Br. J. Pharmac. (1968), 34, 451–474.

THE FIRST GADDUM MEMORIAL LECTURE

CAMBRIDGE: SEPTEMBER 1967

Stimulus-secretion coupling: the concept and clues from chromaffin and other cells

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One of the main influences on John Gaddum's astonishingly fruitful career in pharmacology was his early exposure, at the National Institute for Medical Research in Hampstead, to that borderline discipline between physiology and pharmacology that Henry Dale was to term autopharmacology. At Hampstead, and throughout his life, Gaddum's experiments were concerned mainly with pharmacologically active substances extractable from tissues. His contributions in this field are too many to list here, but I should like to remind you of a few that illustrate the main theme of his work. It was Gaddum, you will remember, who, along with U. S. von Euler (1931), discovered substance P. It was Gaddum, too, who demonstrated, with Chang (1933), that the sympathetic chain was rich in a substance with the physiological properties of acetylcholine and who put forward the idea that " acetylcholine might play a part in the normal transmission of impulses having in view its stimulating actions on ganglion cells." This finding in turn led him to the experiment, with Feldberg (1934), demonstrating release of acetylcholine from the stimulated superior cervical ganglion, an experiment that became the cornerstone of the concept that neurones communicate with one another by chemical means. And, of course, Gaddum was to continue throughout his life in this autopharmacological vein contributing, for example, to knowledge of the physiology and pharmacology of the transmitter substance of adrenergic nerves and other autacoids such as histamine, 5-hydroxytryptamine and bradykinin. Among his many contributions was the discovery, in 1952, of 5-hydroxytryptamine within the brain (see Amin, Crawford & Gaddum, 1954). This was shortly followed by the demonstration that lysergic acid diethylamide (LSD) is a potent antagonist of 5-hydroxytryptamine and by the suggestion that LSD might owe its mental effects to interference with the normal action of 5-hydroxytryptamine (Gaddum, 1953; Amin *et al.*, 1954). It is now evident that these experiments, and the conclusions drawn from them, gave powerful impetus to the study of transmitter substances within the brain and to the development of the field of psychopharmacology.

In this first Gaddum Memorial Lecture it is, then, appropriate that the topic be an autopharmacological one, and the Trustees of the Gaddum Memorial Fund

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doubtless had this in mind when they asked me to discuss the mode of action of acetylcholine as a transmitter at the adrenal medulla, a problem that has been the principal focus of interest in my laboratory for some years now. I am deeply honoured by this invitation and happy to share this honour with those who have participated in different phases of this and related work: Drs Ishida, Kanno, Mikiten, Poisner, Rubin and Sampson. I am particularly delighted to have this unique opportunity of paying tribute to John Gaddum for although I was never privileged to work with him, he early won my affection by encouraging my first faltering steps in pharmacology. This was typical of Gaddum that he used his profound understanding of physiology and pharmacology not as a stick to bring the novice to heel but as an instrument to guide him and support him.

The stimulant effect of acetylcholine on the secretion of adrenal medullary hormones is one of the most familiar phenomena in autopharmacology. From the standpoint of those exploring the workings of the nervous system thirty years ago its importance lay in the fact that it provided evidence for the cholinergic nature of preganglionic sympathetic nerves (Feldberg & Minz, 1934), a conception now firmly established. Today I shall discuss quite another aspect of the phenomenon, one concerned with postsynaptic events rather than presynaptic ones; and my main purpose will be to describe what is known of the way acetylcholine stimulates the chromaffin cells of the adrenal medulla to liberate the catecholamines, adrenaline and noradrenaline. The picture is at present far from complete, but from evidence accumulated in the last few years the main features can be distinguished. To those who are unfamiliar with the field it may seem curious that there has been such a long delay between the discovery that acetylcholine is the immediate physiological stimulus to the chromaffin cells and the emergence of evidence on how it acts. But there are many reasons for this, as will become evident as I describe various facets of the problem. I would like to point out here only that progress in understanding corresponding events in other secretory cells has been no more rapid. Indeed, it may fairly be said that relatively little is known of such processes in other cells: in many instances the chemical nature of the stimulus for secretion is obscure and it must be referred to by some term such as "releasing factor"; and even where the stimulus (or physiological secretagogue) has been identified, there is only fragmentary evidence about how it acts. All this, of course, lends special interest to the findings on the chromaffin cell, for in the knowledge that nature is parsimonious and frequently employs the same basic mechanism to a variety of ends it seems not unlikely that the chromaffin cell may prove to be a model for secretory activity in other cells. This, in any event, was the hope that Rubin and I harboured when we began studying secretion from the adrenal medulla eight years ago. We were encouraged by the historical fact that the adrenal medulla had already contributed several findings of general significance. Not only had it offered an early clue to the existence of cholinergic transmission at sites other than the parasympathetic neuroeffector junction (Feldberg & Minz, 1934) but, still earlier, it had yielded the first chemically characterized hormone, adrenaline, and it was this, in turn, that allowed Elliott (1904) to formulate his prescient scheme that sympathetic nerves act by liberating adrenaline. In retrospect, I suppose Rubin and I might equally well have taken all this as evidence that the adrenal medulla had already provided more than its fair share to the development of biological concepts and should have been less optimistic about its possible contributions to knowledge of the cellular events involved in secretion. But there was no denying that the

adrenal medulla was a good place to begin studying what we were later to call " stimulus-secretion coupling " (Douglas & Rubin, 1961). First, the stimulus to the chromaffin cells was known to be acetylcholine (ACh), a substance readily available, easy to administer, and to which pharmacological antagonists were available. Second, the anatomical arrangements allowed for perfusion so that the extracellular environment of the medullary chromaffin cells could be manipulated at will and secretions recovered quantitatively. Third, the secretory products of the cells were present in enormous amounts and secretion was generally conceded to be a matter of release of preformed materials: thus secretion could be studied uncomplicated by synthesis. And, finally, the secretory products, the catecholamines, were themselves simple chemicals easy to measure.

As it has turned out, several of the factors in the chain of events linking stimulus to secretory response in the chromaffin cell can now be recognized in other secretory cells, and there are experimental grounds for believing that the adrenal medulla may indeed be a useful model for studying " stimulus-secretion coupling." But before I plunge into discussion of chromaffin cells and other secretory systems, perhaps I should offer some explanation of this term. It is, of course, patterned after the phrase " excitation-contraction coupling " applied by Sandow (1952) to related events in the field of muscle physiology. But Rubin and I avoided the word " excitation " since we sought a term that would be as broad as possible and encompass the initial stages of stimulation, including anything that might conceivably be described as " excitation." The term " stimulus-secretion coupling " is thus intended to embrace all the events occurring in the cell exposed to its immediate stimulus that lead, finally, to the appearance of the characteristic secretory product in the extracellular environment.

The adrenal chromaffin cell

One of the principal reasons we adopted the chromaffin cell for the initial studies of stimulus-secretion coupling was that much was already known of the way acetylcholine acts at certain other sites of cholinergic transmission. This information did not derive from the other sites at which acetylcholine acts as a secretagogue, the exocrine glands—for there its mechanism of action was just as mysterious as in the medulla—rather it came from synaptic sites where acetylcholine's function is to elicit quite different responses: stimulation or inhibition of nervous or muscular activity. There was, for example, clear evidence that acetylcholine functions as a transmitter at the neuromuscular junction by acting on the plasma membrane of the receptive cell ; indeed, the elegant experiments of Del Castillo & Katz (1955) had shown that acetylcholine acts on the *outside* of this membrane. And a similar locus of action was generally held to account for acetylcholine's transmitter effects at other sites such as the synapses between neurones, or those between parasympathetic terminals and the heart. Moreover, in each instance the effect of acetylcholine on the membrane was similar in that it involved an increase in permeability to commonly occurring ions. The species of ions to which the membrane became more permeable admittedly differed from site to site, but always it was the movement of the ions across the plasmalemma and the accompanying changes in membrane potential, depolarization or hyperpolarization, that initiated the functional responses of the different post-synaptic elements, end-plate, smooth muscle, AV node, or neurone. To Rubin and me it seemed worthwhile testing

whether similar events might explain acetylcholine's stimulant effect on secretion. Quite soon we had results favouring the idea, for it turned out that the secretory response of the adrenal medulla was critically dependent on the extracellular concentrations of commonly occurring cations (Douglas & Rubin, 1961, 1963). I shall give an account of these results shortly, but I think it better to begin by describing subsequent experiments, of a different sort, that offer more direct evidence that ACh acts on the plasma membrane of the chromaffin cell.

Intracellular recording from chromaffin cells and the effect of acetylcholine on membrane potential

In these more recent experiments my colleagues and I have applied to the chromaffin cell the electrophysiological technique of intracellular recording with microelectrodes which has proved so helpful in analysing synaptic events elsewhere (Douglas, Kanno & Sampson, 1967a, b). Initially we adopted a rather conventional approach and explored the medullary region of halved adrenal glands with microelectrodes. But this proved unsatisfactory. Although we recorded numerous intracellular potentials of about 30 mV we could not prove these came from chromaffin cells; and we were unable, for technical reasons, to study the effects of nerve stimulation or acetylcholine. Similar difficulties were also encountered by Matthews (1967) who used a similar approach. It was thus clear that we had to devise an alternative method. We finally found a solution to the problem in a melding of the techniques of electrophysiology and tissue culture. By applying tissue disaggregation methods to the adrenal medullae of gerbils we obtained isolated chromaffin cells that could be maintained on a feeder culture in a Sykes-Moore chamber. The chamber containing the cells was then placed over an inverted microscope and the chromaffin cells, which were easy to identify through phase contrast objectives, were impaled with microelectrodes under direct visual control. By this method it was possible to establish the responses of chromaffin cells to ACh and various other drugs or alterations in the ionic environment. The resting potentials of the cells were generally about 30 mV and thus similar to those obtained from cells (presumably chromaffin) in the medullary region of the adrenal glands of various species, including the gerbil (Matthews, 1967; Douglas, Kanno & Sampson, unpublished). This potential is somewhat lower than that of nerves and muscle cells but quite comparable with those found in other gland cells (Poulsen & Petersen, 1966). Its ionic basis has yet to be fully defined, but since the potential falls as the extracellular potassium concentration is raised and rises slightly as the extracellular sodium concentration is lowered both K and Na apparently contribute. Presumably, the significant contribution of sodium is responsible, at least in part, for the resting potentials of chromaffin cells being lower than those of nerves and muscles. But our primary concern here is with the effect of ACh. Acetylcholine, we found, depolarizes the chromaffin cell. This effect was shown first by recording the mean value of the transmembrane potential of a population of chromaffin cells before, during and after application of ACh (Douglas, Kanno & Sampson, 1967a); and, later, by injecting ACh close to the cell during intracellular recording. Although depolarization in response to acetylcholine was rapid we never observed any action potentials. Nor were action potentials set up when the membrane was depolarized rapidly by passing current across it (Kanno & Douglas, 1967). It thus appears that spike generation is not involved in stimulus-secretion coupling in the chromaffin cell.

Depolarization in response to ACh seems to be due mainly to inward sodium current since the effect is much reduced by lowering the extracellular concentration of sodium and is linearly related to the logarithm of the extracellular sodium concentration over a wide range. But sodium entry is clearly not the only factor involved for ACh retains some depolarizing action when all sodium is removed and replaced by an osmotically equivalent amount of sucrose. This residual effect seems to be largely due to inward movement of calcium since it increases with the logarithm of the extracellular calcium concentration over the range 1–117 mM (Douglas, Kanno & Sampson, 1967b). This finding harmonizes with earlier evidence showing that ACh increases ^{45}Ca uptake in the adrenal medulla (Douglas & Poisner, 1962).

The main conclusion from the electrophysiological experiments is that ACh acts at the medullary synapse, where its function is to induce secretion, in essentially the same way as it acts at other synapses where its function is to stimulate or inhibit nervous or muscular activity; namely, to increase the permeability of the plasma membrane of the postsynaptic cell to commonly occurring inorganic ions. The most striking consequences of this action on the chromaffin cell are inward movements of sodium and calcium ions and depolarization.

Relation of membrane events to secretion. The question now, of course, is whether this action of ACh on the plasma membrane of the chromaffin cell accounts for stimulation of catecholamine secretion. It seems likely that it does. First, because depolarization increases with increasing concentrations of acetylcholine causing progressively greater secretory responses. Second, because drugs (hexamethonium and atropine*) that block the secretory response to acetylcholine also prevent depolarization. And, third, because various other substances that stimulate medullary secretion also depolarize the chromaffin cells. These include histamine, 5-hydroxytryptamine, bradykinin, angiotensin, excess potassium, and barium (Douglas, Kanno & Sampson, 1967a, b). At this point it is appropriate to summarize the evidence and inferences from these electrophysiological experiments:

Consequences of exposing the chromaffin cell to acetylcholine

Interaction between ACh and the plasma membrane of the chromaffin cell.

↓

Altered membrane chemistry (conformation ?).

↓

Increased membrane permeability.

↓

Entry of sodium and calcium ions.

↓

Depolarization (without impulse formation).

The task confronting us is to determine how the various events depicted in the above schema are related to the secretory response.

* The need for atropine here may seem curious since it is commonly believed that medullary stimulation "belongs to the 'nicotine' aspect of the actions of acetylcholine" (Dale, 1937). But in their classical paper Feldberg, Minz & Tsudzimura (1934) pointed out that although the *principal* action of ACh on the cat's adrenal medulla is of the nicotinic sort, there is also a muscarinic component resistant to the blocking action of nicotine but sensitive to that of atropine. Curiously, this observation has been generally ignored. In recent experiments the presence of muscarine receptors has been confirmed and the further observation has been made that these receptors, at least in the cat, are associated with preferential release of adrenaline (Douglas & Poisner, 1965a).

Sodium and calcium

One of the simplest experiments is to examine how the response to ACh is influenced by removing sodium or calcium from the extracellular environment; and this is precisely what Rubin and I did some years ago in anticipation of the electrophysiological evidence (Douglas & Rubin, 1961, 1963). We used the adrenal gland of the cat perfused with Locke's solution and obtained most satisfactory responses. It was not uncommon for catecholamine output to increase a hundred-fold or more on exposure to ACh. It soon became evident that ACh retained its power to release catecholamines when sodium was omitted from the perfusion medium. Indeed, the omission of sodium potentiated the secretory response. Clearly then, although sodium entry dominates the electrophysiological picture it is not necessary for secretion. By contrast, our experiments showed calcium to be indispensable: within 10–20 min of withdrawing calcium from the perfusion medium the secretory response to ACh was all but abolished. Moreover, the presence of calcium in the extracellular environment proved to be not merely an essential condition for the response to ACh but a sufficient one: all the other ions (potassium, sodium, magnesium, and chloride) could be dispensed with. Furthermore, catecholamine output in response to ACh increased with increasing extracellular calcium over a wide range of concentrations above as well as below the conventional value of 2.2 mM. On these and other grounds Rubin and I proposed that entry of calcium ions might somehow link the acetylcholine stimulus to the secretory response.

Among the other evidence prompting this view was the demonstration that there was nothing unique about ACh, and that other means of promoting calcium entry would also evoke catecholamine secretion. Some years earlier Vogt (1952) had shown that excess potassium stimulates catecholamine secretion from the medulla by some direct action on the chromaffin cells, and there was evidence at the time we began these experiments that excess potassium promotes calcium uptake in tissues such as nerve and muscle (for a review, see Shanes, 1958). We were able to demonstrate that excess potassium increases calcium uptake in the medulla (Douglas & Poisner, 1961), probably as a result of the depolarizing action later observed in the electrophysiological experiments, and that the secretory response to excess potassium is dependent on calcium just as is the response to ACh (Douglas & Rubin, 1961), 1963). Further evidence of the "unspecificity" of ACh and the importance of calcium came from experiments showing that each of several other chemically dissimilar substances known to evoke medullary secretion requires calcium for its action: the substances include, in addition to various ACh-like drugs, the amines histamine and 5-hydroxytryptamine, and the polypeptides angiotensin and bradykinin (Poisner & Douglas, 1966). These diverse secretagogues may also promote calcium entry by their depolarizing actions on chromaffin cells.

Additional evidence that calcium entry might mediate the response to ACh and the other secretagogues came from experiments showing that calcium itself can stimulate medullary secretion. This effect is seen when the medulla is perfused for some time with a calcium-free medium and when there is reason to believe that the chromaffin cells are more permeable to calcium than they normally are. The plasmalemma of the chromaffin cell, like that of other cells (for example, Morrill, Kaback & Robbins, 1964) seemingly requires calcium to maintain its relatively impermeable state and is rendered leaky to ions when calcium is withdrawn from the environment. This leakiness reveals itself by a progressive fall in trans-

membrane potential (Douglas, Kanno & Sampson, 1967b), and by a loss of potassium from the perfused adrenal gland (Douglas & Poisner, unpublished observations). In such circumstances the reintroduction of calcium in the conventional extracellular concentration (2.2 mM) causes a violent secretory response lasting several minutes (Douglas & Rubin, 1961). Our interpretation of this phenomenon is as follows: We assume that the plasmalemma of the chromaffin cell undergoes some structural change increasing its permeability when calcium is withdrawn from the extracellular environment and that calcium, when reintroduced, penetrates the cell to set in motion the processes leading to the extrusion of catecholamines. Secretion, we suppose, passes off as the membrane regains its normal complement of calcium and recovers its normal structure and relative impermeability to that ion, and as the calcium that has entered the cell is bound or extruded. On this view, the calcium normally present in the extracellular environment does not evoke secretion in the absence of ACh or other secretagogues because, in part at least, it penetrates relatively slowly. This led us to study the effects of barium, another alkaline earth metal known to penetrate some cells more readily than calcium. Barium, we found, has a powerful stimulant effect on catecholamine secretion by some action exerted directly on the chromaffin cell that does not require any pretreatment of the cell, neither calcium deprivation nor exposure to any of the other secretagogues (Douglas & Rubin, 1964a). A third alkaline earth metal, strontium, has effects intermediate between those of calcium and barium, while a fourth, magnesium, behaves quite differently—it not only fails to induce secretion, even when the cell is rendered permeable by calcium deprivation, but opposes the stimulant effects of calcium, barium, and strontium (Douglas & Rubin, 1964b). This effect of magnesium provides a further means of testing the calcium entry hypothesis, for clearly if secretion in response to ACh and potassium and the other agents is mediated by calcium then magnesium should inhibit the response. This is in fact the result obtained (Douglas & Rubin, 1963, 1964b).

The findings with the various alkaline earth metals are thus consonant with the hypothesis that calcium entry is a critical event in stimulus-secretion coupling in the chromaffin cell, and with the notion that there exists within the cell some secretory mechanism normally activated by calcium. A clue to the nature of this mechanism may perhaps reside in the spectrum of activities of the alkaline earth metals and other divalent cations. I should point out that barium and strontium can exert their stimulant effects in calcium-free media (in contrast to secretagogues such as ACh, the amines, and the polypeptides) so that the secretory mechanism, although normally calcium-dependent, appears to be responsive to these related alkaline earth metals also. In addition to magnesium, various other divalent cations fail to support the secretory response to ACh and act as blocking agents (Douglas & Rubin, 1964b).

The evidence thus far is that calcium entry has some function critical to the secretory response but that sodium entry has not. What now of the other factors revealed by the electrophysiological experiments ?

Depolarization

The importance of membrane depolarization is not so easily resolved. Although the response to excess potassium indicates that depolarization is an *adequate*

stimulus for secretion, depolarization is clearly not by itself a *sufficient* stimulus. Omission of calcium does not prevent excess K from depolarizing the chromaffin cell yet it blocks secretion ; and the local anaesthetic, amethocaine, does not prevent ACh from depolarizing yet it too depresses secretion (Douglas & Kanno, 1967)*. Indeed, there are grounds for questioning whether a fall in transmembrane potential below the normal value is necessary for secretion to occur. Thus the secretory response to ACh is potentiated when sodium is absent from the extracellular environment (Douglas & Rubin, 1963), yet in such circumstances membrane potential falls relatively little ; in fact, because the resting potential is elevated in the absence of sodium, the response to ACh results in a potential little, if at all, below the normal resting value (Douglas, Kanno & Sampson, 1967b). There is no doubt that ACh can stimulate secretion by some means other than depolarization, for it increases catecholamine output from chromaffin cells already depolarized by isosmotic potassium sulphate (Douglas & Rubin, 1963). This residual effect of ACh on the depolarized chromaffin cell may again be attributable in increased calcium entry: a stimulant effect of ACh on calcium uptake has been observed in analogous studies on depolarized smooth muscle (Durbin & Jenkinson, 1961 ; Jenkinson & Nicholls, 1961).

Perhaps, then, the fall in potential across the chromaffin cell membrane that occurs on exposure to acetylcholine is of little significance in stimulus-secretion coupling and is merely an electrical sign of the interaction of the membrane with acetylcholine, an interaction whose importance lies in its other consequences. Although it is possible that one of these consequences, calcium entry, is sufficient in itself to evoke secretion, it must be borne in mind that in no instance have we succeeded in evoking secretion without there being some change in the chemistry of the plasma membrane as revealed by movement of ions or change in potential. Although we can mask some of the signs of this altered membrane chemistry—for example, diminish depolarization in response to ACh by removing sodium from the extracellular environment—the underlying change in the membrane is doubtless still present. None of our evidence tells whether calcium would be effective in the absence of this unidentified change in membrane chemistry. Here, of course, we are touching on one of the great enigmas of biology, the nature of the molecular rearrangements occurring in membranes on exposure to acetylcholine and other membrane-active substances. Until these molecular events are more clearly understood we cannot hope to define their significance for secretion. One conjecture is that ACh and the other secretagogues decalcify the plasmalemma: this seems the simplest way to accommodate the evidence that the cell exposed to various secretagogues behaves in response to calcium much as does the chromaffin cell previously exposed to a calcium-free environment (Douglas, 1963a, 1965 ; Douglas & Rubin, 1964a). Similar views have been put forward to explain the effects of ACh, excess potassium and depolarizing currents on muscles and nerves (see Gordon & Welsh, 1948 ; Shanes, 1958). Some other speculations will be mentioned later in connection with chemical events associated with secretion in the chromaffin and other cells.

* Amethocaine apparently interferes little, if at all, with inward sodium movement in concentrations blocking secretion, but it strongly inhibits inward calcium movement as is evident from recordings of transmembrane potentials (Douglas & Kanno, 1967) or by ^{45}Ca uptake (Rubin, Feinstein, Jaanus & Paimre, 1967). The analysis of the blocking action of amethocaine is, in fact, fresh pharmacological support for the view that calcium entry is important for secretion while sodium entry is not.

The nature of secretion in the chromaffin cell

What I have so far discussed are events occurring early in stimulus-secretion coupling, events that are somehow responsible for setting in motion the process that leads ultimately to the release of medullary hormones. My task now is to define the release process. This is not easy. Although light microscopists have studied the problem for decades they have failed to agree among themselves, and have left us with a bewildering variety of schemes, including holocrine and apocrine forms of secretion as well as various other processes not involving disruption of the cell (for a review, see Bachmann, 1954 ; Franzen, 1964 ; Grauman, 1956). Nor has the problem been solved with the advent of electronmicroscopy and techniques of cell fractionation. This has extended rather than limited the range of possible mechanisms. It is clear, however, from the cell fractionation studies initiated by Blaschko & Welch (1953) and by Hillarp, Lagerstedt & Nilson (1953) and from electronmicroscopical evidence provided by Hillarp *et al.* (1953), Lever (1955), Wetzstein (1957), De Robertis & Vaz Ferreira (1957), and many others, that most of the catecholamines in the chromaffin cell are present in membrane-limited subcellular structures ranging in size from about 500 to 2,000 Å and known as chromaffin granules (see reviews by Weiner, 1964 ; Coupland, 1965a ; Vane, 1959). But not all medullary catecholamines are recovered in chromaffin granules when the cells are disrupted. About 20% is commonly found in extragranular fractions. Some of this doubtless must come from granules injured during cell fractionation, but Hillarp (1960a) has argued vigorously that there is indeed a functional pool of " free " catecholamines in the cytoplasm of the chromaffin cell. He proposed, as did Blaschko & Welch (1953) in their original study, that stimulation releases amines from this pool while the granules serve as reserve stores. This conception, which has been espoused by many authors, springs not only from the finding of " free " amines but from the attractively simple conjecture that stimulation probably involves some increase in the permeability of the plasma membrane of the chromaffin cell that allows the amines to leak out (compare Blaschko & Welch, 1953 ; Hillarp, 1960b ; von Euler, 1967). Not surprisingly, this view is unpopular with microscopists, for it is inaccessible to experimental test with their instruments which cannot, as yet, resolve free amines. Microscopists prefer to believe that the chromaffin granules are more immediately involved and offer a variety of suggestions, supported by appropriate micrographs, about how release occurs. These suggestions range from leakage of amines from granules to cytoplasm and thence, presumably by diffusion, across the plasmalemma to the cell exterior (for example, Wetzstein, 1957 ; Hillarp, Hökfelt & Nilson, 1954 ; De Robertis & Vaz Ferreira, 1957) to expulsion of intact granules through the cell membrane (for example, Cramer, 1928 ; Smitten, 1965). One of the more obvious limitations of the electronmicroscopical approaches (apart from the impossibility of assessing the involvement of " free " amines) is that while a given image may suggest a means whereby the cell could release its contents, the image cannot answer the question whether this is the mechanism of release, or even an important one: the quantitative evidence and correlation with secretory state that would justify such a conclusion would be difficult to obtain and is certainly not available. I do not wish to labour this limitation of the morphological evidence which I feel is more fittingly expressed by morphologists themselves (see Sjöstrand, 1962).

If the sequence of events involved in stimulus-secretion coupling in the chromaffin cell is to be understood this rich harvest of hypotheses must be winnowed and the correct one selected. A few years ago my colleagues and I reasoned that a possible solution to the dilemma might lie in a chemical approach. We argued that it would be of value in defining the secretory process to know what substances (if any) escape along with the catecholamines from the stimulated cell. For example, if constituents characteristic of the chromaffin granules were found escaping from the secreting gland along with the catecholamines this would indicate that the chromaffin granules are indeed the immediate source of amines. Likewise, the presence or absence of other constituents of the cell might be helpful in assessing those hypotheses that involve some disruption of the cell, such as microapocrine secretion. This approach has turned out to be most helpful. Since our first findings were communicated less than three years ago at a delightful symposium in Stockholm (Douglas, 1965), an abundance of chemical evidence of this sort has been amassed in several laboratories which, when considered along with certain testimony from electronmicroscopy, now appears to offer a coherent account of the mechanism of release of the medullary hormones.

One of the striking chemical characteristics of the chromaffin granule is its high content of ATP: there is close to one molecule of ATP present for every four molecules of catecholamine. (In this ratio the opposite charges of ATP and catecholamines annul one another, and for this reason it is believed that ATP may function to complex the catecholamines and maintain them in osmotically inactive form (see review by Weiner, 1964.) It was natural then, in the first experiments, to seek ATP (or its metabolites) in the effluent from the secreting adrenal medulla—especially since highly sensitive and specific methods were available for measuring these substances. The experiments, performed on perfused cat adrenal glands, showed that whenever catecholamine secretion was induced, whether by splanchnic stimulation, acetylcholine, calcium reintroduction, or other means, traces of ATP and massive amounts of ATP metabolites appeared in the venous effluent along with the catecholamines. The ATP and metabolites, principally AMP with smaller amounts of ADP and adenosine, appeared in the first drop of effluent escaping after stimulation was begun, and the time-course of efflux paralleled that of the catecholamines. Moreover, the molar ratio of catecholamines to ATP and metabolites in the venous effluent was similar to that found within the chromaffin granules. From this we concluded that the classical ATP-rich chromaffin granules provide the immediate source of catecholamines tapped by stimulation, and that other pools—if indeed they exist in the cell before fractionation—are not involved (Douglas, 1965, 1966a ; Douglas, Poisner & Rubin, 1965 ; Douglas & Poisner, 1966a, b). During the course of our experiments, Stjärne (1964) reported finding hypoxanthine in the effluent from perfused bovine adrenal glands ; and other ATP metabolites were later found escaping from the same preparation by Banks (1966a).

This discovery of ATP metabolites escaping from the stimulated adrenal gland intoxicated my colleagues and me briefly with the notion that we had stumbled on the molecular basis of catecholamine secretion and the role of calcium. Some years ago Hillarp (1958a, b) found ATPase activity present in the membranes of chromaffin granules (this has recently been confirmed by Banks, 1965) and suggested that perhaps stimulation activated this ATPase to hydrolyse the ATP within the granules and thus liberate the complexed amines. The new evidence was clearly in line with

this idea: not only had ATP metabolites been found escaping with the catecholamines, but stimulation apparently involved calcium entry and calcium was known to be an activator of ATPases. Our enthusiasm for this charmingly simple scheme was soon dispelled by control experiments. These showed that ATP perfused through the adrenal gland was metabolized to the same spectrum of metabolites as appeared on stimulation, apparently by enzymes in the adrenal blood vessels; and that when we inhibited these enzymes before stimulating the chromaffin cells catecholamines escaped along with massive amounts of ATP, unhydrolysed (Douglas & Poisner, 1966b). From this it seemed clear that secretion is not dependent on the splitting of *intra-granular* ATP and that the metabolites are formed after the nucleotide is discharged. This does not mean that the ATPase in the granules has no function in the release of catecholamines. I shall return to this point later.

In these early experiments we also made some measurements of protein efflux from the cat's adrenal gland exposed to ACh. This was sufficiently low that we felt we could rule out the several postulated mechanisms of secretion that involve substantial loss of cell substances (Douglas, 1965; Douglas & Poisner, 1965b). And subsequent results on lipid efflux to which I shall refer shortly corroborate this. The chemical evidence, coupled with the concensus of electronmicroscopical opinion of recent years (see Coupland, 1965a, b; De Robertis, 1964) pointed clearly to some process allowing the chromaffin granules to discharge without rupture of the cell. It seemed to us most unlikely that the granules discharged ATP and catecholamines to the cell sap, for although ATP and the other adenine nucleotides traverse cell membranes poorly the ratio of catecholamines to ATP and metabolites recovered in the venous effluent in our experiments was similar to that found in the granules. Moreover, under the appropriate conditions ATP itself escaped unhydrolysed despite the fact that the cell and its organelles are rich in ATPases that would be expected to hydrolyse the substance. The only plausible explanation, we felt, was that the nucleotide was extruded directly to the cell exterior, without traversing the cytoplasm, by the process known variously as reverse micropinocytosis, exocytosis (De Duve, 1963) or emiocytosis (Lacy, 1967) in which the membrane of the secretory granule fuses with the plasmalemma and escape of secretory product occurs through an aperture in the fused membranes. This mechanism, advanced by Palade (1958) to explain the release of material from the zymogen granules of the exocrine pancreas, had already been proposed as a possible means of catecholamine release by De Robertis & Vaz Ferreira (1957) and by De Robertis, Nowinski & Saez (1965) on the basis of certain electronmicroscopic images of chromaffin cells. And images consistent with the scheme have also been observed by Coupland (1965b) and by Diner (1967). Further chemical evidence in support of exocytosis was soon provided by the discovery, in the effluent from adrenal glands secreting catecholamines, of a third principal constituent of the chromaffin granules, a characteristic soluble protein (Banks & Helle, 1965; Kirshner, Sage & Smith, 1967). This substance too is not one that would be expected to traverse intact membranes.

There is, of course, an alternative mechanism of secretion that would account equally well for the simultaneous discharge of the various constituents of the chromaffin granules, namely, that chromaffin granules are extruded intact as suggested by various authors on microscopical evidence (Cramer, 1928; Smitten, 1965; Gori, 1964; see also review by Bachmann, 1954). But this seems to be excluded by

other chemical evidence we have obtained more recently which shows: first, that efflux of phospholipid and cholesterol, the principal lipids of the chromaffin granule membrane, rises little, if at all, on stimulation (Trifaró, Poisner & Douglas, 1967; see also Schneider, Smith & Winkler, 1967); second, that there is no fall in the phospholipid and cholesterol contents of a sub-cellular fraction containing the chromaffin granules following medullary stimulation (Poisner, Trifaró & Douglas, 1967); and, third, that electronmicrographs of these fractions recovered after stimulation are rich in profiles with the appearance of empty granules (Malamed, Poisner, Trifaró & Douglas, 1968). All this suggests that the membranes of the chromaffin granules are retained, a conclusion in harmony with electronmicrographs of medullary cells which commonly show, within the cytoplasm, images resembling emptied chromaffin granules (see Coupland, 1965a, b; De Robertis & Vaz Ferreira, 1957).

Although the evidence thus indicates that chromaffin granules are not extruded intact, it is not entirely in accord with the concept of reverse pinocytosis as applied to the chromaffin cell by De Robertis and his colleagues (see De Robertis, Nowinski & Saez, 1965). According to these authors, who adopt the concept of membrane flow expounded some years earlier by Bennett (1956), the membranes of the granules are incorporated in the plasma membrane of the cell. If such incorporation were to occur, however, stimulation should lead to a fall in the lipid content of the subcellular fraction containing the chromaffin granules, and emptied granules should not be found in cell fractions or electronmicrographs. The contrary evidence requires us, I believe, to conclude that after releasing their contents to the cell exterior, either by frank rupture of the adherent plasmalemma and granule membranes, or by some greatly increased permeability at the site of adhesion, emptied granules may remain, for a time at least, within the cell (Fig. 1). Conceivably the granule membranes may be refilled with catecholamines, but it seems more likely that they are

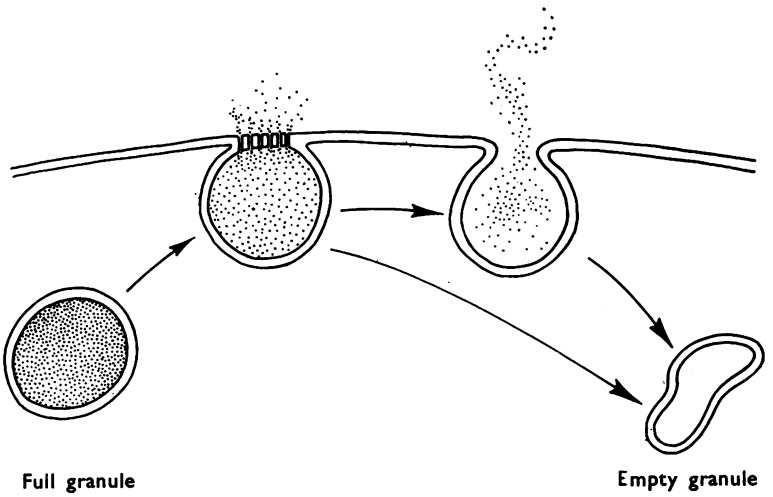

FIG. 1. Two possible mechanisms of catecholamine secretion consonant with the chemical and electronmicroscopical evidence. Both involve release directly from chromaffin granules to the cell exterior either through frank rupture of the adherent membranes of granule and cell (this is the conventional depiction of "exocytosis") or through some greatly increased permeability at the site of adhesion. In both instances granule membranes are retained after evacuation of their soluble contents.

ultimately broken down, perhaps by the lysosomal enzymes in which the chromaffin cell is rich (Smith & Winkler, 1966). Evidence of destruction of secretory granules by lysosomal activity has been obtained from electronmicrographs of other secretory cells (Smith & Farquhar, 1966). But the fate of the granule membranes is somewhat tangential to the main point I wish to make: namely, that there is now compelling evidence that catecholamine secretion from medullary chromaffin cells involves the chromaffin granules directly and occurs by exocytosis or some closely related process as depicted in Fig. 1.

At this point it would seem logical to try to fit calcium into the scheme and thus join together the two major pieces in the puzzle of stimulus-secretion coupling: input and output. Since the evidence from the medulla is fragmentary, however, I intend to broaden the discussion to embrace other secretory cells in which calcium has been shown to be important and where, in some instances at least, there is rather good evidence that secretion also occurs by exocytosis. Although the function of calcium has not been defined in any of these systems it seems profitable to proceed on the assumption that it is similar in all and, by assembling the facts gleaned from studies of the various preparations, to synthesize a working hypothesis. A further, and related, purpose in reviewing the evidence from these other secretory systems will be to substantiate my belief that it is fitting to conceive of " stimulus-secretion coupling " as a general problem, or field of study if you will. I hope to persuade you of this by marshalling evidence which, to my way of thinking, offers a clear hint that rather similar processes are at work in cells that, on first view, appear to have little in common—cells that differ widely in their secretory product, morphology, electrical excitability, and adequate stimulus. I am persuaded that progress in the field of contraction (and with it recognition of the unity of events involved in different muscles) has been speeded by adoption of Sandow's (1952) term " excitation-contraction coupling," and I am hopeful that the same may be achieved in the field of secretion by adoption of a like rallying banner.

Calcium and stimulus-secretion coupling in other systems

Exocrine glands. When it had become evident that the secretomotor nerves to the adrenal medulla act through some calcium-dependent process, it was natural to enquire whether other secretomotor nerves act similarly. To test this we selected the cat's submaxillary gland which, because it is innervated by adrenergic as well as cholinergic nerves, afforded an opportunity to test the importance of calcium for the action of noradrenaline as well as that of acetylcholine (Douglas & Poisner, 1963). We found, in perfusion experiments, that the production of saliva in response to either stimulus could be abolished by withdrawing calcium from the medium. Output of water and electrolytes was depressed only after prolonged calcium deprivation but protein output was quickly lost. Moreover, secretion of protein in response to stimulation increased with increasing calcium concentration over a wide range and was inhibited by magnesium. The dissociation between the effects of acetylcholine on water and electrolyte output on the one hand and protein output on the other is not surprising since the two responses, ion pumping and protein extrusion, are so different. It provides us with some assurance that we are not simply witnessing a need for calcium in the binding of drugs to the receptors on the gland cells—a possibility suggested by the work of Paton & Rothschild (1965) on smooth muscle. Rather, it indicates that calcium has some special function in linking the secretory stimulus provided by ACh or noradrenaline to the

mechanism for protein extrusion. A similar requirement for calcium has subsequently been demonstrated for protein secretion from the exocrine pancreas (Hokin, 1966).

I should like to stress here that the protein secretions of the exocrine glands, like the medullary hormones, are also sequestered in membrane-limited granules (the familiar zymogen granules), and that there is evidence from light and electron microscopy that secretion involves exocytosis (Palade, 1958; Ichikawa, 1965; Ekholm, Zelander & Eklund, 1962). Zymogen granules are sufficiently large that serial sections can be made yielding convincing electronmicrographic images of this form of secretion.

Neurones and neurosecretory cells. Amongst the other cells that clearly have a claim to our attention are neurones. Although they are not commonly classified as secretory cells neurones are, in fact, secretory cells of a rather specialized type, for they elaborate characteristic secretory products, the chemical transmitters, and release them in response to the appropriate stimulus. Moreover, there has long been evidence from cholinergic neurones that this response is critically dependent on calcium and is inhibited by magnesium; and further, that nerve impulses, depolarization, or excess potassium increase calcium uptake in nerves. Indeed, on these grounds both Hodgkin & Keynes (1957) and Birks & MacIntosh (1957) proposed that calcium entry might somehow initiate acetylcholine release; and evidence favouring this view continues to accumulate (Katz, 1962; Katz & Miledi, 1967). These facts, coupled with the evidence of calcium's involvement in the release of catecholamines from the chromaffin cell, made it seem likely that calcium would also be found to be of importance in the release of noradrenaline from adrenergic neurones which are developmental homologues of chromaffin cells (Douglas & Rubin, 1961). Evidence for this was first obtained by Huković & Muscholl (1962) who showed that the stimulated output of noradrenaline from sympathetic nerves to the heart fell as the extracellular calcium concentration was lowered; and others have subsequently corroborated and extended these findings in a variety of adrenergic nerves (see Boullin, 1967; Burn & Gibbons, 1964; Kirpekar & Misu, 1967). The principal effects of calcium and the other commonly occurring ions on release of transmitter from adrenergic nerves can be said to resemble closely their effects on cholinergic nerves and chromaffin cells.

Since neurones and chromaffin cells share a common ancestry it appeared worthwhile testing whether calcium is also involved in the secretory activity of a third developmentally related group of cells: the neurosecretory cells of the hypothalamo-hypophyseal system which secrete the hormones vasopressin and oxytocin. Although the intact hypothalamo-hypophyseal system is rather inaccessible to experiment, the neurosecretory terminals from which the hormones are released lie in a compact mass in the neural lobe of the pituitary gland and it proved a simple matter to remove this lobe, and study secretory activity *in vitro*. Experiment showed that vasopressin output increases upon electrical stimulation or exposure to excess potassium; and that the response to these stimuli fails when calcium is omitted from the incubation medium, increases with increasing calcium concentrations over a wide range, is inhibited by magnesium, and does not require sodium (Douglas, 1963b; Douglas & Poisner, 1964a; Mikiten & Douglas, 1965); and also that excess potassium increases ^{45}Ca uptake (Douglas & Poisner, 1964b). Others who have adopted the isolated neurohypophyseal preparation have obtained similar results when

measuring oxytocin output or when studying different species (Haller, Sachs, Sperelakis & Share, 1965 ; Dicker, 1966 ; Daniel & Lederis, 1967).

When one compares the effects of the commonly occurring ions on neurones, chromaffin cells, and secretory cells it is difficult to avoid the conclusion that these three developmentally related cells have a common mechanism for the release of their different secretory products. There is, admittedly, one feature that distinguishes events in the chromaffin cell from those in the others: in chromaffin cells calcium entry apparently occurs directly as a local response to exposure to the physiological stimulus, acetylcholine ; while in nerves and neurosecretory cells, calcium entry occurs indirectly in response to a wave of depolarization, the action potential, propagated from a remote site, the perikaryon, where the physiological stimulus is received. However, this difference is probably one of detail rather than principle and akin, perhaps, to the difference between certain smooth or tonus muscles which contract directly in response to the local action of ACh, and skeletal muscles which contract in response to action potentials generated by ACh at the endplate region.

The question that naturally arises is whether the release of neurohumours and neurohormones is also by exocytosis. No answer can be given at present. It is generally conceded that much, if not all, of the neurohumours acetylcholine and noradrenaline, is contained in the membrane-limited structures known as synaptic vesicles. But these vesicles are very much smaller than chromaffin granules, and electronmicroscopical evidence is still more difficult to obtain ; although some images consistent with the mechanism have been published (De Robertis, 1964) they are evidently rare, and no correlation with secretory activity has been demonstrated (Birks, Huxley & Katz, 1960). Perhaps the strongest hint that neurohumours are released by exocytosis comes from the electrophysiological finding of quantal release of multimolecular packages of transmitter at various synapses (see reviews by Katz, 1962 ; Martin, 1966) ; but, as pointed out by Katz (1965), this is not in itself sufficient to demonstrate the immediate involvement of the vesicles, let alone the manner of their discharge, and many authors have proposed that neurohumours are liberated from free extragranular pools in the neurones (Hillarp, 1960b ; Paton, 1960 ; Schümann, 1961 ; von Euler, 1966). And there is similar divergence of opinion on the release of posterior pituitary hormones: some authors suppose that release occurs from extragranular pools (Dicker, 1966 ; Ginsburg & Ireland, 1966) while others hold the neurosecretory granules to be involved (Palay, 1957 ; Gerschenfeld, Tramezzani & De Robertis, 1960). Here again, however, no convincing electronmicroscopic images of exocytosis in the neural lobe have been published. Clearly, it would be helpful to have the kind of chemical evidence that has clarified events in the medullary chromaffin cell. This will be more difficult to obtain from nerves and neurosecretory cells which are less favourable test objects, but it should at least be possible, as I have pointed out elsewhere (Douglas, 1966b) to see whether neurophysin, the protein found in the neurosecretory granules escapes along with the polypeptide hormones on stimulation. Although the problem is as yet unsettled, I believe it probable that nerves and neurosecretory cells do " secrete " by exocytosis on the following grounds: (1) they share a common developmental origin with chromaffin cells where the evidence for exocytosis is strong ; (2) like the chromaffin cells they package their secretions in membrane-limited granules (or vesicles) ; (3) they show a pattern of responses to ions closely resembling that of

the chromaffin cells; (4) a few electronmicroscopical images consistent with the mechanism do exist; (5) there is electrophysiological evidence for release of neurohumours in rather uniform multimolecular packages or quanta.

Other "secretory" systems. I have so far discussed representatives of the three major classes of glands: exocrine, endocrine, and neuroendocrine; and, in addition, a highly specialized type of secretory cell, the neurone. But this does not exhaust the classes of cells in which calcium has demonstrably some critical function in "secretion."

The mast cell, for example, which releases histamine (and in some species, 5-hydroxytryptamine) during the antigen-antibody reaction fails to do so when calcium is absent. And the effectiveness of the anaphylactic response increases as the extracellular concentration of calcium is raised. Mongar & Schild (1962) have emphasized the unique importance of calcium and consider it a possible co-factor in some enzymatic reaction set in motion by the antigen-antibody reaction. It is conceivable that the function of calcium in the mast cell is analogous to its function in the medullary chromaffin cell: to link the stimulus, in this instance the perturbation resulting from anaphylaxis, to the secretory response. Once again I believe it noteworthy that the secretory product of the mast cells is held in membrane-limited granules.

The polymorphonuclear leucocyte, too, which releases granule-bound enzymes in response to an appropriate stimulus, such as staphylococcal leucocidin, requires calcium for this response. Moreover, leucocidin demonstrably increases the permeability of the plasmalemma to calcium; and calcium itself (in the absence of leucocidin) releases the granule-bound leucocytic enzymes under conditions where it penetrates the cell membrane readily (Woodin & Wienecke, 1963, 1964). All this is clearly analogous to the evidence my colleagues and I obtained from the adrenal medulla and the submaxillary salivary gland; and like these glands the leucocyte apparently releases its secretory products directly from membrane-limited granules to the cell exterior (Woodin, French & Marchesi, 1963).

It is this accumulation of like pieces of evidence from such diverse sources that leads me to suspect that events identified in stimulus-secretion coupling in the chromaffin cell will be applicable to other cells. I am impressed with the fact that the chromaffin cell is responsive not only to ACh, the physiological stimulus at the medulla, but also to various other chemically distinct substances repeatedly suggested to act as natural secretagogues at various other sites in the body: the amines, histamine and 5-hydroxytryptamine and the polypeptides, angiotensin and bradykinin. Since our evidence is that each of these substances evokes medullary secretion by acting on the plasma membrane of the chromaffin cells to depolarize them and promote inward movement of calcium (Douglas, Kanno & Sampson, 1967b), it is not unlikely that this is a general mechanism whereby stimuli of various sorts evoke different cellular secretions.

A common secretory mechanism and possible functions for calcium

On reviewing the evidence one is astonished by the diversity of cells whose secretory activity is critically dependent on calcium and by the variety of stimuli and secretions involved. The cells range from the aristocratic neurone through endocrine and exocrine glands to the humble leucocyte and mast cell. The stimuli

include action potentials, simple neurohumours released by secretomotor nerves, hormones and autacoids of various sorts, bacterial toxins, and antigens. And among the secretions are molecules as small and simple as amines and as large and complex as enzymes. Clearly, when we search for an explanation for the general involvement of calcium in stimulus-secretion coupling in the various cells logic dictates that we seek it in some feature the cells possess in common. One such feature is the storage of secretory product in subcellular granules which, although of different dimension in the different cells, are always enveloped by a similar sort of membrane ; and allied with this is evidence that whenever a sufficiently complete analysis of the mechanism of secretion has been possible, it has shown secretion to involve release directly from these granules to the cell exterior by exocytosis or some similar phenomenon. Calcium's function may thus have to do with exocytosis. This is plausible since exocytosis in each cell involves only two components —the membrane delimiting the cell, the plasmalemma, and the membrane surrounding the secretory granule—and there is no impediment to supposing that these two components are basically similar in different cells. On this view, then, the chemical nature of the content of the granules, which varies from one cell to the next, is irrelevant to the secretory process ; and calcium's function is concerned solely with the events governing delivery of the contents to the cell exterior. The calcium-dependent process could then be likened to the postman who delivers packages without regard (hopefully!) to their contents.

There are several ways in which calcium might be involved in exocytosis or the related events depicted in Fig. 1. In such schemes the granule membrane must first come into close apposition with the plasmalemma following which the conjoined membranes must undergo some structural change allowing escape of the secretory product. Calcium could facilitate the approximation of granule membrane and plasmalemma by changing the physico-chemical state of the cell sap in such a way as to enhance Brownian motion. Or it could promote adhesion of the two membranes: Woodin, French & Marchesi (1963) have shown that the addition of calcium to leucocytes poisoned with leucocidin causes the enzyme-containing granules to become attached to the plasmalemma ; and Banks (1966b) has speculated that calcium may act similarly in the chromaffin cell by neutralizing net negative surface charges on the granules. The attachment of the granule membrane to the plasmalemma might then lead to reorganization of the two membranes by purely physical forces acting to reduce the total energy of the system by incorporating the granule membrane into the plasmalemma—much as a small soap bubble is incorporated into a larger one contiguous with it. But this seems unlikely. In the first place, granule membranes seem to remain behind after secretion, at least in some cells. And second, the evidence favours a chemical reaction. Thus zymogen secretion is an energy-dependent process (Hokin, 1951), and so also is secretion of posterior pituitary hormones (Douglas, Ishida & Poisner, 1965) and adrenal medullary hormones (Kirshner & Smith, 1966). The nature of the chemical reaction is, however, obscure. One possibility, since exocytosis requires rearrangement of membranes rich in phospholipids (see Fawcett, 1962), is that the reaction involves activation of a phospholipase. My colleagues and I first suspected this on finding an unsaturated fatty acid, a prostaglandin, in the effluent from adrenal glands exposed to acetylcholine (Ramwell, Shaw, Douglas & Poisner, 1966). The phenomenon reminded us of the escape of a chemically similar substance (slow reacting substance, SRS) from the mast cell during histamine release which Högberg &

Uvnäs (1960) had earlier ascribed to phospholipase activity. Our suspicion became stronger when we confirmed (Douglas, Poisner & Trifaró, 1966) the observation of Hajdu, Weiss & Titus (1957) that the adrenal medulla is rich in lysolecithin; and when we uncovered an old report of phospholipase activity in the medulla (Francioli, 1933). Soon, however, we had reason to doubt the scheme for we found lysolecithin in abundance in adrenal glands in which we had taken great care to avoid stimulation (Douglas, Poisner & Trifaró, 1966). Nevertheless, the presence of lysolecithin in the medulla, and in particular the demonstration that it is concentrated to a remarkable extent in chromaffin granules (Blaschko, Firemark, Smith & Winkler, 1967) should be taken into account whenever possible molecular events involved in secretion are being considered. It will be of interest to determine if chromaffin granules are unique or whether secretory granules from other cells are also rich in lysolecithin. There is, of course, the evidence provided by the Hokins over the last 15 years or so (see Hokin & Hokin, 1965) of increased incorporation of ^{32}P into the phospholipid fraction of various secretory tissues on stimulation. But how this interesting effect is involved in stimulus-secretion coupling is uncertain: it is only partially reduced when secretion is blocked by omitting calcium (Hokin, 1966).

An alternative line of speculation, which I find attractive, arises out of studies on the extrusion of enzymes from the polymorphonuclear leucocyte. Woodin & Wiencke (1964) have found that under certain conditions ATP facilitates this response and have speculated that ATP may perhaps be a structural component of the plasmalemma of the leucocyte that is involved, along with calcium, in the extrusion of enzymes. They suggest that removal of ATP from the plasmalemma might result in increased cross-linking in the membrane and facilitate random movements of molecules leading to fusion of the granule membrane and the plasmalemma. Such a sequence, they propose, might result from hydrolysis of ATP in the plasmalemma by an ATPase they have found in the enzyme-containing granules. This ATPase is tightly bound and accessible to substrate and thus appears to reside in the granule membranes. Although the proposed mechanism is highly speculative it contains interesting elements. In the first place, you will remember that an ATPase is also present in the membranes of chromaffin granules; and I find on surveying the literature that ATPase activity has been reported in the membranes of secretory granules from a wide variety of cells including, for example, the histamine-containing granules of mast cells (Schauer & Eder, 1962), the insulin-containing granules of the β-cells of the islets of Langerhans (Lazarus, Barden & Bradshaw, 1962), and the neurohumour-containing granules, or vesicles, of neurones (Hosie, 1965). Second, several other authors have proposed, admittedly on equally indirect evidence, that ATP complexes exist in the membranes of a variety of cells and may participate in structural changes associated with stimulation (Abood, 1966; Kuperman, Volpert & Okamoto, 1964: Maas & Colburn, 1965). Thus the key elements in the scheme proposed for release of enzymes from the leucocyte may be present in more conventional secretory cells.

Against this background, a recent observation made at Osaka University takes on special interest: there it has been found that ATP stimulates release of catecholamines from chromaffin granules if these are suspended not in the commonly used sucrose-containing media but in media rich in sodium or potassium (Oka, Ohuchi, Yoshida & Imaizumi, 1965). In my laboratory Poisner and Trifaró have corrobor-

ated and extended this finding and shown that the effect is inhibited by substances blocking the ATPase activity of the granules. (Their evidence and a fuller account of that of Oka *et al.* has now been published: Poisner & Trifaró (1967), Trifaró & Poisner (1967), Oka, Ohuchi, Yoshida & Imaizumi (1967).)

Here then, in the several pieces of evidence discussed, are hints that an interaction between ATP and ATPase, promoted *in situ* by calcium's effect in causing granules to attach to the plasmalemma, may at one and the same time lead to membrane fusion and to release of the contents of the granules. Although the facts available are insufficient to establish that such events have a pivotal function in even a single secretory cell, the wide distribution of ATPases in granules of different secretory cells makes it tempting to suppose that we have here the outlines of a general mechanism called into play whenever secretion is of the type involving extrusion of granule-bound substances by exocytosis.

The reason I find speculation along this line particularly attractive lies in analogies with molecular events thought to be involved in muscle contraction where, according to Davies (1963), the appearance of calcium ions within the stimulated muscle also serves to neutralize mutually repulsive charges and thus allow approximation of ATPase to its substrate, ATP. Now it may seem extravagantly fanciful to draw this parallel between events involved in secretion on the one hand and those involved in contraction on the other; but a parallelism already exists in the remarkably similar effects of the commonly occurring ions on the two processes. Rubin and I drew attention to it in our report on medullary secretion (Douglas & Rubin, 1961), and Katz (1962) commented on it when discussing release of neurohumours. Subsequently, instances of parallel behaviour have multiplied and my colleagues and I have repeatedly emphasized them: for almost every effect on secretion produced by manipulation of the environment one can find a corresponding effect on muscle contraction. Of course, it may be that the parallel effects of ions and other agents on secretion and contraction merely reflects the common requirement for calcium and the similarity of factors controlling calcium binding and movement; but with indications that ATP and ATPase may be involved in secretion as well as contraction, the parallelism assumes a further significance. In a previous lecture, I suggested it might be profitable for those of us working in the field of secretion to follow closely the development of knowledge in the field of contraction (Douglas, 1965). It is at least conceivable that nature uses similar devices at the molecular level to effect these two seemingly disparate responses, contraction and secretion.

Supported by U.S.P.H.S. Grants RO1-NB04006 and RO1 NB01093.

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