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Review Article

The role of calcium in neurohumoral and neurohormonal extrusion processes

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CALCIUM is a divalent cation, ubiquitous in its biological occurrence, important in its physiological significance. It is an ion of particular interest to investigators of neural and neuro-effector systems. Reviews have appeared that cite the role of calcium in excitation-contraction coupling of striate muscle (Sandow, 1965) and in the maintenance of the nerve membrane during nervous transmission (Abood, 1967). The intention of this review will be to summarize the various experimental evidences that implicate calcium in its role as a necessary factor in the processes of neurohumoral and neurohormonal extrusion. The diversity of physiological foci considered has meant that the discussion could not feasibly cite all primary references. Consequently, an attempt has been made, when applicable, to note recent and well prepared reviews.

The skeletal neuromuscular junction

Synaptology has made its greatest advances in the study of transmission from peripheral nerve to striate muscle. Several authors have made extended comments on the literature (McLennan, 1963; Eccles, 1964; Katz, 1966).

The morphology of the neuromuscular junction has been well described (Birks, Huxley & Katz, 1960; de Robertis, 1964). The nerve ending has a swelling at its terminal in which is contained numerous synaptic vesicles. The vesicles are generally believed to be the containers in which the transmitting substance, acetylcholine, is found. Acetylcholine is released quantally, in packets, from the nerve terminal to produce postsynaptic endplate potentials (epp) and miniature endplate potentials (mepp) (Martin, 1965; Katz, 1966).

As early as the work of Locke, there was recognition that the effectiveness of transmission from nerve to muscle was dependent upon the concentration of calcium ions within the bathing medium. More contemporary work has shown that the amplitude of the endplate potential is affected by calcium (Eccles, Katz & Kuffler, 1941; Kuffler, 1944), and that there is, within a limited range of concentrations, an approximately linear relation between the amplitude of the endplate potential and the concentration of calcium ions in the bathing solution. In the absence of sufficient amounts of calcium, neuromuscular transmission fails (del Castillo & Katz, 1954b).

Even more numerous are the observations on the effect of calcium on

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spontaneous activity at the motor endplate. Spontaneous subthreshold activity was initially described in frog skeletal muscle by del Castillo & Katz (1954b) and Fatt & Katz (1952), and subsequent reports of spontaneous miniature endplate potentials have been noted in mammals (Boyd & Martin, 1956a,b; Liley, 1956b; Hess & Pilar, 1963; Elmqvist, Hofmann & others, 1964), fish (Takeuchi, 1959), birds (Ginsborg, 1960), and invertebrates (Dudel & Kuffler, 1961; Usherwood, 1963). Only the frog and mammal have been subjected to extensive investigations of the relation between calcium and spontaneous activity. For the frog, Fatt & Katz (1952) reported that the frequency of occurrence of mepp's is independent of calcium ion concentrations of the bathing fluid. The calcium independence of spontaneous mepp's, coupled with the calcium dependence of epp's (see above), led to the notion that there exist in the frog two separate processes of acetylcholine release, only one of which was mediated by calcium (del Castillo & Katz, 1954a). Work on the frog involved the toe muscle, m. ext. longus dig. IV. Investigations on *in vitro* mammalian preparations stand in marked contrast to those on amphibian preparations. For both the tenuissimus muscle of the cat (Boyd & Martin, 1956a) and the diaphragm of the rat (Liley, 1956a; Hubbard, 1961), the frequency of mepp's is accelerated by increasing the bathing solution concentrations of calcium. Boyd & Martin (1956a) attempted to reconcile the species difference by suggesting that the alkaline earth metals (group II elements) can accelerate mepp frequency, the mammal being sensitive to calcium and other group II ions higher in the series, the amphibian being sensitive only to strontium and those ions increasing in series number. The notion is consistent with observations made by del Castillo (cf. Boyd & Martin, 1956a) that strontium and barium increase spontaneous potential activity, with barium, the higher of the two ions in the group II series, being more effective.

In addition to work involving increases in calcium, observations have been made on preparations bathed in minimal, or no, calcium. At concentrations below 0·20 mM calcium, no epp is detectable at the frog neuromuscular junction (del Castillo & Stark, 1952). Early work indicated that if calcium were removed from the bathing medium by treatment with ethylene diamine tetra-acetic acid (EDTA), most spontaneous activity at the mammalian neuromuscular junction ceased, but a fraction of spontaneous potentials persisted (Hubbard, 1961). Again, two separate release phenomena were suggested, in this instance both dealing with spontaneous mepp's, but only one requiring calcium as a prerequisite to release. However, more recent work (Elmqvist & Feldman, 1965a) has indicated that if the preparations were given prolonged treatment with EDTA, all mepp production is abolished. Apparently the nerve ending is capable of binding a small store of calcium, and this store, in addition to the free ion in the medium, must be chelated before miniature potentials will cease.

All reports agree that changes in calcium ion concentration do not markedly alter the mepp amplitude unless the change is several-fold above physiological levels, and the effect observed then, is due to a reduction

in the sensitivity of the postsynaptic membrane to acetylcholine (del Castillo & Engbaek, 1954; del Castillo & Katz, 1954a; Boyd & Martin, 1956a; Manthey, 1966).

Thus calcium appears first, to be a necessary synaptic constituent for all neuromuscular transmission, and second, to augment the amplitude of the endplate potential, and third, to increase the rate of occurrence of spontaneous miniature potentials in mammals. At least two sites have been proposed as loci at which calcium could be exerting the listed effects (cf. Katz & Miledi, 1965b). According to one alternative, calcium is intimately involved in the propagation of the nerve impulse (see Abood, 1967). Any alterations in the concentration of the cation would alter the facility with which the spread of depolarization could invade the nerve terminal (e.g., Frank, 1963). A second alternative arises from the combined observations of Fatt & Katz (1952, 1953) and of de Robertis & Bennett (1955) and Palade & Palay; Palay & Palade (1954). The detection of multimolecular yet quantal release of acetylcholine, as indicated by postsynaptic potentials, together with the recognition of subcellular vesicles, has led to the theory that the transmitter is stored in subcellular containers and that the containers spill their contents into the synaptic interspace during transmission. Calcium could be involved in the process in which the vesicles are made to extrude their contents. The evidence is highly in favour of the latter proposal. Three arguments are pertinent to resolving the problem. (i) It is possible to bathe nerve-muscle preparations in calcium-deficient media, and thereby cause severe reduction of the epp (del Castillo & Stark, 1952). If a micropipette is introduced into the calcium deficient endplate environment, and if calcium ions are electrophoretically propelled from the pipette, an enhancement of the endplate response occurs (Katz & Miledi, 1965b). The interval between calcium efflux from the pipette and increase in epp seems insufficient to allow diffusion of the ion to axonal sites of transmission. Rather, the nerve terminal seems the site of activity. (ii) Tetrodotoxin is a biological poison that suppresses bioelectrical potentials in both nerve and muscle, presumably by immobilizing sodium passage across the membrane (Nakajima, Iwasaki & Obata, 1962; Narahashi, Moore & Scott, 1964). Frog sartorius preparations bathed in the poison continue to display spontaneous mepp's, and locally applied current will result in depolarization of the nerve terminal plus a subsequent postjunctional epp. No current spread occurs in the nerve axon, yet addition of calcium facilitates the epp and removal of calcium results in failure of transmission (Katz & Miledi, 1967b). Again, it is the bouton that is implicated as the site of action. (iii) The third argument is perhaps the most germane. The vesicle theory purports that the mepp is the postsynaptic event following the release of acetylcholine from the vesicle content, and the epp is the summed effect of numerous mepp's. It has been reported that appropriate manipulations of the ionic species in the bathing medium of nerve-muscle preparations, particularly of calcium, will produce small but constant increments in the epp (Martin, 1965). Apparently calcium acts to facilitate the release of acetylcholine, and the incremental steps are the result of discrete

increases in the number of vesicles secreting their transmitter substance.

Data on mammalian preparations suggest that both spontaneous and nerve impulse-induced release of acetylcholine are calcium mediated, so both may operate via a common mechanism. The nerve impulse simply increases the probability of the spontaneous, random mepp's occurring synchronously. For the frog, the spontaneous and nerve-induced release are reported to depend on separate mechanisms, only the second being calcium mediated. However, it would be interesting to test the effect of prolonged EDTA treatment of amphibian preparations for possible changes in the frequency of spontaneous activity. Until such work is done, one should be cautious about assigning two processes of acetylcholine release to the frog myoneural junction. The persistence of spontaneous activity by the frog in low calcium media may mean that the creature is capable of binding significant stores of the divalent cation, and only an extended observation in the presence of a chelating agent would uncover depression of spontaneous activity. In the frog, the absence of an accelerating effect by calcium could be explained by the animal relying more upon its stores of bound ion than upon the freely circulating ion.

There is a finite amount of time within which calcium can act in facilitating the neurogenic release of acetylcholine. Measurements of transmission delay at the frog sartorius have produced a modal value of 0·75 msec (Katz & Miledi, 1965a). A short lag time intervenes between nerve terminal depolarization and the subsequent release of acetylcholine. During the lag a sequence of reactions presumably occurs, one step of which involves calcium, that allows the release of transmitter substance (Katz & Miledi, 1965c; 1967c). By bathing nerve-muscle preparations in calcium-deficient solutions, Katz & Miledi (1967c,d) have determined with some precision the point in time of junctional transmission during which calcium is most effective in mobilizing the neurohumoral releasing apparatus. Iontophoretically applied calcium is most effective if it is propelled into the preparation very shortly before a depolarizing pulse, but it is largely without effect if applied during the period of transmission delay. Thus the depolarizing pulse seems to allow the movement of calcium to its reactive site; after the depolarizing pulse wanes the sequence of molecular events transpire that will result in acetylcholine extrusion (Katz & Miledi, 1967a).

Of the various ions that are found in the extracellular space, only magnesium, sodium and potassium have been explored in depth for their interaction with calcium. Magnesium has long been known to interfere with neuromuscular transmission (Jolyet & Cahours, 1869). When preparations are bathed in 20–50 mM magnesium solutions that are otherwise physiological in their content, junctional transmission fails (Engbaek, 1952). The action of magnesium is reported to be the depression of acetylcholine release, and its action can be completely antagonized by the addition of calcium (del Castillo & Engbaek, 1954). Kinetic studies indicate that the amount of acetylcholine liberated from the nerve terminal is directly related to the calcium concentration and inversely related to the magnesium concentration. Hence, quantal emission is

appropriately described in terms of the calcium to magnesium ratio (Jenkinson, 1957).

The frequency of spontaneous activity at the skeletal neuromuscular junction of the frog has been noted as being unaffected by gross changes in the levels of magnesium (del Castillo & Katz, 1954a). A similar suggestion was made for the mammal (Boyd & Martin, 1956a), but subsequent experimentation showed the spontaneous activity at the neuromuscular junction to be depressed (Hubbard, 1961). The latter work also showed the magnitude of depression to be related to the frequency of activity. Consequently, magnesium has a marked effect in the presence of excess calcium and a minimal effect in diminished calcium solutions. Apparently calcium and magnesium compete for some intermediate in the release mechanism of acetylcholine (del Castillo & Katz, 1954a; Jenkinson, 1957; Hubbard, 1961).

Sodium has been discussed as an important factor in the liberation of acetylcholine, the data being based principally upon observations of preparation treated with cardiac glycosides (Birks, 1963). There are two significant drawbacks to such preparations. Firstly, it is difficult to extrapolate evidence from sodium pump inhibitor situations to physiological situations because of the diverse number of actions in which sodium is involved, many of which are not intimately related to junctional transmission. Secondly, sodium pump inhibitors sometimes behave as mobilizers of calcium (Govier & Holland, 1964; Elmqvist & Feldman, 1965b). Other workers have noted a depression of the epp in low sodium solutions, but the lowered response is the result of a reduction in the sensitivity of the postsynaptic element (del Castillo & Katz, 1955). Studies involving the spontaneous release of acetylcholine and sodium levels have been more definite. The frequency of spontaneous mepp's is inversely related to the sodium concentration, and sodium-induced depression is antagonized by calcium (Birks & Cohen, 1965; Kelly, 1965; Gage & Quastel, 1966). Gage & Quastel (1966), in examining the dynamics of the antagonism, proposed either that sodium and calcium compete for a common molecule, or that sodium reduces the ability of calcium to form a complex necessary for acetylcholine liberation. The site of antagonism appears different from that of the calcium-magnesium interaction. Excess potassium has a depolarizing action on the nerve terminal and consequently increases spontaneous activity (Liley, 1956c). The ion has an additional effect of mobilizing the interneuronal stores of acetylcholine (Gage & Quastel, 1965; Parsons, Hofmann & Feigen, 1965). In the presence of raised potassium, increases in magnesium exert a strong depressant effect on spontaneous activity. In comparable situations, calcium is without notable effect (Hubbard, 1961). The absence of an accelerating action by calcium may be due to a masking by the dual actions of potassium.

The adrenergic neuro-effector cell junction of smooth muscle

The juxtaposition of nerve and smooth muscle is somewhat dissimilar

from that of nerve and striate muscle. The cholinergic junction is characterized by a well-defined nerve terminal in which are located the synaptic vesicles (Birks, Huxley & Katz, 1960). The adrenergic nerve, in addition to its terminus, possesses significant numbers of varicosities that impinge upon the adjacent muscle tissue (cf. Burnstock & Holman, 1966). Both the nerve terminal and the individual varicosities contain synaptic vesicles, so both may be able to release catecholamines in transmission (Richardson, 1962; Merrillees, 1963; Burnstock & Holman, 1966). Preparation and analysis of subcellular fractions have provided evidence that noradrenaline and its precursors are associated with vesicles (Potter & Axelrod, 1963; Taylor, Chidsey & others, 1966; Austin, Chubb & Livett, 1967).

Bioelectrical phenomena associated with junctional transmission from nerve to smooth muscle was first described by Burnstock and his coworkers using the guinea-pig isolated vas deferens preparation (Burnstock & Hollman, 1961). Subsequent work has been done on such varied mammalian preparations as the bladder (Ursillo, 1961), the retractor penis (Orlov, 1961), the intestine (Gillespie, 1962), and blood vessels (Speden, 1964). In brief, the potentials recorded at the adrenergic nerve–smooth muscle junction are somewhat similar to those visible at cholinergic neuromuscular junctions. Stimulation of the nerve produces a postjunctional electrical change called an excitatory junctional potential (ejp), which in turn triggers the action potential. Spontaneous electrical activity, termed spontaneous excitatory junctional potential (sejp), can be recorded in the absence of nerve stimulation. Apparently the sejp's summate to produce the ejp, but quantal release of transmitter at the nerve-smooth muscle junction has been difficult to demonstrate. A portion of the problem lies in the inconsistent amplitude of the sejp. The variance may be due to a wide range in the size of the gap between adrenergic nerve terminals or varicosities and the underlying muscle. Such variance in the width of the synaptic interspace would modify the amounts of transmitter diffusing to postsynaptic reactive sites. Presumably the catecholamines are released in quantal form from the prejunctional vesicles.

The effects of calcium on adrenergic nerve transmission to smooth muscle have been described by Kuriyama (1964). If the medium bathing a hypogastric nerve–vas deferens preparation contains less than 0·5 mM calcium, nerve stimulation fails to produce a response in the muscle. Increases in the cation concentration up to 25 mM cause progressive increases in the amplitude of the ejp, as well as prolonging the falling phase of the potential. Repetitive nerve stimulation results in enhancement of the ejp. Elevating the calcium concentration has the effect of further enhancing the facilitatory response during the first few minutes of exposure, while continued bathing causes a diminution of the ejp amplitude. Preparations soaked in a medium with low calcium, with the other ions appropriately adjusted, demonstrated potentials that fluctuated incrementally. Stepwise fluctuations are indicative of quantal release of transmitter. High calcium concentrations first accelerated, then decelerated the frequency of sejp, though the appearance of the spontaneous

activity never entirely ceased. Low calcium concentrations consistently diminished the frequency of the spontaneous potentials.

Other work has shown alterations in the gross behaviour of postjunctional elements in response to changes in calcium levels in the perfusion fluid (Burn & Gibbons, 1964a,b). Stimulation of the periarterial nerves causes inhibition of the pendular movements of the isolated rabbit ileum. Increasing the calcium concentration in the solution from below normal to normal facilitates the inhibitory response produced by shocks to the periarterial nerves. Excess calcium continues to facilitate the inhibition until the cation reaches concentrations of about 17·6 mM. At this concentration calcium no longer magnifies the inhibition of contraction, but it does prolong its duration. Tests with exogenous noradrenaline showed the effect not to be the result of a sensitization of the ileum to the transmitter. Continued repetitive stimulation of the periarterial nerve produces a diminished though constant response in the muscle; addition of calcium provokes further reduction from the contractile response. A noteworthy observation was that preparations bathed for 20 min in medium lacking calcium, upon readmission of calcium, immediately showed inhibition of contraction independent of nerve stimulation. That this event is linked to calcium mobilization of transmitter was shown by pretreating nerve–ileum preparations with reserpine. Those preparations treated with the drug, and thus partially depleted of their cateholamine content, were sluggish in showing the response. Results qualitatively similar to those reported for the isolated ileum have also been noted in isolated rabbit atria (Burn & Gibbons, 1965).

Additional work (Kirpekar & Misu, 1967) described a quantitative relation between the amount of calcium in the perfusion fluid and the amount of noradrenaline released from stimulated splenic nerves. Absence of calcium ions results in near failure of the nerve to liberate any transmitter, whereas replacement of the ion allows normal output. The ratio of noradrenaline output to stimulus could be expressed as a linear function of the logarithm of calcium perfusion levels. Alterations in the calcium ion content of the media appeared to act directly upon the releasing apparatus of the neurotransmitter rather than upon impulse conduction in the nerve fibre. Similar work on the cat colon *in situ* has detected an absence of response in the absence of calcium, plus the spontaneous release of transmitter when the ion is restored to calcium free medium (Boullin, 1966).

Determination of the site at which calcium is active in mediating the release of adrenergic transmitters has not been accomplished. The task is complicated by the presence in the literature of two opposing proposals regarding the liberation of adrenergic neurotransmitters. According to the "classical" theory (see Ferry, 1966), invasion of the nerve terminal by depolarization leads to an influx of calcium. The in-moving cation participates in the sequence of events leading to transmitter release. Opposed to the classical theory is the hypothesis offered by Burn & Rand (1959, 1965). These authors suggest that acetylcholine is released first by the nerve impulse, then in turn provokes the release of catecholamine.

Calcium could act either to promote the release of acetylcholine, as in the cholinergic system, or to promote the release of catecholamine. The authors favour the latter possibility and believe that acetylcholine increases permeability of the nerve terminal membrane to calcium. The choice of the latter rests largely on an analogy with the adrenal gland (see below) rather than on empirical evidence. Nevertheless, the weight of observations appears not to favour the Burn–Rand hypothesis. In two recent reviews, one by Burn & Rand (1965), the other by Ferry (1966), the suggestion is made that the most important evidence for the cholinergic-link hypothesis is the blocking action of adrenergic transmission by botulinum toxin and by hemicholinium-3, both cholinergic blocking agents. Neither observation seems pressing. There are several reports that contest the universal blocking action of botulinum toxin on adrenergic transmission (Ambache, 1949; Vincenzi, 1967), and even the original positive report did not find paralysis of all preparations (Rand & Whaler, 1965). In addition, the cholinergic blocking effect of hemicholinium-3 does not necessitate a similar action at the adrenergic synapse. As Paton has pointed out, hemicholinium-3 is structurally similar to a phenylethylamine, and thus may exert a blocking action peculiar to adrenergic junctions (Paton, 1963). The literature seems to support the assumption that calcium acts at the adrenergic nerve–smooth muscle junction like it does at the cholinergic junction. Nerve depolarization causes calcium to move to some site at which it can greatly increase the probability of synchronous release of transmitter packages.

An interaction between calcium and magnesium has been noted at many of the adrenergic neuro-effector systems studied. Excess magnesium reduces the frequency of spontaneous electrical activity at the vas deferens. Reduction in the amplitude of the ejp follows elevation of the magnesium concentration, and the effect is countered by calcium (Kuriyama, 1964). The response of the ileum to stimulation of sympathetic fibres is antagonnized by magnesium (Burn & Gibbons, 1964a); output of noradrenaline by both the splenic nerve and neural plexus of the colon is diminished in perfusion fluids with high magnesium levels (Boullin, 1966; Kirpekar & Misu, 1967). All the effects are reversed by calcium.

Little work has been done in relating the action of other common physiological ions to that of calcium. Altering the levels of sodium and potassium does not modify cateholamine output at the splenic nerve terminal until such time as nerve conduction is blocked (Kirpekar & Misu, 1967).

Autonomic mediators at cardiac muscle

Reports that elucidate the mechanism of action of calcium at sites of cardiac innervation are few (Midrio & Sperti, 1963; Vincenzi & West, 1965). The negative chronotrophic, or cholinergic, response is augmented by increasing the calcium ion concentration of the bathing fluid. That the effect is cholinergic was proved by reserpinization and resultant inactivation of adrenergic influence. On the other hand, atropinization removed cholinergic influences and showed the positive chronotrophic,

or adrenergic, response to be enhanced by calcium. Apparently, release of transmitter from both the vagus and sympathetic cervical nerves is increased by calcium. Also, results indicated that spontaneous release of neurotransmitters was accelerated with high concentrations of the cation. Huković & Muscholl (1962) have introduced specific evidence favouring a noradrenaline release that is calcium influenced.

Autonomic ganglia

Both the morpology (de Robertis & Bennett, 1955; Taxi, 1961; Elvin, 1963a,b; Hunt & Nelson, 1965) and the pharmacology (Volle, 1966) of autonomic ganglia have been described. The frog and the cat sympathetic ganglion have been the subject of much study. In the mammal, the terminal arborizations of the preganglionic fibre intertwine with the dendritic processes of the postganglionic cell, and numerous functional contacts are obvious. In the amphibian, the postganglionic cell possesses few dendritic ramifications. The principal contact is between preganglionic boutons and postganglionic somas. Several terminals from one fibre may impinge upon a single soma. In all instances, the presence of synaptic vesicles in the prejunctional terminals have been witnessed.

Bioelectrical events at the ganglion are closely analogous to those noted at nerve-striate muscle and nerve–smooth muscle junctions. Both synaptic potentials and spontaneous miniature synaptic potentials have been recorded in the frog (Nishi & Koketsu, 1960; Blackman, Ginsborg & Ray, 1963b,c; Hunt & Nelson, 1965) and the squid (Miledi, 1966). Release of transmitter appears to be quantal, and observations on the ganglion are in keeping with the vesicle theory. Eccles failed to detect miniature synaptic potentials in the mammalian sympathetic ganglion (1955), but Blackman & others suggest the absence of activity results from an insufficient depolarization of the presynaptic terminals. Others have reported that transmission at the mammalian ganglion is quantally composed (Martin & Pilar, 1964). Like the various neuromuscular junctions, ganglionic synapses are markedly affected by fluctuations in surrounding calcium. Synaptic potentials vary directly with the levels of bathing calcium; transmission fails at low levels (Blackman & others, 1963c).

Work on the cat superior cervical ganglion was the first in which the depression of acetylcholine output in low calcium perfusion fluids was reported (Harvey & MacIntosh, 1940). Removal of calcium from the environment produced two distinct effects. Firstly, there ensued a period of rapid and spontaneous firing of the ganglion cells which appeared to be independent of the action of any transmitter. Secondly, synaptic transmission failed. Stimulation of the preganglionic fibres, whether by external pulses or by addition of potassium, failed to produce a peripheral action. Bioassay of perfusion fluid showed the sympathetic trunk did not release acetylcholine whenever transmission was blocked by calcium deficient medium. An additional interesting finding was that the ganglion cells showed transient behaviour that was dependent upon the previous ionic environment. Changes in calcium did not produce constant and

absolute differences in the contractility of the nictitating membrane; rather, differences were relative and a function of the previous concentration of the ion in the bathing solution. Perhaps the cells were binding some amounts of the cation, or, since the experiments were made *in situ*, there may have been a delay in reaching equilibrium with other tissues. Hutter & Kostial (1954) have extended the observations made on the cat ganglion. These workers have noted that increases in calcium from 2·1 to 6·3 mM result in a 40% enhancement in acetylcholine output; raising the cation concentration to 8·4 or 10·5 mM doubled the amounts of transmitter released.

A particularly important set of experiments was reported by Lipicky, Hertz & Shanes (1963). Working with radioactive calcium, they compared the influx of the ion in vagal and superior cervical trunks both at rest and during depolarization. The evidence indicates that calcium enters the nerve from the external environment rather than from binding sites in the membrane. Furthermore, the magnitude of calcium influx can be augmented by adding depolarizing levels of potassium; acetylcholine release is directly proportional to the magnitude of calcium influx. Addition of magnesium to the treatment medium partially prohibits the movement of calcium into the nerve during depolarization. Resting influx of calcium is unaffected by magnesium. These experiments present one of the clearest relations between calcium movement and the mobilization of transmitter. Also, the work indicates that magnesium exerts its depressant action on neurohumoral release not by interfering with the actual release mechanism, but rather by preventing calcium from reaching its reactive site within the nerve.

Several investigators (Hutter & Kostial, 1954; Blackman & others, 1963b,c) have likewise noted ganglionic depression by magnesium. Concentrations of 25 mM magnesium frequently reduced the acetylcholine output to levels too low for bioassay. Transmitter release suppressed by 15 mM magnesium was nearly relieved by 6·3 mM calcium. Low calcium–magnesium ratio fluid diminishes the synaptic potential. Raising magnesium decelerates miniature potential frequency, the magnitude of deceleration being relative to the recurrence frequency. At high recurrence frequencies, magnesium exerts a maximal depression. Potassium depolarization facilitates calcium-induced transmitter release (Lipicky & others, 1963), but by itself potassium is unable to mobilize the releasing apparatus (Harvey & MacIntosh, 1940). Treatment of ganglia with ouabain or digoxin results in increased intracellular stores of sodium and augmented spontaneous release of acetylcholine (Birks, 1963). Whether results like these directly implicate sodium in the extrusion of neurohumoral substance is difficult to decide (see p. 893).

The central nervous system

Though not as accessible to experimentation as the peripheral nervous system, the central nervous system (CNS) has been penetrated by several investigators. Reports of the presence of synaptic vesicles within brain structures are well documented (de Robertis, 1964; Whittaker, 1965).

Although several substances have been suggested as CNS transmitters, none has fulfilled the criteria for acceptance as a brain transmitter. Acetylcholine appears to be the neurohumour secreted onto Renshaw cells in the spinal cord (cf. Kuno & Rodomin, 1966). Electrical activity in the brain has been recorded by authors too numerous to cite; much credit goes to Bremer for his initial recognition of EEG activity. Spontaneous activity has been noted in both the brain (Li, 1959) and the spinal cord (Brock, Coombs & Eccles, 1952; Katz, 1966; Kuno, 1964). Transmission in the cord seems quantal in nature (Katz, 1966; Kuno, 1964).

Reports of cationic influence on CNS synaptic transmission are scarce. Distortions in body fluid levels of calcium and magnesium provoke a variety of disturbances that may be associated with disruption of synaptic activity, though alterations in membrane stability may also be implicated (see Katzman, 1966). Hypocalcaemia frequently results in seizures and modification of the normal EEG. Hypercalcaemia may provoke mental imbalance, and less often seizures and cerebellar ataxia. Magnesium is well established as a CNS depressant (Engbaek, 1952), and excessive amounts will suppress the EEG as well as producing an anaesthetic state. Administration of calcium acts as an antidote to hypermagnesia. Krnjević, Randić & Straughan (1966) have described an inhibitory process in the cerebral cortex that is impervious to iontophoretically applied magnesium, though Randić & Padjen (1967) have shown the release of acetylcholine from the exposed cerebral cortex to be strongly influenced by the presence of calcium.

In the spinal cord of the frog, synaptic transmission between dorsal roots 7–9 and the associated motoneurons is depressed by magnesium; high magnesium Ringer solution prohibits reflex activity (Katz & Miledi, 1963). Elevation of calcium to 6–10 mM was "partially successful" in ameliorating the magnesium block. It is intriguing that in the frog spinal cord, as at the frog neuromuscular junction, raising the magnesium concentration does not alter the frequency of spontaneous activity. The effects of calcium have not been reported.

Glandular secretion

The adrenal medulla is the paradigm of a sympathetic neuron and consequently is a liberator of catecholamines. Catecholamine storage vesicles have been obtained upon differential centrifugation of the homogenized organ (Blaschko & Welsh, 1953; Hillarp, Lagerstedt & Nilson, 1953). A simplified version of junctional events seems to be: (i) depolarization of the adrenal nerves and subsequent release of acetylcholine, (ii) stimulation of the adrenal gland by acetylcholine, and (iii) release of catecholamines from the chromaffin cells of the adrenal medulla. Calcium is the necessary factor for chromaffin cell extrusion of sympathomimetic agents.

Work describing the action of calcium in mobilizing the release mechanism of adrenal catecholamines has been presented largely by Douglas & Rubin (1961, 1963), Douglas & Poisner (1962) and Douglas

(1966, 1967). The investigations show mainly the amounts of catecholamine released from the adrenal medulla as a function of the type of fluid with which the organ is perfused. Glands were typically treated either by acute denervation or by hexamethonium to insure that effects were the result of direct actions rather than indirect actions via the nerve. Output of catecholamine by the resting gland bathed in Locke solution is between 0·05–0·1 μg/min. Addition of acetylcholine to a concentration of 10^{-5} μg/ml may evoke up to a 200-fold increase in amine output. The augmented output by acetylcholine is unaffected or enhanced by the removal of potassium or sodium from the perfusion fluid. Removal of calcium results in a dramatic loss by acetylcholine in its ability to evoke secretion of catecholamines. Maintaining acetylcholine at a concentration of 10^{-5} μg/ml while elevating calcium to 17·6 mM doubled the adrenal output. Addition of potassium to levels sufficient to cause depolarization of the chromaffin cells acted like acetylcholine in promoting the output of amines, but only if calcium were also present. Although both acetylcholine and excess potassium excite large secretions into otherwise normal Locke solution (tonicity adjusted), the excitatory effect is transient and eventually output falls to lower levels. Doubling and redoubling calcium ion concentration in the medium once output has stabilized will again provoke increases in medullary secretion. Finally, addition of calcium, even in the absence of any stimulation, to a preparation that had previously been calcium deprived, results in the spontaneous release of amines.

Studies with ^{45}Ca have indicated that there is a movement of calcium into the gland during activity. The influx of calcium in the resting medulla is 0·50 μmole/mg/sec. Stimulation of the organ (10^{-5} μg/ml acetylcholine) causes an eightfold increase in calcium influx.

The summed data of the various experiments have led the authors to propose that calcium is a stimulus-secretion coupler. Acetylcholine stimulates the chromaffin cells and thereby causes an increase in the permeability to calcium. Calcium moves to a reactive site at which it promotes the extrusion of catecholamine; thus the cation serves as the link between chromaffin cell stimulation and chromaffin cell secretion. The hypothesis had been advanced that calcium acts directly upon the storage granules to cause release of the granule contents into the cell and then diffusion outward (Philippu & Schumann, 1962), but the idea has been contested (Greenberg & Kolen, 1966). The granules apparently release their amine directly to the exterior of the cell.

Exposure of the adrenal glands to magnesium results in suppression of medullary output whether the stimulus is acetylcholine or excess potassium. Addition of significant amounts of calcium overcomes the suppression. Observation on the movement of ^{45}Ca in the presence of magnesium showed the movement into the gland to be severely depressed (Rubin, Feinstein & others, 1967). The observation closely parallels that made by Lipicky & others (1963) (see p. 898) and suggests that magnesium interferes with the movement of calcium rather than the mobilization of hormone. When magnesium is added together with calcium to previously calcium-deprived glands, spontaneous output of catecholamines is

reduced; when magnesium is in the calcium deprived preparation, reintroduction of calcium provokes no sponataneous output (Douglas & Rubin, 1963).

Secretory phenomena in the hypothalamo-hypophysical system may be similar to that of the secretomotor-adrenal system. Granules that probably contain the secretory substance have been reported (Palay, 1957; Gerschenfeld, Tramezzani & de Robertis, 1960). More particularly, the secretion of vasopressin appears to be calcium dependent (Douglas & Poisner, 1964a,b). Experiments on the isolated posterior pituitary show that vasopressin is secreted from the gland after electrical or excess potassium stimulation, providing that calcium is in the preparation. Acetylcholine is without effect, but since the isolated gland contains only the neurosecretory terminals rather than the somas, the absence of an acetylcholine action does not remove the possibility of the cells being cholinoceptive. However, several disparities do exist between calcium-provoked hormone extrusion in the adrenal and in the posterior pituitary. In contrast to the former, there is a definite limit to the secretory augmenting effect of calcium in the pituitary. Vasopressin release first increases, then decreases as calcium levels are elevated. A second disparity is that the reintroduction of calcium into calcium-free Locke solution does not cause the spontaneous release of vasopressin. Additional dissimilarity appears in noting the movement of ^{45}Ca. As might be expected, depolarization of the cells by potassium leads to ^{45}Ca influx. Addition of magnesium slightly depresses the influx of calcium, yet it markedly depresses vasopressin secretion. The conclusion drawn by the investigators is that calcium acts at the pituitary in the same way as it does at the adrenal to promote the secretion of hormone, but other mechanisms must also be involved.

The submaxillary gland parallels the behaviour of the adrenal and the pituitary glands sufficiently to warrant brief comment. The gland receives both adrenergic and cholinergic innervation. Solutions devoid of calcium failed to evoke the normal secretory response to either acetylcholine or noradrenaline. The magnitude of glandular output upon cholinergic stimulation was directly related to the calcium concentration within a 0·8 mM limit. Magnesium did decrease the response to cholinergic stimulation, but the effect was not impressive (Douglas & Poisner, 1963).

Proposed mechanisms

The available literature seems overwhelming in evidencing that chemical extrusion following excitation, whether from gland, neurosecretory terminal, or ordinary nerve bouton, is calcium dependent. In a number of instances, even the spontaneous release of chemical mediators is modified by the amounts of calcium within the environment. The level of existing awareness has moved from one of attempting to implicate calcium in extrusion processes to one of attempting to decipher the nature of the calcium link in the processes. At least two calcium-linked mechanisms have been sufficiently often witnessed to warrant proposals regarding the molecular activities involved: (i) the mode of action of calcium in

evoking, upon its reintroduction, spontaneous release of catecholamines from preparations that had previously been bathed in calcium-deficient medium, and (ii) the mode of action of calcium in promoting, after excitation, the synchronous release of large numbers of preformed packages of transmitter.

The first mechanism has been reported to be operative at both the adrenergic nerve terminal (see pp. 893–896) and at the adrenal medulla (see pp. 899–901). That these two sites demonstrate a similar behaviour may be more than coincidental. All peripheral adrenergic neurons are postganglionic sympathetic structures, and indeed, the adrenal medulla is a structural analogue of a postganglionic sympathetic neuron. Consequently, the mechanism proposed by Douglas (see Fig. 1) for the adrenal chromaffin cell may be very similar to those that function at the adrenergic nerve terminal. As Fig. 1 indicates, calcium is located both free in the

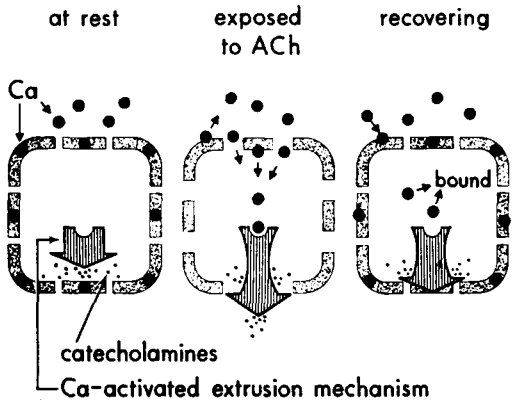

FIG. 1. Proposed role of calcium in catecholamine release. When calcium is located in the membrane, the membrane is stabilized and little transmitter substance is released. Upon exposure to ACh, the membrane loses calcium, thereby allowing the outward passage of transmitter. Recovery involves a relocating of calcium in the membrane. (After Douglas, 1967.)

extracellular space, and bound in the chromaffin cell membrane. Acetylcholine released from secretomotor nerves acts upon the membrane in such a way that the bound calcium is loosened. The result of the breakdown in membrane calcium binding is that the membrane subsequently becomes highly permeable to extracellular calcium. The in-moving cation proceeds to the reactive sites at which it can promote the extrusion of hormone. Recovery involves the rebinding of calcium by the membrane and accompanying loss of cell permeability to the cation. During those unusual experimental periods when no calcium surrounds the gland, some of the ion is lost from the membrane. Thus, when normal medium is reintroduced, the membrane is "leaky" until it can resequester its quotas of calcium. During the interim, the ion moves through the leaky membrane to evoke hormone extrusion. The proposal is compatible with the observation that readmission of calcium to deficient medium

promotes a transient, spontaneous release of catecholamines. It also explains the interaction between calcium and magnesium in relation to the observed phenomenon. When both ions are added to deficient medium, the amount of amine released is less than if only calcium is added. In this instance, magnesium acts by prohibiting calcium from participating in amine release. When magnesium is included in the deficient medium, readmission of calcium causes no spontaneous release. In the second case, magnesium simply substitutes for calcium in stabilizing the membrane. The ability of magnesium to act as a substitute in the membrane has been noted by others (Frankenhaeuser & Hodgkin, 1957).

Though the mechanism may be similar to that evoking release of amines from adrenergic nerves, it would be unwise to extend the notion to other structures. At the cholinergic myoneural junction, insufficient experimentation is available for drawing conclusions. Investigation of the hypothalamo-hypophysial system has revealed no such mechanism (Douglas & Poisner, 1964a).

A statement of the molecular events by which calcium links excitation and extrusion has not yet been elaborated. In fact, slight modification of the middle diagram in Fig. 1 adequately describes all systems thus far investigated. Alter the phrase "exposed to ACh" to "exposed to synaptic transmitter", and redesignate the catecholamines as being any neurohumour or neurohormone, and one has a universal scheme. Synaptic transmitters promote the influx of calcium, and calcium promotes the efflux of another chemical mediator. For the cholinergic neuromuscular junction, several diagrammatic variations on the scheme have been advanced. Del Castillo & Katz (1954a) have proposed that within the sequence of reactions leading to neurohumoral release, there is a reactive site or carrier molecule for which calcium and magnesium compete. Only the Ca complex is effective in transmitter release. To account for the calcium-independence of spontaneous mepp's in the frog, they further proposed that the hypothetical reactive mechanism could spontaneously revert to an active form which would promote extrusion. The work of Gage & Quastel (1966) introduced another variation to the basic scheme. Besides the antagonism shown by magnesium, calcium is also antagonized by sodium, at least in terms of the recurrence frequency of mepp's. And even excess calcium will depress the mepp frequency. The model intended to explain the additional antagonism indicates that the interaction is of a second or higher order. Consequently, Na_2Y or Ca_2Y (Y being the hypothetical site or molecule) will depress quantal release. One easily sees that either excess calcium or sodium could depress output. Another point to note is that the authors allow that the hypothetical mechanism for which calcium and magnesium compete may be distinct from the hypothetical mechanism for which calcium and sodium compete.

It was remarked above that no molecular scheme has been advanced to explain the specific mechanism by which calcium effects extrusion. However, if various lines of research tangential to that being reviewed are brought into perspective, a feasible mechanism appears. The mechanism is offered within the context of the assumption that extrusion from

all neural and glandular structures considered is roughly equivalent. The common appearance of vesicles, of quantal release of substance, and of excitation-extrusion dependence on calcium, make the idea not too presumptive.

The first line of research to be considered is that dealing with the structure of synaptic vesicles. Electron microscopic evidence has been presented that the vesicle is composed of an orderly array of osmiophilic and osmiophobic units, but the array does not conform to the unit membrane design (di Carlo, 1967). The observation lends credence to a hypothesis offered earlier by Burton, Howard & others (1964) and Howard & Burton (1964). Studies on the subcellular distribution of bound acetylcholine and ganglioside showed the two to be localized similarly in the brain, both within the nerve ending. This work, together with their observations on the physical characteristics of ganglioside, led Burton & others to propose the hypothesis illustrated in Fig. 2. The vesicle is

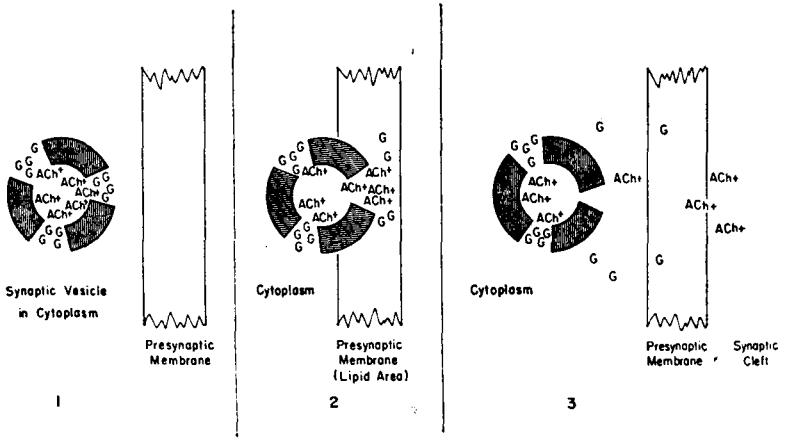

FIG. 2. Proposed mechanism for the vesicular release of ACh. 1. The resting state: the nerve membrane and a vesicle before release of the transmitter. 2. After the arrival of the nerve impulse: the vesicle has migrated to the nerve membrane in which it is partially soluble allowing transmitter release. 3. Recovery: migration of the vesicle away from the nerve membrane. (From Burton, Howard & others, 1964.)

built of ganglioside aggregates about which is a rigid structure, perhaps protein. As a result of the nerve impulse, the vesicle migrates towards the internal surface of the presynaptic membrane. The ganglioside of the vesicle is soluble in the lipid of the terminal membrane, so the ganglioside aggregate serves as a potential pore through which the transmitter can diffuse.

Quarles & Folch-Pi (1965), in a different set of experiments, have reported the effects of several cations, including calcium, on the distribution of gangliosides in an organic-aqueous biphasic system. The data show that when Ca is in the upper (organic) phase at concentrations below

5 or above 160 mM, all the ganglioside is found in the organic phase. At calcium concentrations between 5–160 mM, the ganglioside partitions to the aqueous phase. Sodium, potassium and magnesium have virtually no effect on the distribution of ganglioside. The most intriguing finding was that the ganglioside, when contaminated with protein, was induced by calcium to localize at the interface of the aqueous–organic phases.

Finally, some scant evidence is available to suggest the site at which calcium may be physiologically active. Botulinum toxin suppresses the release of acetylcholine from the cholinergic nerve terminal, and the poisoning can be partially antagonized by calcium (Thesleff, 1960; Simpson & Tapp, 1967a). Toxin and calcium act at separate steps in the release mechanism of acetylcholine (Simpson & Tapp, 1967a), and the toxin localizes within the synaptic interspace (Zack, Metzger & others, 1962), which suggests that calcium must act at the membrane or within the neuroplasm. In fact, papers have been cited in this review that reported the inward movement of calcium associated with subsequent chemical extrusion. Owing to the frequently noted release of transmitter in packages, it seems appropriate to assume that calcium acts at the membrane to promote release of transmitter from the vesicles.

The various pieces of research listed suggest the following scheme. The synaptic vesicles, composed of ganglioside and protein, are in constant intracellular movement. Collision with the internal surface of the membrane, be it neural or glandular, is frequent. On the occasion of a nerve impulse, calcium moves into the cell. For a brief time, the concentration of calcium at the surface of the membrane is high enough to allow the collision between ganglioside and the organic component of the membrane to persist. In addition, the presence of protein in the vesicle promotes the ability of calcium to orientate the vesicle at the interface of the neuroplasm (aqueous phase) and the membrane (lipid phase). Thus calcium evokes the extrusion of transmitter. The mechanism can be expanded to explain other events. During periods when the nerve or gland is at rest, only small amounts of calcium would reside near the internal surface of membranes. Again, at very low concentrations of calcium the effectiveness of ganglioside-lipid contact is facilitated. Spontaneous secretory activity could be viewed as a consequence of low intracellular, or low intramembranous, concentration of calcium. Those intermediate concentrations of calcium at which ganglioside is partitioned into the aqueous phase also have a physiological counterpart. Katz & Miledi (1967c) have applied lengthy depolarizing pulses to nerve terminals in studying the release of acetylcholine (see pp. 889–893). If the duration of the pulse is not excessive, the amount of acetylcholine released is a function of the pulse length. However, as the pulse is lengthened, there is an increasing time lag before liberation of acetylcholine. Apparently lengthening the pulse augments the influx of calcium, the ion moving down both concentration and electromotive gradients. According to the proposed mechanism, as long as the pulse persists, calcium can flow through the membrane. Its concentration may be such that it promotes the stabilization of vesicles in the aqueous neuroplasm. Upon cessation of the

pulse, the concentration of calcium at the membrane increases, thereby enhancing extrusion as depicted above.

In view of the earlier scheme, namely, that of the calcium–magnesium and calcium–sodium interactions, the proposed mechanism would be too simple if it relied only on the physical presence of calcium to evoke extrusion. Obviously calcium must be situated in some critical relation to a reactive site on the membrane. During periods of rest, there are a finite number of transmitter releasing sites available (Gage & Quastel, 1966). During activity, the number of releasing sites must increase. Calcium is known to bind to artificial membranes composed of phosphatidylserine and phosphatidylethanolamine (Rojas & Tobias, 1965). Both phospholipids are accepted as constituents of membrane. It may be conjectured that temporary fixation of calcium by a lipid component of the membrane exposes calcium for its augmentation of secretion, and the calcium-lipid complex allow the conformational changes in the membrane that will promote outward diffusion of the transmitter.

An attempt can now be made to consider the implications of the ionic interactions listed earlier. The suppression of calcium influx by magnesium indicates competition for a carrier molecule. 5-Hydroxytryptamine has been suggested as a calcium carrier at smooth muscle (Woolley, 1958) and perhaps at the nerve terminal (Boroff, DasGupta & Fleck, 1963). Work in our laboratory (Simpson & Tapp, 1967b) has not favoured the latter idea. In any event, the principal action of magnesium is to prevent calcium from reaching the site at which it promotes transmitter release, and the hypothetical molecule remains unidentified. In addition, it should be recalled that magnesium only slightly reduces calcium influx in the posterior pituitary, but the ion dramatically reduces vasopressin output. A discussion of this phenomenon will be momentarily deferred. Work at the frog myoneural junction introduced the possibility that sites for spontaneous transmitter release in the frog can promote release in the absence of calcium. This is not an unreasonable proposition. The sites at which calcium is temporarily fixed during nervous activity may spontaneously make the conformational changes necessary for transmitter diffusion. Also, there is little difference between low-calcium and no-calcium in terms of effective ganglioside retention in the lipid. The only difference would be in the ability of calcium to bind with the ganglioside-protein moiety at the neuroplasm–membrane interface. Yet, before attempting extended explanations, the experiments with EDTA offered on page 892 should be undertaken to insure that spontaneous events in the membrane could actually occur.

Two loci readily suggest themselves as potential areas in which calcium and sodium could compete. The locus could be extracellular, and competition between sodium and calcium would determine influx of calcium. Or, alternatively, the locus could be the releasing site in the membrane. The latter is suggested by various observations. If sodium and magnesium do compete with calcium at sites of intracellular movement, then a resultant sequence would be: magnesium prevents calcium from moving to the site at which sodium prevents calcium from moving to a releasing site,

or vice versa. The sequence seems teleologically unsatisfactory. Some experimental support is offered by the fact that phospholipids which bind calcium also bind other inorganic ions, sodium and magnesium included (Rojas & Tobias, 1965). Sodium fixed to the appropriate membrane structure could represent a nonfunctional releasing site. This could also explain the action of magnesium in the pituitary. A portion of its action is to depress influx of calcium; a supplementary action could be to fix to the releasing sites and thereby render them nonfunctional. Caution has been exercised thus far in not using the term "reactive site". Rather, it seems wise to assume that there are a number of sites from which release can potentially take place, but only when calcium is fixed there is the site reactive. This caution allows the possibility that when one calcium ion fixes to the site, the site is functional. When more than one calcium ion, i.e., Ca_2Y, or some other inorganic ion becomes bound to the site, release is prevented because the site is not reactive. In this context "reactive" means the ability to lodge the vesicle for a sufficient time for humoral or hormonal release.

Recent observations by Rahaminoff (Dodge & Rahaminoff, 1967; Rahaminoff. 1968) indicate that the rate of packet release in a single epp is nearly proportional to the fourth power of CaY. Consequently, there may be as many as four, or some multiple of four, effective CaY bindings involved in the emission of a single packet of transmitter.

Admittedly an assumption was made in assuming that all extrusion processes are roughly comparable. However, the universal excitation–extrusion effect of calcium, the calcium influx antagonism by magnesium, and the quantal release of transmitter, all make the assumption tempting. They also encourage the comparison of data from dissimilar preparations. Gage & Quastel delineated a calcium–sodium antagonism at the mammalian myoneural junction (Gage & Quastel, 1966), and the mechanism drawn here from the available literature attempts an explanation involving the releasing site. Douglas & Rubin (1961, 1963), Douglas & Poisner (1962) and Douglas (1966) showed that removal of sodium facilitated catecholamine release from the adrenal medulla. Certainly a common mechanism at both sites is suspect. The proposed mechanism also facilitates the explanation of several physiological observations. One pertinent to the discussion will be considered here. In examining the kinetics of the calcium–sodium interaction Gage & Quastel (1966) suggested that the resting nerve must have available only a small number of sites for calcium-ion action. Increasing calcium to high levels depresses spontaneous secretion. The constant release of small amounts of transmitter is necessary for maintaining the functional integrity of receptor structures (see Katz, 1966). Were more than a limited number of releasing sites available, the level of calcium at the membrane could reach a concentration sufficient to stabilize the vesicles in the neuroplasm. Obviously this situation would not promote release. The existence of few rather than many releasing sites is actually necessary for maintaining the constant secretion of mediators. Only on the arrival of a nerve impulse does the

number of releasing sites increase enough to allow adequate calcium for promoting vesicle-in-membrane stabilization.

A summing-up

There can be no question of the involvement of calcium in the release of several chemical mediators. The ion has been implicated at various neural and neuro-related structures. Evidence presented thus far has been largely quantitative, i.e., modifying the calcium concentration to a specific degree will result in measurable alterations in humoral or hormonal output. Explanations to account for the action of calcium are few and unspecific. An attempt has been made here to collect from the literature a mechanism that is amenable with the quantitative observations. The mechanism explains processes of extrusion in terms of critical calcium concentrations at or within the cell membrane. Both the presence of calcium and its physical relation to phospholipid in the membrane are necessary for effective extrusion. In view of the relative naivety of the literature with respect to molecular procedures and membrane architecture, the mechanism discussed may well be largely of theoretical value only. It therefore seems appropriate to indicate at least one possible course of investigation into the model. Since it is possible to isolate synaptic vesicles in a fraction of some purity, and since phospholipid monolayers are constructed without great difficulty, it would be appropriate to expose vesicles to an artificial system in which there is a phospholipid monolayer. Application of a modest electrical bias could be used to mobilize the vesicles. It would be important to determine whether calcium, when fixed to the artificial lipid membrane, does in fact have the capacity to stabilize vesicles at the interface of an aqueous-lipid biphasic system. Even if such a demonstration were not forthcoming, study of the system would possibly suggest alternatives to the mechanism proposed herein.

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