarizing effect of potassium escape when E_m is less than E_K, enhances transmitter hyperpolarization instead (107); apparently the action of $[K]_o$ on E_m determines its effect.

An important advance in the study of these inhibitory potentials is the finding by Edwards and Kuffler (109) that γ-aminobutyric acid and other, shorter amino acids to a lesser extent, duplicate the action of the inhibitory transmitter with

respect to potential and conductance changes in the stretch receptor cells. The action is stated to be upon dendrites, where increase in G_m serves to short out the spread of depolarization arising more peripherally. Such an effect, as well as mere cancellation of the potentials of excitatory post-synaptic potentials, appears to be required to account for the two stages of i.p.s.p. which have been observed to neutralize the e.p.s.p. of motoneurons (54).

2. Crustacean muscle. A different form of inhibition is apparent at crustacean muscle fibers, where the transmitter induces a *decrease* in G_m (124). The magnitude of the inhibitory potential is exceedingly small and probably not responsible for the marked reduction in e.p.p. Rather, the action is most easily explained as being that of a stabilizer, which as usual prevents the increase of P_{Na} during the e.p.p. The behavior of the inhibitory potential with E_m qualitatively, but not quantitatively, resembles that described before; in the light of the associated decrease in G_m, it may be accounted for by a small decrease in P_{Cl}. Ionic studies may be expected to clarify the situation further. In any case, these findings show that the mechanism of transmitter inhibition need not be restricted to an increase in P_K and/or P_{Cl}.

F. Epinephrine and skeletal, heart, and smooth muscle

In mammalian skeletal muscle the sympathomimetic amines cause a transitory hyperpolarization and decrease in net potassium leakage which involves the electrically excitable membrane rather than the junctions (23, 174, 176). The temporary electrical and leakage effects can be accounted for by a decrease in P_{Na}. The absence of an electrical effect in frog skeletal muscle would be a consequence of the low value of P_{Na} in this preparation, reflected by the proximity of E_m to E_K. In keeping with these proposals, a hyperpolarization also occurs in dog auricles which is more marked when E_m is initially lower (*i.e.*, P_{Na} initially larger) and, as might be expected from the involvement predominantly of P_{Na}, the membrane conductance does not change (119). Smooth muscle, too, is hyperpolarized by epinephrine (25); in this case the effect cannot be attributed simply to decreased P_{Na}, for the change in E_m alone should slow potassium outflux and accelerate potassium influx, whereas only the latter occurs with a net uptake of potassium (16). An explanation for the ionic and electrical effects could very well be the synthesis of indiffusible anions—possibly the organic phosphates as described for frog skeletal muscle (304) and rat diaphragm (110, 111)—which we have seen may be associated with reduced leakage of potassium and phosphate as well as with better sustained membrane potentials in frog muscle (304, 352); H^+ released during their synthesis would augment I_K by H_i-K_o exchange. Whether this and/or P_{Na} is the basis of action of the amines in skeletal and heart muscle can be checked further by measurements of both potassium fluxes and of the sensitivity of the E_m changes to alterations in $[Na]_o$.

G. Summary

The response of the excitatory synaptic membrane to its transmitter resembles that of the myoneural endplate in producing an increase in permeability to all

ions which have been examined; the response of most inhibitory synaptic membranes duplicates that of heart fibers to Ach insofar as P_{Na} is not appreciably altered. Thus, the difference between an excitatory or inhibitory effect usually involves the presence or absence of an increase in P_{Na} in addition to the increase of other permeabilities. Since this difference is obtainable with the same transmitter, *viz.*, Ach, it is clearly a membrane property. It is tempting to propose that the presence or absence of a response of P_{Na} reflects the presence or absence of a membrane constituent. The fact that certain uterine muscles are electrically excitable only when supplied with estrone (see 184 for references) is suggestive of this situation.

The significance of P_{Na} changes for the actual process of excitation and inhibition must be left for Part II. Here we may note again that the change in membrane potential is but a sign of the alteration in membrane properties reflected by the ion permeabilities [our familiar equation (I)]. Moreover, the presence of an electrical response, as well as its direction, hinges on the initial membrane potential and on the parameters of our equation (I), *viz.*, the relative magnitudes of the permeabilities and the ion concentrations inside and outside the cell.

VII. THE CONCEPT OF PERMEABILITY

The many facts and interrelationships which have been described indicate that the general electrochemical principles which have been developed here do indeed underlie the electrical characteristics of the excitable cell at rest and its electrical and ionic changes in response to ions and other physiological and pharmacological agents.

With respect to the intimate details of the interactions of these agents with the cell, particularly the cell membrane, much remains to be learned, as the flux measurements already have indicated. Up to this point the concept of permeability has been kept as general as possible, an approach that has proven convenient for an analysis and integration of the many phenomena dealt with.

A number of features of membrane permeability have been noted which indicate the possibility of a more detailed characterization of this important property, particularly with respect to the relative permeabilities to cations and anions and their alteration by physiological and pharmacological agents. Many of the hypotheses and models which have appeared on this subject are summarized by Beutner (11a), Davson and Danielli (76), and Höber (215), and more recently by Butler (33) and Mullins (340a). Only a brief discussion of certain aspects bearing on the findings covered in this review can be attempted here. Following this, a somewhat modified approach will be presented which seems to integrate the facts summarized in this review and suggests several additional avenues of experimental study.

A. Permeability in model systems

Artificial membranes which have received the greatest attention have been of the oil or non-aqueous solvent type and rigid, porous structures such as collodion. In the case of the former, penetration by ions is governed by the relative solu-

bility or distribution coefficient, which in turn depends in part on the hydration and solvation energies of the ions, *i.e.*, the ease with which water attracted to the ions can be removed and the extent to which the oil interacts with the ion. Davies (74) has systematically studied the relative rates of penetration of cations and anions into nitrobenzene from water. These conform well to the relative hydration energies (Table 3); thus, the rate of entry increases with decreasing hydration energy, so that the sequence is Li < Na < K < Rb and Cl < Br < I. When the solubility of anions is less than cations, the polarity of electrical effects with the physiological ions conforms with those observed in living systems (74a, 354). It will be recalled that living membranes differ in that the cations Rb and especially Cs penetrate less readily and depolarize less effectively than potassium (61, 131, 215); also, the relative penetrability of the anions in muscle is the reverse of that in nitrobenzene (49, 315).

In the case of porous membranes, the ions are visualized as passing through channels of diameters, at least along certain points in each channel, which are close to those of the *hydrated* ions (*i.e.*, the effective diameter of the ions including water molecules which remain intimately associated with the ion) so that the relative velocities in water are exaggerated within the membrane. Moreover, the difference in the velocity of cations compared to anions can be controlled by altering the charge on the walls of the channels, a net negative charge making the membrane selectively permeable to cations, a net positive charge favoring anion selectivity (150, 215, 450, 451, 515).

In cation-selective artificial membranes the effectiveness of cations in pene-

TABLE 3

*Some physical properties of unhydrated and hydrated ions (279)**

Ion	Crystal Ionic Radii	Polarizability $\alpha_0 \times 10^{24}$	Hydration Energy	Effective Hydrated Radii†
	Å	cm^3	kcal	Å
Li	0.6	0.075	131	4.5
Na	0.95	0.21	116	3.4
K	1.33	0.87	92	2.2
Rb	1.48	1.81	87	1.9
Cs	1.69	2.79	63	1.9
F	1.36	0.99	94	2.6
Cl	1.81	3.02	67	2.2
Br	1.95	4.17	63	2.2
I	2.16	6.28	49	2.2
Mg	0.65	0.012	460	5.9
Ca	0.99	0.531	395	4.5
Sr	1.13	1.42	355	3.7
Ba	1.35	1.69	305	3.7

* Tables II, 5; II, 3; 10; III, 18; respectively

† Kielland values converted to Å on the assumption I is unhydrated; figures about ⅓ larger would conform better to theoretical derivations (200).

trating and in causing depolarization conforms well to the sequence of estimated hydrated diameters (*cf.* 98, 215, 515, with our Table 3), which is the same as in nitrobenzene.

Anion penetration in artificial anion-selective membranes does not conform to the hydrated diameters. Thus, although among the halides hydrated diameters do not differ appreciably (Table 3), and the greater weight of larger anions makes them less mobile in free solution, it has long been known that the electrical effectiveness and presumably penetrability of the larger anions, in artificial membranes, is greater (98,215), again as in nitrobenzene. It is generally assumed that this is due to ion polarizability (*i.e.*, the ease with which an external charge distorts the electron cloud of the ion to make the latter behave like a dipole), which is particularly marked in anions and is in the necessary direction, as may be seen in Table 3. Since the polarizability of cations is also in the proper order, it might be a factor but less important. In any case, the permeability of rigid porous membranes to cations and anions is similar to that of nitrobenzene and conforms only in part to that of living membranes. Mullins (340a) suggests that the entry of ions into membrane pores may involve a solvation with the pore walls which makes larger as well as smaller pores inaccessible to the ions. The distribution of available pore sizes estimated on this basis, however (his figure 2), would make Rb^+ more effective than and Cs^+ almost as effective as K^+ in causing depolarization, which is out of keeping with the experimental findings. Therefore, other considerations not within the framework of conventional pore or solvent membranes are called for to account for such findings.

B. A Pore-solvent hypothesis

Reasons were given in Section III D 1 c for considering the membrane to be not a rigid but rather a semi-rigid, semi-fluid structure, with lipid molecules held together by intermolecular forces with an effectiveness dependent somewhat on the vibrational, thermal energy of the molecules. Protein has been suggested to contribute at the aqueous interfaces to rigidity, but the fact that large oil drops can "snap" into cells (Arbacia eggs, from which extraneous coats can be removed) without undergoing constriction and the continuity of the membrane is then reestablished (38), shows that such protein may not be important at least on the outer surface in some cases; rather, the intermolecular forces of membrane lipid, especially in the presence of small amounts of calcium, would appear to suffice for the low surface tension known to exist, for the entry of the oil with reconstitution of the membrane (much as dissolved collagen can be reconstituted), and for the electrochemical characteristics of the excitable cell.

In keeping with the properties of compressed lipid films on solutions, we may expect a certain flexibility in spacing, depending on how normal to the surface the molecules are oriented, on the entry or attachment of foreign molecules and ions, on alterations of the intermolecular forces (*e.g.*, due to coulombic effects or modified interaction of the lipid molecules with water), and on temperature (*e.g.*, 195, 447). These will be considered shortly in connection with experimental effects and drug action.

Here let us restrict our attention to the semi-rigid aspect of the membrane, which must provide certain more or less fixed spacings for ion entry, and consider the sequence of ion permeability which would be obtained if ion penetration involved only the *unhydrated* ions. In this case entry would be governed inversely by both the hydration energies and by the crystal ionic radii. Thus, although the decrease in hydration energy which occurs with increasing crystal ionic radius would favor the uptake of the larger ions, a restriction in the number of larger channel diameters would ultimately limit the entry of larger unhydrated ions despite their more favorable hydration energies. Entry of ions under such conditions has been referred to as "interstitial solution" (187).

Thus, the permeability sequence $P_{Li} < P_{Na} < P_K$ may be due to the decreasing hydration energies of the respective cations, and the sequence $P_K > P_{Rb} > P_{Cs}$ to the fewer pores of large size available to the larger *naked* cations (Table 3, p. 135). The failure of NH_4^+ to cause much depolarization in muscle (163) is also easily understood on this basis, for its naked diameter is about that of rubidium (295).

In the case of the anions, although the hydration energies are particularly favorable for membrane entry, the crystal ionic radii of anions which have been studied run substantially larger than those of cations (Table 3). Hence, the complete electrical indifference of vertebrate nerve to anions could be attributed solely to their larger size. Because F^- is comparable to K^+ in size and hydration energy, if it could be shown that KF, and particularly RbF, depolarize strongly and rapidly, the presence of a negative charge in the channels to retard F^- entry would be indicated. Anions penetrate muscle according to their naked radius.

The sequences of cation and anion permeabilities therefore are accounted for in terms of hydration energies and naked ion diameters. The process whereby water is removed from the ions cannot be specified. It probably does not involve metabolic work, for it will be recalled that the depolarizing effectiveness of potassium, which depends on its selective entry, is unaltered by metabolic inhibition in the squid axon (226). A physical process, for example thermal fluctuations in the state of hydration, seems more likely. The complete removal of water by this means cannot be expected (340a), but if the number of water molecules which remains associated with each ion is limited to the same minimum, say one as suggested by Mullins (340a), then the relative ionic radii will still vary proportionally with the naked ionic radii, but increased by a term governed by the average dimensional increase caused by the attached water molecule. If solvation (*i.e.*, interaction with membrane components) occurs, then complete water removal is a possibility, and steric hindrance would still limit the entry of the larger naked ions. In the last case, the smaller penetrability to Rb^+ and Cs^+ than K^+ would indicate that pore radii diminish appreciably above 1.5 Å (Table 3).

Measurements of the conductivity of picrates of the alkali metal ions Li^+, Na^+, and K^+ in nitrobenzene indicate that the cations are unsolvated, *i.e.*, do not interact with the solvent (280). This unusual situation is no doubt related to Osterhout's observation (354) that nitrobenzene is unlike most other solvents because of the great selectivity it exhibits for potassium over sodium, reflected by the much greater effectiveness of potassium in causing

depolarization and by the approximately ten-fold greater uptake of potassium by nitrobenzene from aqueous solutions. Osterhout's findings have since been confirmed and extended (74, 74a). The absence of solvation accounts for Davies' finding (74) that the rate of exit of ions from nitrobenzene to water varies little with ion diameter, for solvation should decrease appreciably as diameter increases and thereby increase exit rates correspondingly; the rate of uptake of the ions by nitrobenzene from aqueous solution, on the other hand, shows marked differences among the ions in keeping with the hydration (*i.e.*, water interaction) of the ions (74).

Thus, nitrobenzene appears to differ from other solvents in that solvation contributes less to the free energy changes involved in ion uptake. The net effect is that hydration energy (difficulty of removal of water from ions in aqueous solution) is a dominant energy barrier to be surmounted for entry of the ions into nitrobenzene. Therefore, uptake will vary inversely with the hydration energies, as actually found (*cf.* data in Table 3 with data in reference 74, table 2).

From these considerations, then, ion entry in living membranes appears to involve the hydration energies—a factor of importance for a solvent such as nitrobenzene—but also the crystal ionic radii because of pore size limitations. For this reason the suggestions made are referred to as a pore-solvent hypothesis. The limitation of the size of the channels offers a basis for the "single-file" passage of potassium ions through the squid membrane suggested by the interaction of influx and outflux observed by Hodgkin and Keynes (Section V C 1 a).

Several questions that may be raised are as follows:

(a) Is there an interaction of the hydrated ions with the membrane surface which favors water removal, such as solvation or "complexation" (Part II)?

(b) Are several distinct phases normal to the membrane surface involved? At least two are certainly indicated by the effectiveness of Ach, carbachol, and d-tubocurarine applied ionophoretically to myoneural junctions from *outside* the cell, and their ineffectiveness when similarly applied but from *inside* the same cell (82, 87), a difference also suggested by injections of inorganic ions. The ineffectiveness of cocaine on sodium and potassium outflux, except for a transitory depression of the latter, while influx remains reduced, indicates that ion uptake can be affected only at the extracellular interface—a result strongly suggestive of an altered distribution coefficient at the outer interface (Section V C 4 b).

(c) Are the channels, and the regions immediately around them, distinct, specialized areas lying scattered in a more homogeneous lipid film, or are they merely intermolecular gaps governed by a balance between attractive and repulsive forces? More will be said about this when stabilizers are discussed below.

C. *Applications of the pore-solvent hypothesis*

On the basis of the hypothesis presented, permeability changes to ions may result from alteration in the size of the channels or from development of an interaction (*e.g.*, solvation) with membrane constituents which thereby reduces the energy barrier to entry. Only channel size changes will be considered since these appear to suffice for the permeability changes which have been described in this review.

1. Stabilizers. Many different kinds of compounds, including multivalent ions, have been shown to reduce resting membrane permeability to sodium and potas-

sium ions to a varying extent, but chiefly to *prevent an increase* in ion permeability. The very variety of the agents suggests a physical interaction rather than a chemical one which is specific. Stabilization is exhibited by agents which, because of other more prominent effects [*e.g.*, cholinesterase inhibitors such as physostigmine and neostigmine, junction depolarizing drugs such as acetylcholine and decamethonium, and metabolic inhibitors such as neopyrithiamine (290)], are frequently assumed to act by a more specific mechanism; however, a common nonspecific action appears to be more appropriate for an interpretation of their mode of action, for, as we have seen, the same results are obtained at other sites where their specific effects are not demonstrable and with agents which do not exhibit their "specific" effects.

a. Lateral pressure. The studies of Skou on a series of local anesthetics and on butyl alcohol (443–448) are the most systematic and extensive available; they lead to the conclusion that stabilization is linked with the tendency of the lipid phase of the membrane to expand. Skou carefully compared the blocking[4] potencies of 6 compounds (a) with the myelin-water distribution coefficients, (b) with air-water surface and with water-hexane interfacial activity, (c) with the spreading pressure developed in stearate monomolecular layers and in monomolecular layers of myelin extract. Only with the last was the sequence of effectiveness not only the same, but over a concentration range of equal anesthetic potency covering a factor greater than 13,500, the effect on spreading pressure agreed within a factor of about 2. This correlation is a further indication of the similarity of myelin to nodal membranes (see Section III D 1 c).

Accordingly, one may conclude that solubility in or penetrability of the membrane and the effective molecular volume of the stabilizer, which determine the spreading tendency, are involved. The high lipoid solubility of the many compounds which have been used in the past for narcosis certainly is consistent with this view.

That lateral pressure rather than stabilizer charge is of primary importance in stabilizer action is revealed also by pH studies. Thus, it has long been known that local anesthetics are more effective at higher pH, at which the predominant form is the free base rather than the cation. Skou showed quantitatively that the relative uptake of procaine by nerve lipid is increased by raising pH (445); his table of the ratio of the partition coefficient to the free base in solution, corrected for the different aqueous activity coefficients of the base revealed by his solubility curves (444), shows that the anesthetic content of nerve lipid is directly proportional to the free base, not the cation. A similar activity correction applied to his estimates of aqueous base concentration for cocaine (443) reveal that the minimum blocking concentrations at different pH correspond to the same activity of base in solution. Thus, a specific mechanism can be attributed to the demonstration of the equal effectiveness of narcotics on the basis of their thermodynamic activities (22). Of course, if more than one mechanism is involved, the equality of thermodynamic activities will not necessarily hold.

[4] Since we shall see in Part II that action potential production also reflects an increase in P_{Na} and at least in some cases an increase in P_K, the results are pertinent.

These findings are in keeping with Straub's (468) that the protective action of procaine against an *increase* in P_{Na} in low calcium is enhanced at higher pH, when the free base is predominant.

Of course, a wider variety of compounds remains to be studied to establish the general applicability of the idea of lateral pressure to the many agents which act as stabilizers. It is desirable, too, to demonstrate that drugs such as veratridine behave differently. But accepting lateral pressure tentatively as the primary factor, we may inquire how this might be translated into stabilization. If we accept the evidence discussed in Section III D 1 c that the channels through which the ions pass represent only a small part of the total surface area of the membrane, and consider that the diameters of these channels cannot decrease much further but increase with greater permeability, then we may postulate that stabilizers "dissolve" or enter in the region *surrounding* or *between* channels because of intermolecular forces, rather than *in* the channels themselves as frequently assumed. The increase in lateral pressure would then compress the channels, if they can be reduced in diameter any further, and certainly would interfere with the enlargement of these channels such as may be presumed to occur during an increase in permeability. In contrast to previous efforts to account for stabilization in terms of entry into membrane pores, this viewpoint has the advantage that the weakness or lack of an effect of stabilizers on membrane permeability at rest is easily accounted for as due to the pores being close to the minimum they can attain (due to repulsive forces and/or to rigidity), so that unless they have been previously enlarged (*e.g.*, by a labilizer), compression will have little further effect.

The concept of an effect by entry into the region *between* pores which represents a major part of the membrane surface offers a reasonable basis for understanding the effectiveness of drugs which act at such low concentrations that the probability of their encountering the "sites" with which they are usually presumed to combine is very low. The wide variety of compounds, including inert gases (340), which may function as narcotics appears explainable in terms of membrane solubility and molecular volumes. The possibility of a more intimate association of some stabilizers with the regions immediately around the pores is of course by no means ruled out, especially in those cases where the charge of the stabilizer may be important (see below). Stabilizers may be expected to affect membrane capacitance and high angle X-ray diffraction lines when the region between channels is markedly involved, although much of a change may not take place at usual pharmacological levels. Handovsky (193) noted sharpening of high angle X-ray lines in the lipid of the spinal cord of frogs killed with chloroform; this he interpreted as improved alignment of fatty acid molecules normal to the membrane surfaces, the effect to be anticipated from the lateral pressure exerted by foreign molecules on lipid molecules originally somewhat less oriented in the membranes.

b. Charge. Many stabilizers carry a positive charge by virtue of which, it has been pointed out for muscle, permeability is reduced more to potassium than to chloride (403, 410); this effect of stabilizers as well as of many other bases has been demonstrated with negatively charged artificial membranes (*e.g.*, 150, 515).

That stabilizer charge may be of importance for potassium permeability is in-

dicated by the greater effectiveness of procaine in depressing depolarization by potassium at low pH, at which the anesthetic is largely a cation (468).

c. *Dielectric constant.* It has been pointed out that lower members of the aliphatic alcohols depolarize, whereas the upper members hyperpolarize vertebrate nerve (292, 360). As alcohol length increases, the dielectric constant decreases markedly. The mixing of miscible solvents of different dielectric constants gives a mixture with an intermediate dielectric constant which affects electrolytes accordingly (*e.g.*, 280). If we now consider that the "solubility" or distribution coefficient (solvation?) of the ions in the membrane may vary with the effective dielectric constant, it follows that the penetrability (and depolarization) will increase with the smaller alcohols, which raise the coefficient for the membrane, and decrease (causing hyperpolarization) with the larger alcohols. The exact significance of "dielectric constant" for a semi-fluid, semi-rigid structure, such as the membrane is visualized to be, remains to be elucidated, but its possible role should be explored.

d. *Temperature.* Low temperature may also stabilize by limiting channel expansion. The reduced thermal vibrational energy of molecular components would augment the effectiveness of intermolecular forces and promote rigidity. The protective action of low temperature against secondary depolarization (augmented permeability) in high concentrations of drugs is attributable to these more effective intermolecular forces, which would also tend to exclude the drugs. The restoration of conduction of cocaine-blocked axons by low temperature (482) may be in keeping with reduced "solubility".

e. *Calcium.* This ion, at low concentrations, shrinks stearate and myelin films and makes them more rigid (195, 294, 448). The rigidity may be expected to contribute to stabilization by interfering with an increase in permeability, while the shrinkage will tend to make the system leaky through membrane rupture; if ionic channels were within the shrunken areas, the net effect could be an increased constriction of the channels as well. The hyperpolarization of nerve at lower concentrations and the depolarization at higher concentrations may represent the last two effects, rigidity preceding rupture. The double positive charge and the tendency of calcium to bind with organic constituents are undoubtedly of importance, but the ineffectiveness of this ion at junctions except at high concentrations suggests a high dissociation constant with endplate membranes. Calcium may also reduce permeability to Na^+ and K^+ by making negative sites in the membrane unavailable for their transfer; this is discussed in Part II.

The expansion of calcium stearate films when the pH of the adjacent solution is lowered (294) provides a basis for the stabilization noted under these conditions in terms of lateral pressure.

f. *Membrane potential.* Improved alignment of dipolar or polarizable lipid molecules of the membrane in a stronger electric field may, like low temperature, improve rigidity by intermolecular forces. On the other hand, there is also evidence that calcification of the membrane can be involved (*e.g.*, 157), although some experiments indicate this is not the only factor (Section V C 4 a).

To summarize, then, it appears possible that stabilization may be the consequence of at least two processes which interfere with a permeability increase involving enlargement of membrane channels: (a) Increased lateral pressure, due to the entry of foreign molecules in the region between pores and (b) increased rigidity of the interpore region.

2. Depolarizing agents. We have seen that veratrine and its alkaloids, on excitable membranes of nerve and muscle, and acetylcholine (Ach), other excitatory transmitters, and other depolarizing agents on myoneural, ganglionic, and motoneuron endplates, exert the same effects, *viz.*, an increase in permeability to all ions studied. Whether the mechanism whereby the increase in permeability is achieved is actually the same for veratrine and junctional drugs cannot be stated with absolute certainty. Certainly in the case of Ach and nicotinic drugs the fact that depolarization and increased permeability precede the stabilizing action, presumably due to actual entry into the membrane, suggests a more superficial influence (adsorption?) at first. In the light of the opposing action of stabilizers, the simplest explanation seems to be that Ach and other excitatory transmitters cause an opening of channels by reducing the spreading force between interchannel molecules, perhaps by neutralizing adjacent negative charges in the membrane with their cationic charge.

The ineffectiveness of transmitter agents on the nerve and muscle membranes could be due to chemical specificity; another possibility is a greater rigidity of the membranes, perhaps because of a greater effectiveness of calcium in inducing this rigidity than in junctional membranes. It will be recalled that only high calcium concentrations affect the depolarization of junctions by Ach. From this standpoint, veratrine and its alkaloids may be able to act where the transmitters cannot be virtue of an additional effect, *viz.*, displacement of membrane calcium, perhaps because of the strong surface adsorption suggested by the tenacity of its action on ion permeability (469) and on the action potential (Part II), and indicated by other studies (179, 405, 406, 413, 469, 513). It would be of interest to test for an effect by Ach on nerve or curarized muscle in the absence of Ca_o^{++}, preferably with calcium precipitants present, and also in the presence of veratrine.

It is noteworthy that histamine is effective on curarized muscle when calcium is lacking from the medium, but not when calcium is present (26a).

The permeability increase brought about by depolarizing agents is most easily attributed to an enlargement of the channels to a point which permits penetration of hydrated ions. This would explain the effectiveness of Na^+, Li^+, and NH_4^+ in such depolarization.

3. Inhibitory transmitters. The concepts which have been introduced above are clearly speculative, but because they give promise of integrating the available facts and of suggesting further avenues of study they are exposed to public view. A few more possible applications will be briefly considered.

The increase of motoneuron permeability to monovalent anions and potassium but not to sodium by the hyperpolarizing transmitter may be accounted for by an increase of channel diameters to above that of all the hydrated ions but sodium (Table 3, p. 135). In the case of the crustacean receptor, where P_{Cl} and P_{Br} in-

crease, but not P_I and perhaps not P_K, pore size would have to increase in a very restricted fashion, too restricted, it seems, to account for the results. Information on P_{Rb} and P_{Cs} might clarify the situation, perhaps by indicating whether solvation or complexation (*i.e.*, interaction with the membrane) may play a part.

4. Active transport. It was pointed out earlier that active transport of sodium in giant axons appears to involve combination with an anion which must accompany it out of the cell. It is not unlikely that one or more of the organic anions present in high concentration in invertebrate axoplasm, or those formed in the metabolism of vertebrate cells, represent, are the forerunners, or are products of anions which combine with membrane sodium and transport it outward by diffusion. Such ion pairs or complexes may be expected to be "soluble" in the membrane and hence to pass readily through it, in contrast to the free ions.

Since the organic products commonly formed by metabolism are acids, they could function effectively as carriers in the membrane only if their associated H^+ were tied up by a suitable base, $B^{(-)}$, within the membrane which would diffuse as a complex to the outer cell surface. There $B^{(-)}$ could give up H^+ in exchange for K^+, and then diffuse back as $BK^{(+)}$, or as free $B^{(-)}$ and K^+. $B^{(-)}$ may or may not have the indicated negative charge. Figure 2 shows how this system would work to give the Na_i—K_o (sodium extrusion) and Na_o—K_o (sodium exclusion) type of active transport (see Fig. 1).

The net effect of this model, in addition to the transport of sodium outward and potassium inward, is the appearance of the organic acid in the medium which is removed as a waste product. On the basis of the small ionic radius of

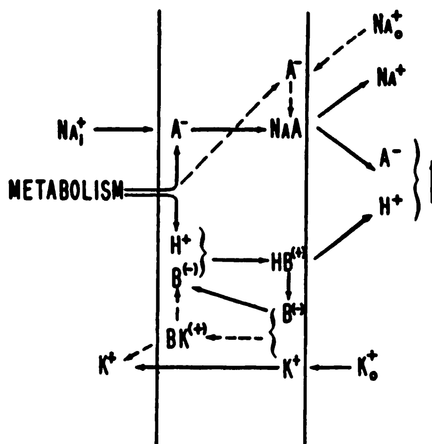

Fig. 2. Acid (HA) production and diffusion as a source of energy for active transport. Dashed arrows represent the additional pathways in vertebrate nerve (Fig. 1B) which (a) exclude extracellular sodium by its combination with A^+ near the outer surface and (b) transfer extracellular potassium by its combination with the base, $B^{(-)}$, which may or may not have the indicated charge and which also transfers H^+. The possibility that $B^{(-)}$ and K^+ are not associated in cephalopod membranes could account for what is now regarded as a fortuitous agreement between the ratio of potassium influx and outflux of uninhibited preparations and that predicted by simple theory [equation (V)].

lithium, one might expect poor reversibility of its combination with the carrier anion. The accumulation of lithium in frog skin (528) suggests that this occurs.

The concept of organic acids produced metabolically for transport of ions is by no means hypothetical. In yeast H_i^+—K_o^+ exchange is clearly evident (50, 378); in the absence of K_o^+ succinic and other acids gradually appear in the medium (378). An important element in the H_i^+—K_o^+ exchange is its rapidity compared to the rate of escape of the organic acids, and hence such exchange may be considered of importance for the buffering of the cell and probably also for the removal of H^+ from the membrane, where its accumulation could impede reversible H^+ generating reactions (376).

The experiments of Fenn and Cobb (136) have already been mentioned. These showed that in nerve and muscle CO_2 leads to H_i^+—K_o^+ exchange, HCO_3^- becoming part of the fibers' store of indiffusible anions. These are, of course, the familiar elements of the CO_2, potassium-accumulating model of Osterhout and Stanley (355).

The rapidity of the H_i^+—K_o^+ exchange may account for the considerable evidence that intracellular pH is higher than to be expected from a Donnan equilibrium (*e.g.*, 37, 48, 211). The removal of metabolically generated H^+ by K_o^+ may well underlie the general enhancement of respiration and heat production by the increase of extracellular potassium in invertebrate (434) and vertebrate (351) nerve and in muscle (207, 449). Available evidence indicates that potassium acts at the surface. Thus, heat production in muscle is accentuated by potassium far too rapidly to be attributed to penetration (449). Moreover, suspension of tissues in air, which causes some accumulation of potassium in the extracellular space from the intracellular store, also gives rise to increased metabolic activity (133, 190, 449).

According to the model proposed here, the situation in yeast and the effects of CO_2 in nerve, muscle and the Osterhout-Stanley model differ in that ion pair or complex formation does not take place between the anions (succinate or bicarbonate) and sodium. It may be suggested, moreover, that such sodium complexes, by neutralizing the charge of the anions, would facilitate the escape of the anionic portion of the metabolically generated acid (improved "solubility"); this in turn, might be expected to enhance reactions leading to such anions. In keeping with this, all conditions which raise $[Na]_i$—*e.g.*, veratrine, low extracellular calcium, electrical stimulation, and prior metabolic inhibition— increase respiration (21, 126, 387), which thereby can function as a governor to limit potassium loss and sodium gain.

To test for the concept of acid production in active transport one must first determine whether acids are produced in sufficient quantity to satisfy the proposed hypothesis. If not, a more complicated system requiring recycling within the membrane (*e.g.*, 173, 489), or multiple ion formation such as can be demonstrated in solvents of low dielectric constant, must be evolved.

The acid most easily checked is that derived from CO_2 production, which conceivably might appear in the membrane in a manner much more favorable for ion pair or complex formation than when CO_2 is applied externally. In Table 4 the best estimates of maximum equivalents of sodium or of potassium transported are compared with the maximum equivalents of $2H^+$ or $CO_3^=$ which might be available from the oxygen consumed for transport of sodium and potassium. In the case of nerve such hypothetical CO_2 production might suffice, but in muscle it would take care of only half the transport. It is desirable, then, to determine whether other organic acids which appear under aerobic conditions—

TABLE 4

Comparison of sodium or potassium active transport with $CO_3^=$ produced as estimated from O_2 consumption

Fibers	O_2 Consumption	$CO_3^=$		Transport	
	$\mu l/g\ h$	$\mu eq/g\ h$	$\mu eq/cm^2\ sec$	$\mu eq/g\ h$	$\mu eq/cm^2\ sec$
Loligo giant axon	80[1], 150[2]		18, 34		30[3, 4]
Sepia giant axon					38[4]
Frog, toad nerve	50[5]	4		2.4[6]	
Frog muscle	34[7]	2.8		4.4[7*]	
Rat diaphragm	120[8]	10		24[9]	

References: [1](48); [2](219); [3](429); [4](225); [5](524); [6](423); [7](274); [8](529); [9](35).
* Taken as half the sodium outflux (see 108, 473).

e.g., lactate, as in intact brain (170) and in heart slices (518)—may contribute appreciably to the balance sheet. It will be recalled that the equivalents of lactic acid produced in vertebrate nerve under anaerobic conditions can account for the additional equivalents of potassium retained and sodium excluded (414). It is interesting in this connection that in the human red cell the equivalents of acid produced do not exceed the cations transported (101).

Obviously, these possibilities can be considered only suggestive. Careful measurements of oxygen consumption, or better still of production of CO_2 as well as of organic acids, coupled with sodium and potassium flux measurements, should indicate more clearly the extent to which active transport may involve only diffusion of acid metabolites. The action of insulin in stimulating muscle metabolism and potassium uptake (262, 331) appears to offer interesting potentialities for such studies. It is to be hoped they will be extended and include an examination of the labile, indiffusible organic anions which may appear. The acceleration of aerobic acid production by brain slices in elevated $[K]_o$ (95) may also be significant from the standpoint of transport.

5. Cardiac glycosides. Metabolic inhibition is by no means implicated with any degree of certainty in the action of these drugs. In fact, in the light of their effects which indicate a more specific membrane action (Section V C 5), it would be desirable to examine alternatives such as interference with sodium ion pair or complex formation or with $H_i^+—K_o^+$ interchange. The similarity of the action of ouabain on O_{Na} to removal of K_o^+ in muscle (108), whereas intense metabolic inhibition is ineffective (274), certainly is more suggestive of the physical mechanism. According to the model suggested in the section immediately above, ouabain may render the base $B^{(-)}$ unable to combine with H^+ (or possibly K^+); therefore H^+ associates with its organic anion, that normally combines with sodium, hence prevents sodium transport; the organic acid would appear as before, perhaps more slowly, but now without having done any work. Absence of K_o^+ would have the same final effect, with the difference that $B^{(-)}$ becomes unavailable because of its saturation with H^+. Glynn's observation (172) that glycosides affect ionic fluxes in red cells over and above those contributed to by metabolism certainly indicates the desirability of exploring physical effects such as have been proposed.

D. Summary

Natural membranes appear to combine the properties of a solvent of low dielectric constant as well as those of a porous structure. Available data suggest that the penetration of ions into the untreated membrane is governed by the ease with which water can be removed from them (hydration energies) and their unhydrated diameters; and that permeability changes may be due (a) to changes in diameters of the channels through which unhydrated, or incompletely or fully hydrated ions move, and possibly (b) to changes in the membrane-ion interaction (solvation). Stabilization by local anesthetics and related compounds may be accounted for by entry of the molecules into the membrane between the pores or channels, thereby tending to compress the channels but especially interfering with their enlargement under conditions which cause increase of permeability. The stabilizing action of agents such as calcium and low temperature seems more likely to be due, at least in part, to increased rigidity of the membrane. Observations have been noted which suggest that at least some of these phenomena are restricted to the outermost layer of cellular membranes.

Considerably more data are needed to establish the validity of the concepts which have been introduced. The approach presented is susceptible to analysis in living membranes by studies of the permeabilities of and changes of permeability to cations and anions, inorganic and organic, which cover a wide range of diameters and of associated and other physical properties. In such studies, metabolic disturbance should be minimized and its possible role considered when interpretations are attempted. An extension appears desirable of studies such as Skou's to membranes and surface films composed of different fractions of lipid extracts, preferably from tissues which contain the membranes of interest in adequate amounts (*e.g.*, abdominal muscle for endplate lipids), and to a wider variety of drugs as well as to transmitters.

Observations have been described which suggest that sodium transfer in the membrane—active and passive—is at least partly in the form of ion pairs or as part of an uncharged complex. It is suggested that acid products of metabolism may contribute to transport during diffusion if H^+ is kept separate from the anion so that the latter can combine and diffuse with sodium; the former would have to be combined with a base which remains in the membrane but diffuses with it to the surface where membrane selectivity favors H^+—K^+ exchange. The possibility of a recycling carrier within the membrane or of another of the many mechanisms which have been proposed can be entertained only after the rate of total acid production can be shown in specific cases to be unrelated to or inadequate to account for active transport.

VIII. CONCLUSIONS

A. The undisturbed cell

The available facts indicate excitable cells to be labile electrochemical systems—with respect to intracellular composition and particularly with respect to membrane characteristics—carefully adjusted to and quite different from the

environment. This difference from the surroundings is preserved by the selectivity of the membrane for ions which greatly favors the entry of potassium over sodium, and by energy turnover that performs the function of active transport, *viz.*, extrusion or exclusion of Na^+ and uptake of K^+ at a rate sufficient to balance the downhill leak of these ions in the opposite direction. The relative permeabilities to *ions* and their concentration gradients determine the membrane potential. Permeability is governed in large part by hydration energies and unhydrated diameters of the ions. The rate of leak of the ions into and out of the cell is governed by the membrane potential, the difference in ion concentrations, and permeability to the *ions* and also to *ion pairs* if these form.

Energy turnover does not contribute directly to the membrane potential but does so indirectly insofar as it affects membrane permeability and maintains the labile and "fixed" anionic pools and ionic gradients. The importance of metabolism in the maintenance of the steady state increases with the leakiness of the systems (*e.g.*, with temperature). The major function of metabolism in the ionic and electrical phenomena which have been discussed is therefore storage of potential energy in the form of ionic gradients.

Unlike the aerobic metabolism of activity, which increases with the surface-to-volume ratio of fibers to meet the greater relative drain on ionic reserves by activity (421), resting respiratory rate is little different on a wet weight basis for large and small fibers (*e.g.*, 47, 434, 521). In the case of medullated fibers this reflects the low leakage rate made possible by the myelin. Whether other cellular devices reduce dependence on resting aerobic metabolism for transport must await more comparative studies of unidirectional fluxes and their dependence on anaerobic as well as aerobic processes.

Further exploration appears desirable of the possibility that diffusion of acids, produced metabolically, occurs at a rate sufficient to accomplish active transport. It has been pointed out that H_i^+—K_o^+ exchange, accompanied by diffusion of Na^+ with the organic anion in the form of an ion pair or complex, not only would accomplish transport but would contribute to the escape of end products. Other mechanisms are by no means ruled out, however.

The cell membrane, particularly its outermost layer, is visualized as a semi-rigid, semi-fluid structure (the "smectic mixed fluid-crystalline structure" of Schmitt). The diameters of channels through which the unhydrated ions move are considered to be predominantly those of intermolecular spaces that vary somewhat because of thermal agitation of surrounding molecules, but not to the extent possible in a fluid. The entry of ions therefore takes on the characteristics of passage into highly polar nonaqueous solvents but is limited by the semi-rigidity of intermolecular spaces.

B. The treated cell

1. Monovalent ions. Extracellular potassium causes depolarization because of the generally high permeability to this cation, a permeability which is augmented as $[K]_o$ increases because of depolarization. An increase of P_{Na} is also likely with sustained depolarization (2) and possibly of P_{Cl} as well, for in muscle

at lower levels of depolarization (135, 438) and in nerve at higher levels (209, 426) swelling occurs as $[K]_o$ increases, largely through the osmotic ineffectiveness of KCl. The high permeability to potassium is also shown by the considerable increase in membrane conductance with increase of $[K]_o$, an effect due to entry of K^+.

Intracellular changes in potassium concentration of muscle brought about osmotically or by entry of potassium are in accord with its bioelectrical action via selective permeability. Additional data are considered necessary to establish the significance of the negative findings with microinjections of potassium and other ions.

The relative depolarizing effectiveness of cations in nerve and muscle has long been known not to conform to the hydrated diameters. The concept of the membrane as a semi-rigid solvent accounts for the sequence as follows: Cations of small crystal ionic radii (Na^+, Li^+) are kept out by their large hydration energies, those of larger crystal ionic radius enter because of their lower hydration energies (K^+), but much larger (unhydrated) ions (Rb^+, Cs^+) lose the advantage of more favorable hydration energies because of the limited number of large enough membrane channels. The very large crystal ionic radii of anions may suffice to account for the low permeability to them without the need to postulate negative charges in the channels; the presence of negative charge cannot be ruled out until experiments are carried out with smaller anions. In muscle, anion penetration with potassium is again in keeping with unhydrated ionic radii; the relative electrical effects of anions in this tissue suggest an additional factor, possibly adsorption or reduced potassium penetrability, which remains to be clarified.

The importance of ion pairs in sodium and chloride fluxes must also be resolved. A careful analysis of the effect of E_m and of intracellular and extracellular concentrations on these fluxes, in the absence as well as presence of active transport, should do much to establish their relation to the permeability to the ions and ion pairs. Obviously, only the permeability to ions can be of significance for electrical behavior, but that to ion pairs would contribute to ion distribution.

2. Metabolic alterations. Conditions which increase $[Na]_i$ or $[K]_o$ have been pointed out to accelerate metabolism, possibly by facilitating the escape of organic acids which contribute to their transport. Obviously, studies of alleged metabolic effects of physiological and pharmacological agents must be examined for the possibility that metabolic changes are secondary to alterations in the ion distribution. A similar problem of distinguishing cause and effect has been noted in connection with the action of drugs in brain slices, where metabolic changes appear to be secondary to changes in spontaneous activity (24).

When *bona fide* inhibition occurs, the general effect is a decrease in potassium influx. Sodium outflux may decrease or sodium influx may increase depending on whether active transport involves sodium "extrusion" or sodium "exclusion". The latter is a new principle of sodium-potassium transport revealed by studies on vertebrate nerve.

3. Drug action. The vast majority of agents studied change the permeability to ions—some selectively, as in the case of transmitters which interfere with

transmission, some non-selectively, as in the case of stabilizers and depolarizing (excitatory) transmitters and agents.

The non-specific stabilizing action of local anesthetics, antihistaminics, and many other lipid-soluble compounds is attributed to the development of increased lateral pressure by entry of stabilizers into the inter-channel region of the membrane; this in turn tends to compress the channels through which the free ions normally move and interferes with the opening of such channels induced by depolarizing agents. Stabilization may also be brought about by changes in rigidity of the membrane, as with alterations in temperature and calcium content of the medium, by changes in the effective dielectric constant of the membrane, and by the charge of the stabilizers; additional studies are needed to distinguish these and effects due to simple adsorption from the type of stabilization due to entry into the lipid phase of the membrane.

Transmitters and depolarizing agents can exert their effects by acting on the interchannel region to enlarge the channels sufficiently to admit all hydrated ions (non-specific permeability increase), by a more limited increase in channel diameter or by modifying hydration or solvation energies of the ions (specific permeability increase). These possibilities and the nature of the interaction remain to be resolved by studies particularly of junctions and of lipid films with appropriate cationic and anionic series and by use of alcohols which may modify effective dielectric constants.

Cardiac glycosides and related drugs resemble metabolic inhibition with respect to their effects on active transport. But other effects, on fluxes and other membrane properties, make more likely a physical interaction within the membrane which decouples metabolism from ionic movement. In terms of a specific mechanism for active transport—that involving organic acid production by the cell—these may act on sodium combination with a carrier, the anion, or on an H^+–combining (or K^+–combining) component in the membrane. Careful comparison of stabilizer and glycoside action on passive and active ionic movement should contribute to an elaboration of the mechanisms involved.

4. "Specific" drug effects. The physical effects which have been described are so general that great caution is necessary in interpreting the action of experimental agents (anticholinesterases, antihistaminics, *etc.*) on living cells in terms of what are purported to be their specific properties. Careful consideration of the concentrations necessary for their "specific" effects and for physiological or pharmacological effects is undoubtedly helpful. But in the final analysis only a systematic demonstration of the absence of the phenomena associated with permeability and other physical changes, by criteria such as noted in this review, can be considered convincing evidence that their specific action actually is involved.

5. Implications for regenerative activity. This will be discussed in detai iln Part II, but we may note here that considerable evidence is now at hand that excitation and the action potential reflect specific alterations in ion permeability which can be understood in large measure from the principles which have been elucidated for the resting cell. Since the radius of the unhydrated sodium ion is

smaller than that of K^+, the concept of entry as unhydrated ions offers a basis for the rise in permeability to sodium prior to that to potassium during excitation.

6. Anions in skeletal muscle (added in proof). The electrical effects on single muscle fibers of application and removal of K^+ and Cl^-, recently reported by Hodgkin[5], indicate that Cl^- usually redistributes itself quickly between the interior and exterior of the cell according to E_m, which is governed by [K] and [Na] and by $P_{Na} = 0.01\ P_K$. Thus, when $[Cl]_o$ is reduced, a transitory depolarization occurs, presumably due to outward diffusion of Cl^- (with K^+); upon restoration of the original $[Cl]_o$, a transitory hyperpolarization is obtained. The relative electrical effectiveness of K^+ and Cl^- leads to the conclusion that the anion conductance is twice that of potassium. Hence, G_m will be less affected by a given change in G_K than usually assumed, and changes in G_{Cl} will have an appreciable effect. The decrease in R_m when Cl_o^- is replaced by more polarizable anions is therefore due in part to the poorer penetration by the latter; in addition, the other anions reduce O_{Cl} (Adrian, communicated by Hodgkin; communication from Harris).

Single fibers do not exhibit the hyperpolarization, when Cl^- is replaced by other anions, described in the older literature on whole muscle; the older observations may have been due to a reduction in the transitory depolarization caused by a slower exit of Cl^-.

Data on G_m and on the fluxes, especially as affected by E_m, still remain to be obtained, particularly in systems where permeability to the free anions appears to be low.

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