STUDIES OF ACETYLCHOLINE STORAGE AND RELEASE:

AN ION EXCHANGE HYPOTHESIS

BY

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A THESIS

Presented to the Department of Biochemistry and
Graduate Division of the University of Oregon Medical School in
partial fulfillment of the
requirements for the degree of
Master of Science

June, 1969

ACKNOWLEDGEMENTS

The author would like to acknowledge the inspiration, assistance and moral support of his major thesis advisor, Dr. Jack Fellman and also that of Dr. Geoffrey Seaman, especially for his advice on electrophoresis and assistance in writing the Discussion Section.

A special thanks to Tom Fujita for his glass blowing, organic synthesis, and constant encouragements.

Assistance with the statistical analysis were provided by Dr. Victor Milstein and my wife Nelda.

In addition, helpful suggestions were made by Dr. Michael Litt , Donald Brooks and Lavelle Jackson.

The manuscript was typed by Pat Pageles, Pamela Bentley, and Timmie Gayton (a special thanks to the latter for sticking by me through long nights and weekends and always being so positive).

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AN ABSTRACT OF THE THESIS OF

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Date of receiving this degree: June 1969

Title:

STUDIES OF ACETYLCHOLINE STORAGE AND RELEASE-AN ION EXCHANGE HYPOTHESIS

Approved: ______________________________

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LIST OF ABBREVIATIONS

ACh	-	Acetylcholine
AChE	-	Acetylcholine esterase
AChI	-	Acetylcholine iodide
$AgNO_3$	-	Silver nitrate
ATP	-	Adenosine triphosphate
Ca	-	Calcium
Ch	-	Choline
ChAc	-	Choline transacetylase
CoA	-	Coenzyme A
Cs	-	Cesium
GABA	-	γ- aminobutyric acid
Hcl	-	Hydrochloric acid
K	-	Potassium
Kcl	-	Potassium chloride
Li	-	Lithium
M	-	Molar
ml	-	Milliliter
mM	-	Millimolar
mepps	-	Miniature endplate potentials
N	-	Normal
Na	-	Sodium
NaCl	-	Sodium chloride
Na_2HPO_4	-	Sodium phosphate dibasic
NaH_2PO_4	-	Sodium phosphate monobasic
NaOH	-	Sodium hydroxide

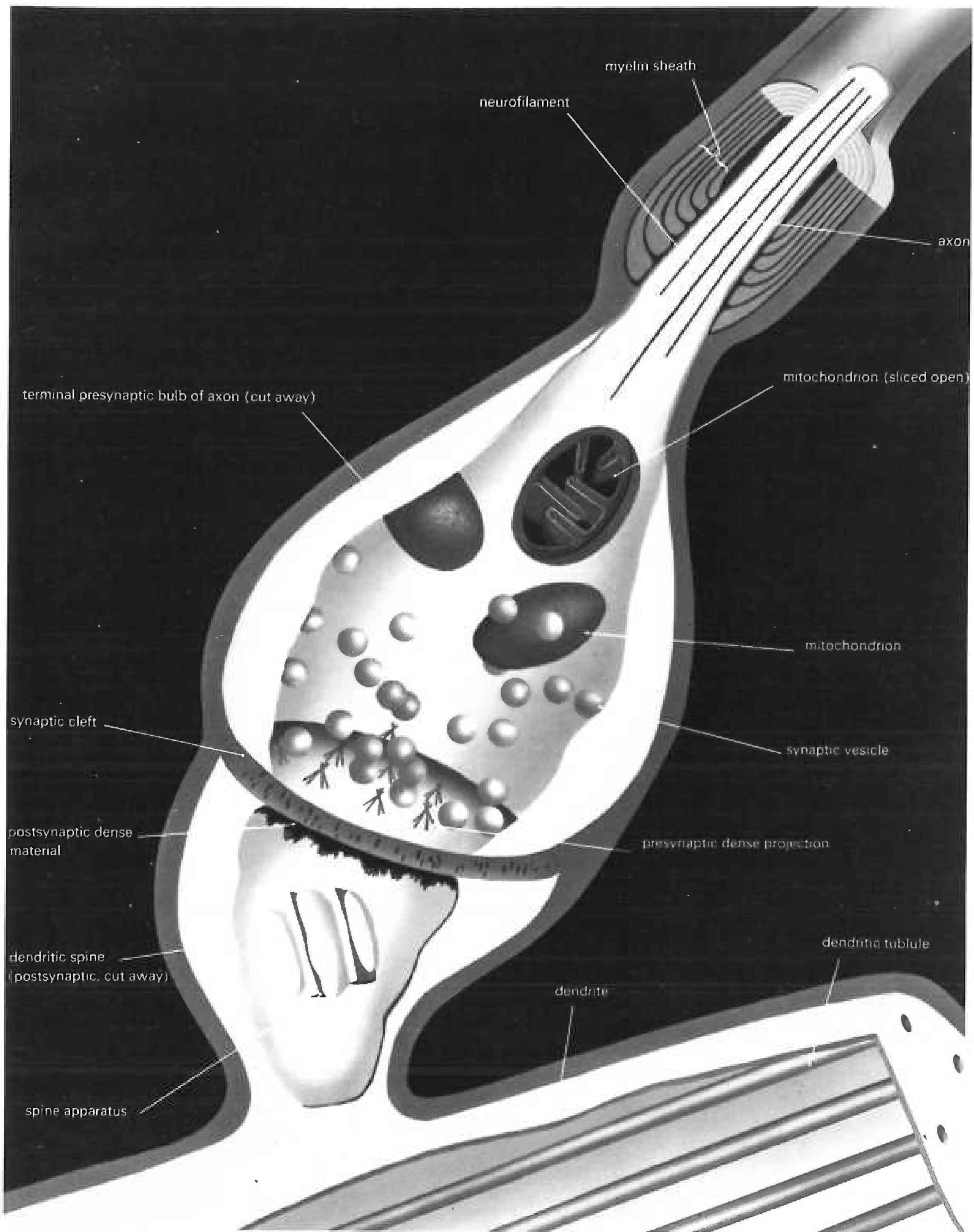
NEP	-	Nerve ending - particle (synaptosome)
POPOP	-	2,2-p-phenylenebis (5-phenyloxazole)
PPO	-	2,5 - diphenyloxazole
Rb	-	Rubidium
TMA	-	Tetramethylammonium
TMABr	-	Tetramethylammonium bromide

INTRODUCTORY REMARKS

With the advent of electron microscopy and microelectrode monitoring of pre-and post-synaptic resting and activation potentials, a whole concept of the rudiments of storage and release of acetylcholine at cholinergic synapses has evolved which remains relatively untested and unexplained on a firm molecular or neurophysiological basis. To be sure, numerous hypotheses have been advanced, nonetheless the whole concept of chemically-mediated synaptic transmission remains to be firmly established. At any rate, the state of the art appears most ripe for a least preliminary examination of possible mechanisms.

What of the state of the art? Beginning in the late 1950's with the work of Castillo and Katz (1), Eccles (2), and Hebb (3), chemical synaptic transmission was being increasingly discussed in terms of the "synaptic vesicle" theory. Synaptic vesicles are an aspect of electron microscopic topography of the cytoplasm of the bouton terminale or pre-synaptic region of the axon. These organelles appear as spherical-shaped structures varying in diameter from 359-569 Å (4). Several morphologic types have been characterized, the large mulberry or clustered type with electron-dense cores seen predominately at biogenic amine nerve terminals (5) and the smaller, electron-lucent centered type which are the only type found at the cholinergic synapses (6) being the two best established examples (Fig. 2). It will be the details of the latter type of vesicle which shall be discussed for the most part in the remainder of this work. Careful observation of these structures has appeared to

Fig. 1 An Artist's conception of the cholinergic synapse.
From Science Journal, 3, 67. May, 1967.


myelin sheath
neurofilament
axon
terminal presynaptic bulb of axon (cut away)
mitochondrion (sliced open)
mitochondrion
synaptic cleft
synaptic vesicle
postsynaptic dense
material
presynaptic dense projection
dendritic spine
(postsynaptic, cut away)
dendritic tubule
dendrite
spine apparatus

rule out the possibility that they represent artifact. They appear to have either a bilayered wall (7) or a honeycombed substructure resembling a rosette, possibly representing a more basic subunit of tetrahedral shape (8). The bulk of these structures appear to be concentrated along the pre-synaptic membrane itself, suggesting to some investigators perhaps an ultimate fusion or some permanent connections which may exist with that structure (as yet, however, none can be firmly demonstrated).

It was Fatt and Katz (9), who initially noted a peculiar arithmetic quality to the so-called background noise detected with microelectrode monitoring of the resting potential post-synaptically. These miniature endplate potentials (m.e.p.p.'s) all appeared to be small whole number multiples of some basic subunit of millivolts of depolarization. These investigators thus suggested that perhaps the pre-synaptic terminal was leaking discrete packets or quanta of transmitter substance secondary to some sort of spontaneous release (thermodynamically responsive as later demonstrated) of the tenuously held transmitter agent. Application of neostigmine enhanced the monitored post-synaptic responses which represented this phenomenon and thus further supported the above contention.

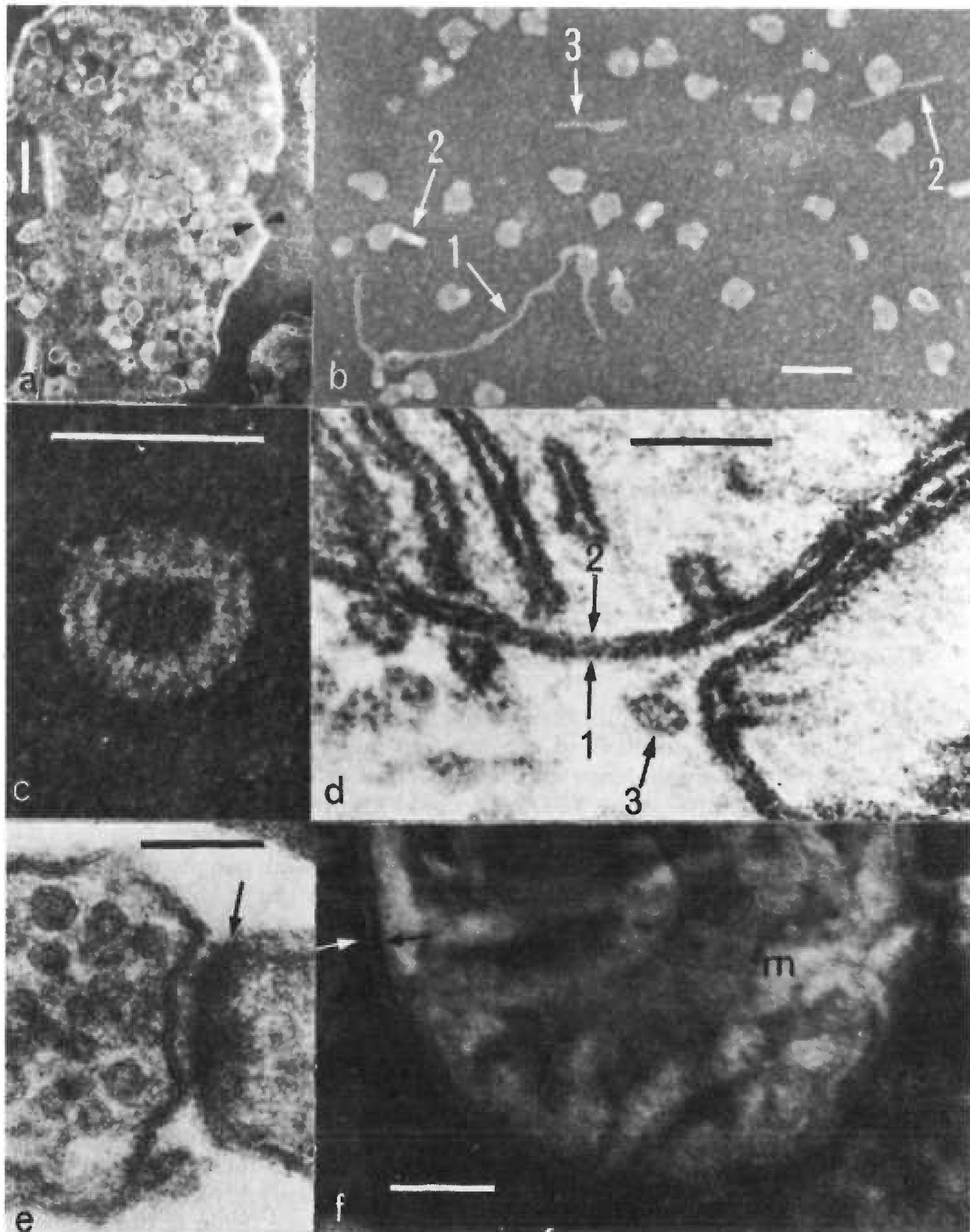
Coupling these two pieces of data one arrives at the appealing hypothesis that the vesicles are the storage sites and that their "breaking down" or individual spontaneous release of their complement of transmitter substance represents the microanatomic equivalent of m.e.p.p.'s. Calculations based upon the mean dimensions of the cholinergic vesicles indicate an appropriate order of magnitude of

acetylcholine molecules relative to the amplitude seen as the basic subunit of m.e.p.p.'s (the latter being arrived at by micro-pipette applications of acetylcholine to the cleft region). Furthermore, the amplitude and frequency of the m.e.p.p.'s can be appropriately altered by manipulation of the pre-synaptic potential below the firing level (increasing in both parameters with increasing depolarization). The only source of ambiguity to this view stems, perhaps, from the lack of any supportive evidence from experiments designed to alter the population densities of vesicles by use or disuse (for example, intense stimulation at neuromuscular synapses has failed to demonstrate any depletion whatsoever) (10).

With such a crucial role assigned to the vesicle, it was only natural that a number of investigators should embark upon the development and perfection of techniques designed to yield varying degrees of isolation of this subcellular particle as a prelude to intensive studies of its inherent properties. Whittaker's group (11) (12) in England and De Robertis' group (13) (14) in the Argentine seemed to match each other paper for paper throughout this period stretching from the late nineteen fifties through to about 1964, affording an excellent system of constant checks and critiques of one another's work and hence, no doubt, contributed much to the high relaibility and reproducibility that can now be assigned to their techniques.

In essence, they both evolved a procedure whereby rat or rabbit brain cerebral cortex was homogenized in an isosmolar

Fig. 2 A collection of electron micrographs representing the fine structure of the synapse, utilizing several different techniques. From Ann. N.Y. Acad. of Sci., 137, 995.



(*a*) Portion of negatively stained synaptosome showing external membrane (between arrows) and synaptic vesicles. (*b*) Isolated synaptic vesicles (fraction *D*) negatively stained without prior treatment. Note: (1) connections between vesicles; (2) vesicles with "tails"; (3) lengths of fibrillar material. (*c*) Isolated synaptic vesicle from fraction *D* after suspension in phosphate buffer and negative staining. Note bilayered appearance of membranes. (*d*) Portion of synaptosome cytoplasm after permanganate fixation, thin section and lead staining showing (1) outer, (2) inner mitochondrial membranes, (3) synaptic vesicles all with triple layered structure. (*e*) Portion of synaptosome as in (*d*) showing triple-layered structure in synaptic vesicles and external synaptosome membranes (arrow indicates adhered postsynaptic membrane). (*f*) Portion of negatively stained mitochondria showing thin external membrane (between arrows) and unpenetrated matrix (m) with cristae filled with stain.

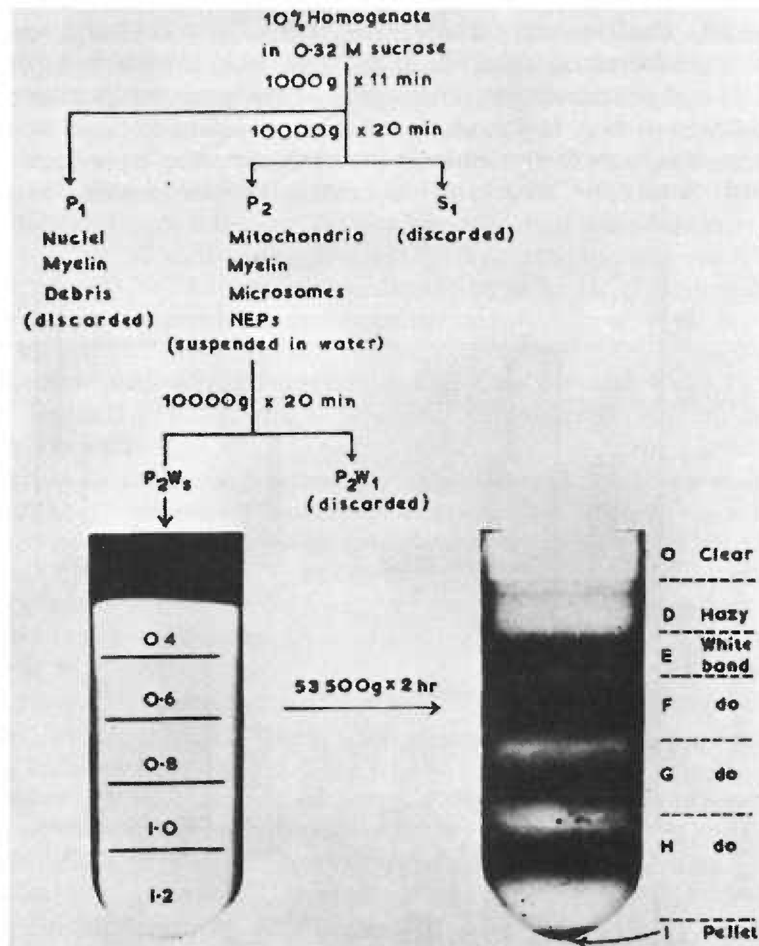
sucrose solution and then differentially centrifugated; first at low speeds to sediment the fraction rich in mitochondria, microsomes and nerve-ending particles (N.E.P.'s or synaptosomes are the pinched-off axon terminals entrapping the vesicle rich cytoplasm in a millieu nearly comparable to that in vivo for at least a brief span of time); next the N.E.P.'s are lysed in hypoosmotic solution thus releasing the mitochondria, microsomes, and vesicles into the solution where they remain suspended during the high speed centrifugation which follows. Next, the suspension is placed atop an interrupted sucrose gradient and spun at ultra-high speed with the result that the vesicles, owing to their unique density, become entrapped relatively free of all other subcellular debris in a readily isolatable fraction (Fig. 3) (15). (The development of this technique depended heavily upon electron microscopy as a laboratory tool.) There exist numerous variations of this basic format by this time which reflect the specific interests of a particular investigator and the kinds of equipment at his disposal.

Following the perfection of such procedures the following information has been derived from scrutiny of the resultant fraction: (a) the vesicle fraction is richest of all subcellular fractions in acetylcholine and Acetyl-CoA: Choline-O-acetyl transferase (E.C. 2.3.1.6), (the enzyme responsible for acetylcholine synthesis); (b) this acetylcholine was bound to the vesicle and could be released by (i) allowing the vesicles to stand even at 0° C., (ii) by decreasing the pH, (iii) by ultrasound, (iv) by heating (16);

(c) one could exchange label vesicles with ^{14}C-acetyl-choline or ^{14}C analog (e.g., choline) which displayed the same tendencies to release under the same conditions as the bioassayable endogenous vesicular acetylcholine (17); (d) careful chemical analysis indicated that the vesicles were comprised of some 43% protein and the remainder principally the phospholipids phosphatidyl choline and phosphatidyl ethanolamine as well as cholesterol and gangliosides (Fig. 4) (18).

Burton *et al*. have more recently proposed a mechanism of release utilizing the polar properties of gangliosides in order to bring the vesicles into juxtaposition with the neural membrane and its mutually miscible phospholipid bilayer (Fig. 5). At the time this hypothesis was proposed gangliosides were thought to occur in much higher concentrations in the vesicle fraction than subsequent analysis would indicate. It was while pursuing a demonstration of proof of this proposition, that they showed the labelling properties of vesicles which, as will be seen later, affords a very sensitive and specific means then of monitoring the phenomena of binding, release and the factors which influence both. Furthermore, Burton, *et al*. (19) have successfully demonstrated binding of ^{14}C-choline to a 1:2 mixture of cholesterol and phosphatidyl choline as well as how this binding is influenced by the presence of gangliosides (a possible detergent effect) (Fig. 6). Finally, Burton, *et al*. have extracted the lipid from the

Fig. 3 The technique for separation of the subcellular components of the nerve ending particle, including the synaptic vesicle is outlined. From Ann. N.Y. Acad. of Sci., 144, 397.



Preparation and density gradient fractionation of hypotonically disrupted NEPs. Left: SW 25 lustroid tube of Spinco ultracentrifuge showing water-treated preparation and sucrose density gradient (numbers give molarity of sucrose layers). Right: same after centrifuging, showing fractions taken (Whittaker, Michaelson & Kirkland, 1963, 1964).

Morphological Appearance of Fractions Obtained by Density Gradient Separation of Water-Treated Crude Mitochondrial Fraction of Guinea-Pig Brain

FRACTION	DENSITY*	MORPHOLOGY
O	0	No organized structures
D	0.4	Synaptic vesicles, occasional microsomes
E	0.4–0.6	Microsomes, clumps of synaptic vesicles
F	0.6–0.8	NEP ghosts, myelin fragments, postsynaptic membranes (?)
G	0.8–1.0	NEP ghosts, membrane fragments, postsynaptic membrances (?)
H	1.0–1.2	Damaged NEPs, NEP ghosts
I	1.2	Small mitochondria, some shrunken NEPs

* Expressed as the sucrose concentrations in molar units in which the particles float.

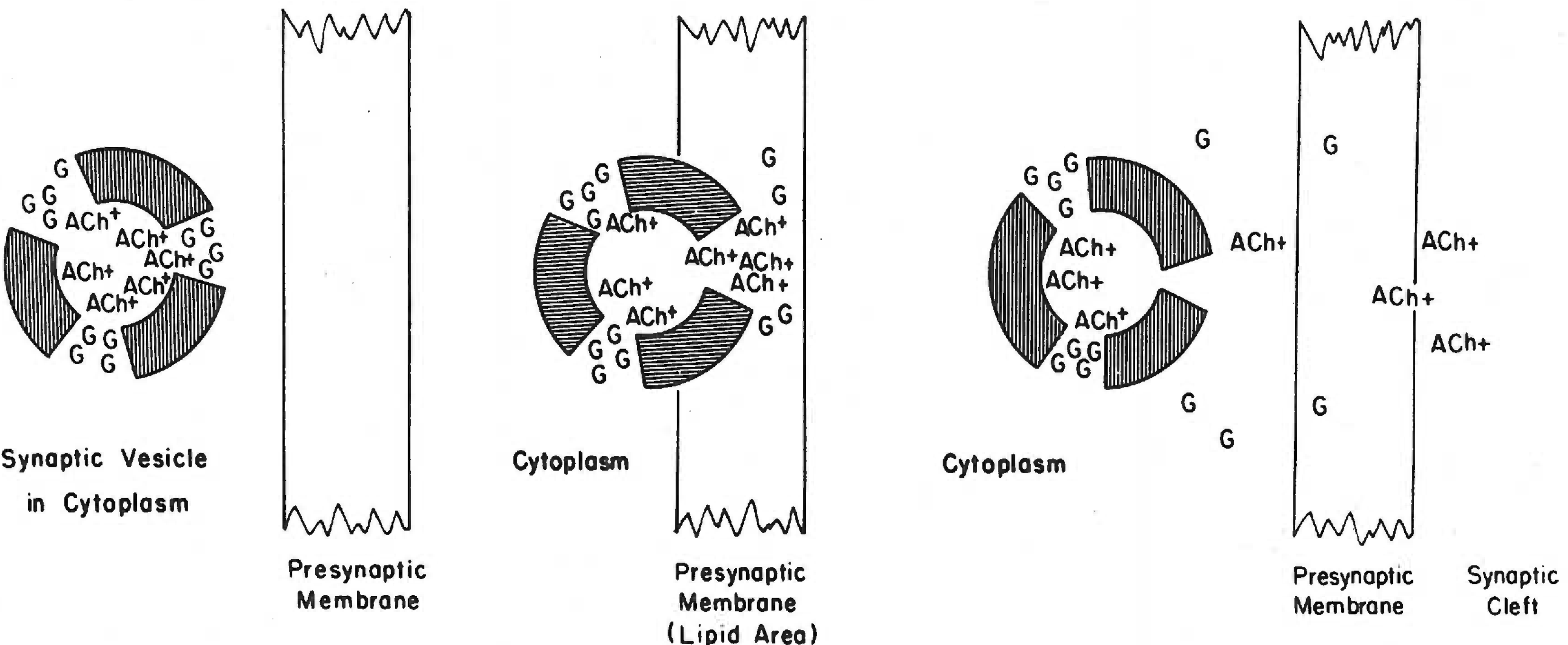
Fig. 4 An Analysis of the phospholipid composition of the various subcellular components of the nerve ending particle, including the synaptic vesicle. From Ann. N.Y. Acad. of Sci., 137, 992.

Phospholipid Composition of Brain Fractions

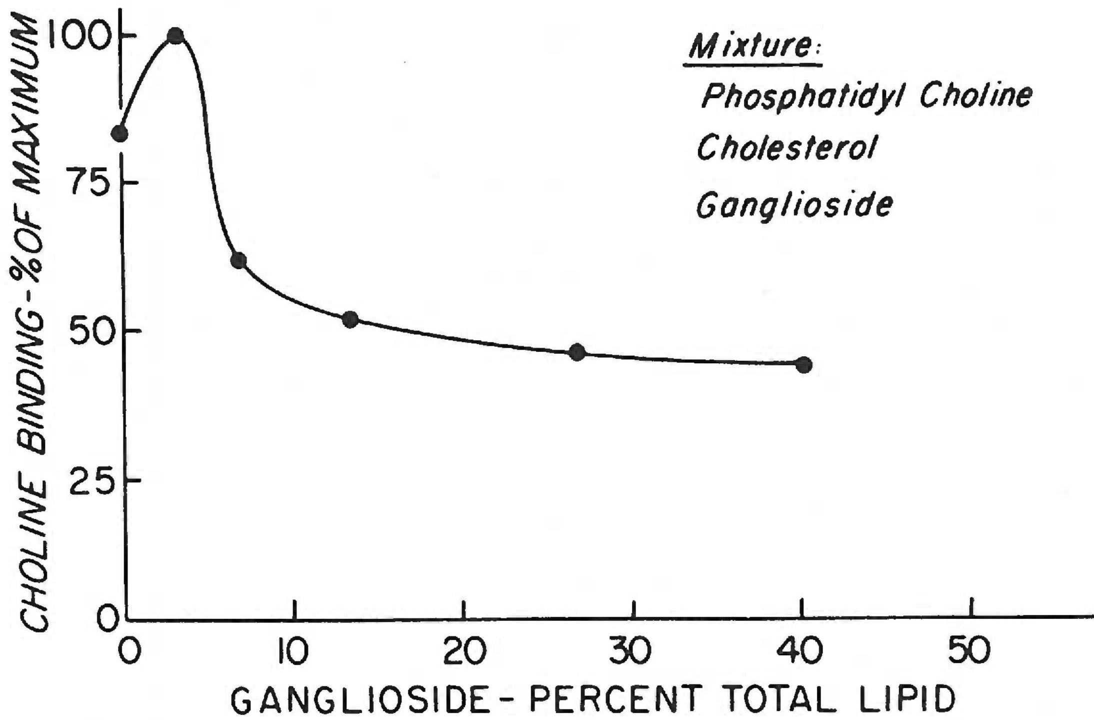
Lipid	1. Large myelin (P_1A)	2. Small myelin (P_2A)	3. Microsomes (P_3)	4. External synaptosome membranes (G)	5. Synaptic vesicles (D)	6. Mitochondria (P_2C)	7. Synaptosomes (P_2B)
Phosphatidylcholine	26	32	41	24	41	40	39 (35)
Phosphatidylethanolamine	9	14	15	10	15	23	18 (15)
Phosphatidylserine	13	14	12	3	12	6	13 (7)
Monophosphoinositide	3	3	4	1	5	5	4 (4)
Phosphatidic acid	2	1	0	6	0	1	1 (2)
Cardiolipin	1	0	1	0	1	11	2 (4)
Choline plasmalogen	1	0	0	0	0	0	0 (0)
Ethanolamine plasmalogen	26	24	15	20	15	9	16 (15)
Serine plasmalogen	1	0	0	0	0	0	0 (0)
Sphingomyelin	12	7	8	6	11	4	5 (7)
Alkylether	4	2	2	4	3	2	2 (3)
Recovered	93	97	98	74	94	101	99 (93)

Fig. 5 The Burton model for release of acetylcholine from synaptic vesicles and the role of gangliosides. From Ann. N.Y. Acad. of Sci., 144, 416.

Burton & Howard: Gangliosides and Acetylcholine



Hypothetical mechanism for bound acetylcholine release and transport through the pre-synaptic membrane. Events being represented are: (1) polarized membrane prior to transmission, (2) during depolarization and acetylcholine release, and (3) repolarization after acetylcholine is in the synaptic cleft. Acetylcholine – ACh^+; gangliosides (or other complex lipids) – G; and synaptic vesicle proteins matrix – cross hatched areas. (From Burton *et al.*, 1964).

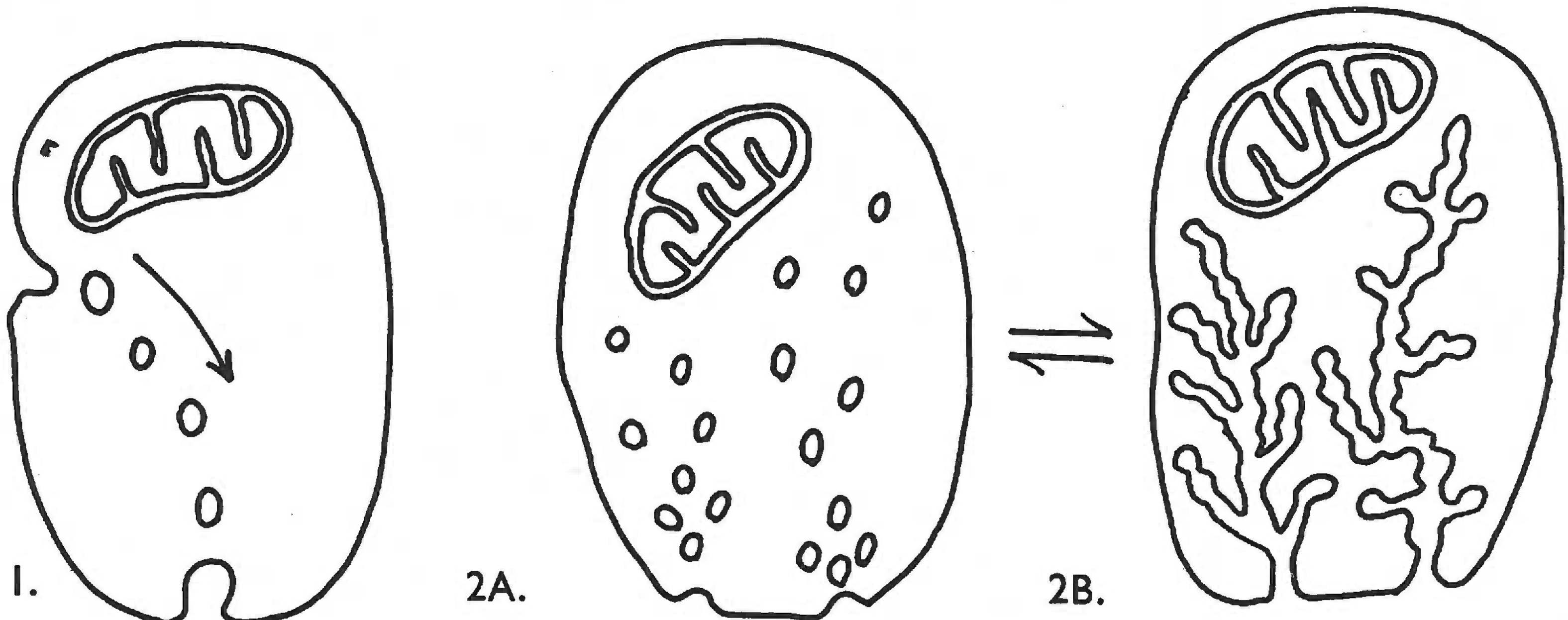


Effect of gangliosides on choline-C^{14} binding by phosphatidylcholine : cholesterol (3 : 1) suspensions. Details are described in the text and under Methods. The phosphatidylcholine and cholesterol were used in a 3 to 1 ratio by weight. The gangliosides were added and reported as percent of total lipids, i.e., phosphatidyl choline, cholesterol, and gangliosides.

synaptic vesicle fraction and demonstrated loss of label into the lipid layer (20).

Whittaker (21) has proposed an alternate possibility in that the vesicles may represent the resting phase in a two-phase system in which the active phase is the neural tubule. These tubules are envisioned as having connections and openings through the cell membrane into the synaptic cleft. When depolarization reaches the bouton terminale, fusion of vesicles to form tubules would occur. As supportive evidence, electron micrographs demonstrating tubules in large synaptosomes obtained from cerebellar cortex (presumably the large mossyfiber endings of the granular layer) as well as vesicles. Israel and Whittaker (22) also obtained synaptosomes from highly purified preparations in which only tubules, no vesicles were to be found. The presence of intact mitochondria in these synaptosomes seemed to preclude that these subcellular structures might be responsible for such tubular artifact. The idea of ejection of the entire vesicle structure out into the synaptic cleft, as some initially suggested, now appears unlikely simply upon the basis of the size of the cleft and absence in all so far reported electron micrographs of anything even remotely resembling a vesicle outside the presynaptic membrane (Fig. 7).

Fig. 7 The Whittaker hypothesis of vesicle formation and transmitter release. From Mechanisms of Release of Biogenic Amines, Proc. Int. Werner-Gren Cent. Simp. Stockholm, 1965.



Hypothesis of vesicle formation and transmitter release. 1. Vesicles are formed by withdrawal of external membrane material in regions distal to the area of synaptic contact, are charged with transmitter in the cytoplasm and fuse with the membrane in regions proximal to the region of synaptic contact. For reasons given in the text, the patches of membrane resulting from vesicle fusion must retain their identity. What maintains a unidirectional flow of vesicles? 2. Vesicles are present only in resting endings (A). On activation, they fuse to form tubules (B) which discharge transmitter accumulated during phase A. The vesicles readily reform from the tubules and the membrane of the tubule-vesicle system retains its identity. Possibly the vesicles are linked even in the resting phase by occluded lengths of tubule.

HISTORICAL CONSIDERATIONS

The earliest reference to the possibility that some sort of chemical substance might be responsible for the bridging of synaptic clefts by a neuronal action potential was by DuBois Reymond in the year 1877, when he suggested ammonia or lactic acid might be such transmitter agents (23). Later, at the turn of the century, Langley and Lewandowsky were struck by the close similarity between the effects of injections of adrenal gland extracts and discharge of sympathetic neurons (24). Finally, while still an undergraduate student at Cambridge, England, Elliot postulated that sympathetic neuronal impulses released minute amounts of an epinephrine-like substance in immediate contact with end organ cells (25). In addition, he noted that such end organs were still responsive to epinephrine after sympathetic innervation had been deliberately interrupted. Dixon (26), utilizing the alkaloid muscarine, was able to simulate vagal stimulation and, therefore, posited that, "excitation of a nerve induces the local liberation of a hormone which causes specific activity by combination with some constituent of the end-organ, muscle or gland". This first clear ennunciation of the neurohumoral release met with considerable resistance and the counter hypothesis that transmission was purely upon an electrical basis remained in vogue.

The year 1907 saw the initial serious work on acetylcholine by Hunt (27) and later in 1914 by Dale (28). The latter investigator coined the term "parasympathomimetic" to describe its action and made the important observation that the brevity of ACh's action was most likley explainable on the basis that there existed a

potent tissue esterase capable of very rapid hydrolysis of the substance to acetic acid and choline hence its inactivation.

It awaited, however, for Loewi (29) in the nineteen twenties to establish rigorously that end organ stimulation is the result of the secretion of chemical substances. His now classic experiment of stimulating the vagus nerve of a perfused (donor) frog heart and perfusing a second dennervated frog heart with this same perfusate with a resultant slowing the heart rate, and his equivalent series of manipulations in relationship to the cardiac sympathetics with resultant acceleration of heart rate taken together remain, perhaps, the most powerful of demonstrations of the concept of so-called reciprocal innervation: that numerous organ systems receive both parasympathetic and sympathetic innervation with the former acting to produce an inhibitory or braking action and the latter acting to produce an excitatory or accelerating action (and, in some instances, such as the pupillary muscles of the eye, both excite and it is the structural arrangement of the two organ systems that are, in fact, in opposition). Loewi referred to the material released by the vagus as Vagusstoff (parasympathin) while that released by sympathetic stimulation simply bore the name sympathin.

With the passage of time, mammalian systems were utilized and found to possess the exact same arrangements. Then, investigators turned their attentions towards an even more difficult biologic preparation to work with, which yet still held forth the promise of even greater prize: the sympathetic chain ganglion. Here the

classic preparation was perfected by Feldberg and Gaddum (30), utilizing the unusually large cervical sympathetic ganglion of the common domestic cat with its equally prominent and intimate blood supply being utilized as an isolated perfusion bed. Neuronal synaptic transmission was easily monitored via the response of the nicitating membrane, post-synaptically innervated, to presynaptic stimulations electrically. In the early 1930's Dale and Feldberg (31) and their collaborators established that ACh was released not only at peripheral terminal synapses of mammals, but also at preganglionic nerve endings in sympathetic ganglia. Thus interneuronal transmission as well as at neuron-end-organ synapses (e.g., myoneural junctions) was demonstrated as being at least possibly mediated by ACh.

The reason why this secretion of ACh at the synapse fails to make its role as a transmitter something less than ironclad, relates to the not inconsiderable evidence amassed in advocacy of a strictly electrotonic[1] form of synaptic transmission. There are well-documented examples of synapses which transmit their activity by direct electrotonic spread of current from one cell to another. Some of these are rectifying, i.e., the current can pass in one direction only (e.g., the crayfish giant motor synapse and between the club endings and dendrites of the Mauthner cell in goldfish),

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[1]Electrotonic transmission: Transmission of a nervous impulse effected by the depolarizing action of an electric current that is generated either by impulses or by synaptic potentials in the pre-synaptic component. Transmission is either direct via septum or by induction of an electrical field post-synaptically and no chemical mediator substance then plays a role.

while others are not and pass current in both directions (e.g., the crayfish giant motor synapse). In all of these instances, micrographs show that either the synaptic cleft is quite narrow or nonexistent. In the case of the Mauthner axon cap, there is a branching of twigs filled with vesicles and enclosing the axon of the post-synaptic cell in a field without making contact with it. Inhibition is apparently brought about by a marked change of the ionic environment adjacent to the post-synaptic membrane instead of via the release of a bolus of transmitter molecules. If then, electrotonic synaptic transmission is known to occur in nature, what differentiates the chemical synapse from this sort of synapse (which some refer to as an ephaptic junction in contradistinction to an actual synapse) other than on the grounds of histologic second guessing?

Direct evidence for the possible mechanism of excitation-secretion coupling remains sparse indeed. The phenomenon of _synaptic delay_ coupled with the seeming morphologic differences noted above and subsequent use of chemical inhibitors are the cornerstones of this hypothesis. Synaptic delay represents the finite time interval between the activation potential arriving at the bouton terminale and becoming regenerated in the post-synaptic region of the adjacent neuron. This finite event distinguishes ephaptic from synaptic transmission. Originally, it was thought due to the time required for diffusion of the transmitter substance across the cleft, but it is now recognized as being related to time required for effecting of transmitter release primarily (this was elucidated

by implantation of microelectrodes into giant synapses and determining delay secondary to diffusion alone as opposed to that occurring following presynaptic electrical stimulation) (32). In addition, as neuropharmacology developed, innumerable inhibitory substances apparently acting at the various stages of transmission (i.e., synthesis, storage, release and inactivation) have been shown to produce the exact pathophysiologic alteration predicted upon the basis that far-and-away the bulk of synaptic transmission chemically is mediated. Many clinical applications have been found for such agents which argues empirically for the validity of the chemical concept. More detailed discussion relating to these latter evidences shall be postponed until the sections dealing with the phenomena of synthesis, storage, release and inactivation.

Finally, Fatt and Katz's (33) exhaustive studies referrable to synaptic transmission and their characterization of miniature end-plate potentials (again discussed in detail at a later time) have at least solidified the evidence in favor of a predominance of chemical transmission in peripheral synapses. Regrettably, at the present stage of investigation (the technical difficulties in working with the physiologic preparations being one of the chief barriers) very little concrete can be stated concerning synaptic transmission in the central nervous system, and thus the assumption is made for the time being that what is known of peripheral synaptic transmission adheres centrally with the only proviso being that the transmitter substances involved may be different.

What are the hypothetical ideal properties of a transmitter substance? (a) it must be synthesized and stored in such a manner as to be physiologically inactive; (b) it must be released in response to an activation potential from the pre-synaptic region of an axon; (c) it must diffuse across the synaptic cleft with sufficient rapidity so as to reach the post-synaptic membrane and its hypothetical receptor site in a physiologically meaningful space of time; (d) it must induce an alteration in permeability in the post-synaptic membrane such that an action potential is generated and propagated in that cell membrane; (e) it must be inactivated with sufficient rapidity so that the synapse is cleared for a new and discretely separate event of transmission in a physiologically meaningful space of time.

How then does ACh match up with these five criteria? First of all, as will be described in greater detail later, the synthesis of ACh appears to be stimulated to a considerable degree by the very changes which occur with repetitive nerve stimulation (i.e., the influx of sodium with a gradual build-up of the ion being sustained between action potentials). The synthesis occurs within the cytoplasm of the axon and the product of this process is either bound such that it is physiologically inactive or destroyed by the intracellular ACh esterase. As Whittaker (34) and De Robertis (35) have demonstrated, this bound fraction appears, at least in part, to reside in the structure known as the synaptic vesicle. Much earlier, investigators such as Bodian (36) and Elliot and Brodkin (37) could demonstrate a fraction in crude homogenates of brain

that appeared resistant to esterase degradation and was, therefore, presumed to be bound or sequestered in some physiologic and/or anatomic manner. The issue of quantal release was principally elucidated by Fatt and Katz (38), and later by Katz and Miledi (39). The key factors here were the increasing miniaturization of micropipette and microelectrode assemblies coupled with refined methods of placement in appropriately macroscopic biologic preparations such as _Aplysia_ and giant squid. In this experimental design, exogenous boluses of ACh or known pharmacologic releasing agents were introduced presynaptically and activation monitored downstream in a quantitative fashion. In this way, these investigators arrived at a calculated value of about 10^{-17} moles of ACh as the amount liberated at each nerve terminal per physiologic impulse (40). They documented a form of background "noise" at the post-synaptic membrane that was indicative of a gradual leak of ACh producing subthreshold pulses of depolarization. Curiously, this phenomenon was observed to be increased in small multiples as to amplitude of the depolarization wave produced and failed to be simulated by a steady flow of a low concentration of ACh in a bathing solution in which the synapse was immersed (41). These "bursts" of small depolarization waves with discrete beginnings and terminations could not then be explained by a few molecules leaking across the synaptic cleft intermittently (this only produces a lowering of the resting potential a few millivolts and had no effect whatsoever on the other phenomena). Later work (42) showed

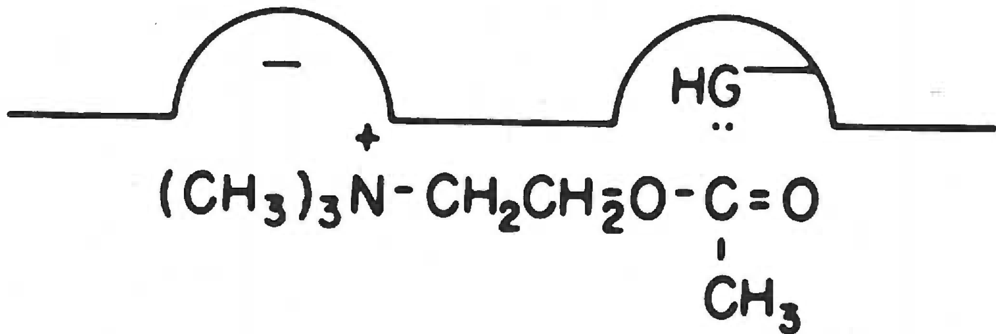
that 300 of these discrete packets of depolarization (and, therefore, 300 discrete quanta of ACh) go to make up a single fullblown action potential, i.e., since about 10^{-17} moles of ACh are thought to be liberated with each action potential, it must contain roughly 3×10^{-20} moles per packet.

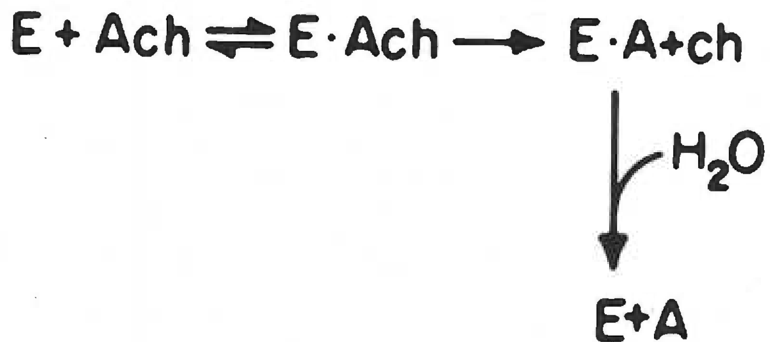
Finally and most briefly, the post-synaptic events in all of their complexity and present ambiguity: the effect of ACh on smooth muscle preparations or in vivo, using the sorts of sophisticated physiologic preparations described earlier, is one of initiation of an alteration in ion permeability associated with depolarization or hyperpolarization (i.e., depending apparently upon the nature of the receptor site, it has been shown to act as either an excitatory or inhibitory substance...). By the use of structural analogues of ACh, pharmacologists have acquired considerable knowledge of the nature of the specialized receptors that they have used in their particular experiments (i.e., the battery cells of the electric eel electricus Electrophorus (43), smooth muscle myoneural junction, striated skeletal myoneural junction and cardiac myoneural junction). Admittedly, they all show certain similarities upon which basis some generalizations as regards the character of the cholinergic post-synaptic receptor are made; however, it is always well to bear in mind the fact that little of such work has been done utilizing mammalian interneuronal synapses and morphologically as well as physiologically to refer to a single cholinergic receptor would seem far too much of a generalization.

The pharmacologic cholinergic receptor, then, has been found to possess two primary sites: (a) an anionic site to which the choline's quaternary ammonium binds and (b) an esteratic site capable of donating an electron pair to the acetate portion of the molecule (44) (Fig. 8). Beyond this, the mechanics by way of which this enzyme-substrate complex alters membrane permeability remains as yet unknown (presumably it relates to transient conformational changes in membrane protein). Curiously, the exact same description exists for the character of the inactivating enzyme ACh esterase. The only difference being that when the acetate combines with the esteratic site, it is cleaved from the choline leaving a transient acetylenzyme intermediate, which is then, in turn, hydrolyzed. While not the purpose of this discussion, one cannot avoid the obvious question of whether the esterase and receptor are not in fact identical. The evidence pro and con is voluminous, the techniques and reasoning behind much of the work quite involved and at times rather speculative. Therefore, let it suffice to say that as yet no one has been able to isolate or truly distinguish in a rigorous manner between the two and a severe handicap lies in the fact that different investigators use rather specially suited or traditionally accepted preparations rather than some uniform mammalian preparation involving true interneuronal synapses. One curious phenomena associated with the receptor site is the way in which its localization appears to be induced in response to the proximity of end fibers of the presynaptic neuron.

Ingenious nerve cell degeneration studies indicate that when innervation is cut to a muscle fiber, the sensitivity or "receptivity" to ACh spreads out over the entire membrane, leaving the old receptor site no longer unique or dominant (45).

Fig. 8 The pharmacologic receptor site. From Neruoscience, p. 437.





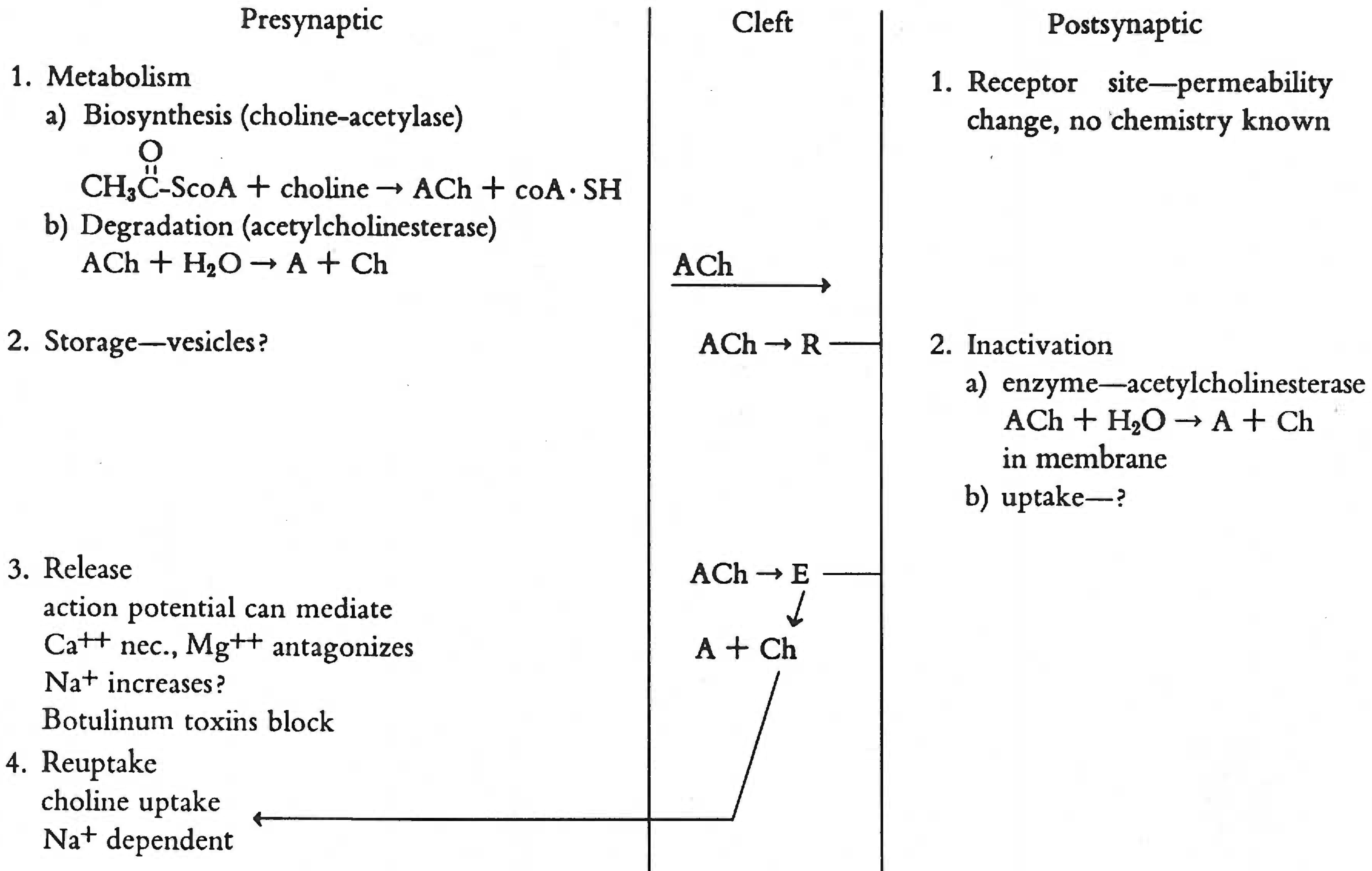
Acetylcholinesterase: enzyme surface and reaction sequence.

THE PHENOMENON OF SYNTHESIS, STORAGE, AND RELEASE OF ACETYLCHOLINE:

These three phenomena: (a) synthesis, (b) storage, (c) release are intimately intertwined and much labor and contemplation have been expended in an effort to elucidate the precise mechanisms entailed in accomplishing these three functions. Furthermore, at least in part, consciousness and all that term encompasses must be predicated on this or a highly similar biological device.

The approaches to the study of synthesis have been perhaps more successful, since they involve more classical biochemistry, than the last two processes. The substrates for synthesis in their most elemental forms are ATP and acetate which combine to form adenyl-acetate which in turn reacts with coenzyme A via mediation of the enzyme E.C. ATP: acetate phosphotransferase 2.7.2.1. (acetyl kinase) to form acetyl CoA (this acetate has recently been shown, through brain slice tracer experiments to arise from glucose or pyruvate primarily) (46). That this process occurs throughout all cell population in the body is well-known and therefore it is the steps beyond this which prove unique for the cholinergic neuron. Acetyl CoA interacts with choline via mediation of the enzyme choline transacetylase (Fig. 9). This enzyme is unique to nervous and primate placental tissues (47). Choline is suspected of being transported actively into the neuron (this concept being based upon studies with hemicholinium, tetramethylcholinium and tetraethyl-cholinium which all seem to block choline uptake presynatpically (48), and it is choline which is recognized as the rate limiting substance

Fig. 9 Synthesis, storage, release and inactivation of acetylcholine at the cholinergic synapse. From <u>Neuroscience</u>, p. 439.



Synaptic chemistry of acetylcholine.

in the synthetic process (49). Questions of the influence of pH and temperature have been explored, but *in vivo* appear to have but small influence save, as will be later noted, in altering the degree of dissociation of various ions felt to play active roles in storage and release as well as synthesis.

Sodium ion influence on synthesis has been explored by Keynes (50) and more recently by Birks, (51) with the following conclusions being drawn: by utilizing the fact that the superior cervical sympathetic ganglia bilaterally in the same cat have an equal amount of total assayable ACh $\pm$ a few percent, one can determine the resting or baseline ACh content on one side and then proceed to perfuse the contralateral ganglion with perfusates of equivalent osmolarity, but varying concentrations of sodium and then determine the assayable ACh remaining at the end of a fixed period of time and compare the baseline and this value (this includes ACh released in perfusate). Subtracting final from the baseline values equals depletion exceeding synthesis and by subtracting baseline from final + released equals the amount synthesized. Results of such experiments indicate that by increasing the sodium concentration one increases synthesis such that a maximum three times resting levels is achieved by perfusion with a 154 mM NaCl concentration.

As to the mechanism of sodium stimulation, there are three major hypothesis at present: (1) choline and glucose are acquired by the cell by active transport in which the pump is primed by sodium (analogous to the Na^{+}-K^{+} ATPase dependent pump). (2) Choline transacetylase (ChAc) is activated or rendered accessible to its substrates

(perhaps the compartment sequestering this enzyme is collapsed in the absence of sufficient intracellular levels of sodium or at least becomes swollen or somehow altered in shape. (3) Sodium may promote an increase in the number of ACh binding sites and/or more efficient vesicle formation and in this way stimulate synthesis.

The question of location of synthetic machinery is naturally raised by these last remarks and fortuitously a partial answer is at last forthcoming. Initially Whittaker, et al. (52) localized ChAc in the soluble cytoplasmic fraction while, utilizing differential centrifugation rather than interrupted sucrose gradients, De Robertis et al. (53) showed ChAc in the synaptic vesicle fraction. Later when ruptured synaptosomes from rat brain were subjected to density-gradient centrifugation ChAc was found associated with neither cytoplasm or vesicles but rather with heavier membranes (54). At last, Fonnum demonstrated that particulate ChAc could be made soluble by increasing ionic strength and pH to values more like they are in vivo within the bouton terminale. More recently, Fonnum (55) has gone on to demonstrate that ChAc possesses activity in either bound or free states and that temperature and hypoosmotic states fail to release ChAc from its binding sites (56). Perhaps the most suggestive finding was that acetylcholine and choline do release ChAc. It remains unclear whether ChAc has a high affinity for the vesicle under these more in-vivo-like conditions which would clearly go a long way towards explaining how ACh gets into vesicles, if in fact they do represent the true storage depots of the transmitter substance in vivo.

As MacIntosh underscores repeatedly in his papers, that not just ChAc and ACh are found in close proximity at the terminal portion of the axon, but strikingly ACh esterase occurs in high concentrations intracellularly in the exact same region. How then, does the newly synthesized ACh survive long enough to serve its transmitter functions? This latter query was tentatively answered by hypothesizing that freshly synthesized ACh is stored or sequestered in such a way as to be protected from hydrolysis by the esterase. Perhaps it is a bit teleologic, however it would then seem to follow that the enzyme's presence is to insure that no stray ACh molecules accumulate in the synaptic region outside of this storage compartment (one need only contemplate the havoc that would result were significant amounts of free ACh allowed to leak-out into the synaptic clefts in between physiologically meaningful releases of transmitter substance). As time went by, electron microscopic evidence has suggested a subcellular structure, i.e., the synaptic vesicle, which is a likely candidate for the role of an ACh repository.

Whittaker finds three separate and distinct biologically characterizable forms of ACh in the bouton terminale (57): (a) 20-30% is released by homogenization of cortical tissue, (b) a labile fraction (either a high concentration of ACh inside, but not or only loosely bound to, synaptic vesicles or the residual of the free axoplasmic ACh, (c) a stable fraction held in some way to the vesicles (within or bound to the surface). MacIntosh (58) refers to this stable fraction as bound ACh, labile as depot ACh, and free as surplus ACh. Category (a) is felt by both a manifestation of the continuous syn-

thesis and breakdown of ACh. MacIntosh's bound ACh by definition is not released by depolarization while his depot category is. In this sense his categories do not agree with Whittaker's since the latter's are not characterized in any way in terms of how they respond to depolarization or hyperkalemic solution stimulation. MacIntosh also describes a <u>readily releasable</u> ACh which is that portion of the depot ACh released immediately upon maximum or supramaximum stimulation of a nerve electrically. One might speculate that this corresponded to those synaptic vesicles advantageously juxtaposed relative to the presynaptic membrane making them susceptible to immediate breakdown.

Birks has examined the fine structure of motor nerve-endings at frog myoneural junctions using osmium tetroxide which he incubated with his biological preparations (59). He found evidence on electron micrographs of uptake into the neural tubules (structures thought to represent smooth, nongranular endoplasmic reticulum in axon). Birks hypothesized that the explanation for the uptake was something of an analogy with Huxley's model for the T junctions of striated skeletal muscle. He speculated as to whether this system represented a transport mechanism for choline. The major difficulty, however, with such speculation is that no connections have been so far demonstrable between axolemma and neural tubular apparatus. In support of the choline transport hypothesis, hyperkalemic stimulation of the nerve-endings resulted in increased uptake of osmium tetroxide particles (just as choline uptake might be assumed to increase with the increase in turnover of ACh with stimulation). Birks also reported that

osmium tetroxide stained particles were to be seen inside of synaptic vesicles. This prompted him to speculate as to whether synaptic vesicles were budded off of the endings of the neural tubules (a similar suggestion has been put forth by Whittaker upon the basis of suggestive electron micrographic pictures). Finally, Birks raised the question, and a very valid one, as to whether synaptic vesicles are nothing more than mere artifact, a by-product of the harsh procedures of fixation in electron microscopy which may cause a certain amount of fragmentation of the neural tubular system. An objection to this premise may be found in experiments in which nerves have been transected at midlength several weeks prior to removal and fixation with the net result that synaptic vesicles are seen to pile up in the iatrogenic "presynaptic region." (60). Chemical assays show low concentrations but still reproduciby measurable amounts of ACh along the axon, which might be argued for as evidence that the true source of ACh is upstream. This piece of evidence is the keystone of Nachmansohn's chemical hypothesis for axonal transmission (61).

Release of ACh has been looked at in relation to sodium ions, by Birks (62), using the sympathetic ganglion preparation described earlier. He could demonstrate not only increase in total synthesis, but also increase in release with increasing concentrations such that at 154 mM (Na^+) release was increased four-five fold over perfusion with a salt-free medium (utilizing the isolated vascular bed of the ganglion). Likewise, he noted that ACh release was deficient from the beginning if the perfusate were deficient in sodium, relative

to the physiologic, but contained adequate amounts of potassium to stimulate depolarization and hence release.

When hyperkalemic solution served as the stimulus, and sodium added to the perfusate after perfusing for a time with a sodium-free solution, increase in ACh release would occur only following a measurable and reproducable interval of time. This time delay was felt to represent the time required for sodium ion to penetrate to an intracellular site of action in order to produce the phenomena observed.

Without further supportive experimental evidence, Birks was lead to suggest that sodium may facilitate release of ACh by altering the movement or binding of cations (especially Ca^{++}). Katz has argued the analogy between the muscular contraction role of calcium and ACh's role which is well established by Hodgkin, Frankenhauser, Huxley and Katz[2]; in both instances depolarization of an excitable membrane is the prerequisite to calcium having an effect. It may be that in all these cases the primary action of the electrical potential change is to displace calcium from the inner surface of the presynaptic membrane (Fig. 7). Birks has argued for a competition between influxing sodium ions and already bound calcium ions for some anionic site which is also subject to the same alteration secondary to the act of depolarization (perhaps depolarization rendering the site less anionic by redistribution of charge, and the normal physiologic concentrations of sodium and calcium competing for it). Recent evidence has demonstrated a sodium

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[2] Quoted from R.I. Birks in: The role of sodium ions in the metabolism of acetylcholine. Can. J. of Biochem. and Physiol., 1963, 41, 2555-2571.

dependent glucose pump and a calcium dependent amino acid pump. Perhaps then, sodium activates ACh synthesis also by calcium displacement (63).

There exists, as alluded to in the previous paragraph, quite a strong case for the key role in ACh release going to calcium. By varying pH one varies calcium dissociation (on the alkaline side, i.e., over pH 7.8, ACh release is decreased). Barium ion acts as a reasonably good substitute for calcium in effecting ACh release. Castillo and Katz have shown that magnesium ion, on the other hand, acts to inhibit calcium and prevents release (64). Using giant squid axons and labelled barium and calcium, it is possible to localize uptake of these ions during depolarization secondary to either hyperkalemia or electrical stimulation (65). It has also been demonstrated that electrical stimulation is reflected by uptake primarily at the presynaptic membrane during synaptic transmission (66). Both using calcium-free or deficient baths Castillo and Katz (67) and Hutter and Kosteil (68) with electrical stimulation-perfusion experiments have shown a mathematical correlation between calcium concentration and amount of ACh released.

Birks performed a series of experiments in which cat sympathetic ganglia were bathed in digoxin (a cardiac glycoside) and extracellular potassium was removed (69). One of the actions attributed to the glycosides is that of inhibition of the ATPase dependent Na^+-K^+ pump, thus with an external deficiency of potassium the net result would be a considerable efflux of that ion from the neurons as an equilibrium was once again established. Similarly, an influx of

sodium ion would also be expected to occur. As the intracellular sodium concentration steadily rose over time, naturally increased ACh release occurred. However, by lowering the calcium concentration to one-tenth its physiologic concentration, a significantly increased amount of ACh was released above that occurring when calcium concentration was normal. Finally, when calcium concentrations were lowered even further, the release of ACh sharply declined to almost nil. No explanations for this well-documented phenomena were ever offered. It was also noted that the size of the endplate-potential evoked by the same sized electrical stimulus increased with increased intracellular sodium concentration. As sodium concentration grew even greater, transmission blockade occurred; presumably secondary to a loss of a sodium gradient across the cell membrane. Finally, at an estimated intracellular sodium concentration of 50 mM, spontaneous multiquantal release of ACh occurred.

The phenomenon of post-tetanic potentiation (a larger endplate-potential following tetanic electrical stimulation presynaptically) has been explained as a build-up of calcium ions intracellularly and/or secondly as a build-up of Na^+ ions (perhaps secondary to the former) intracellularly as suggested by results such as discussed in the last paragraph.

The sodium hypothesis, based upon the ionic mechanism of Nernst and Bernstein, was evolved by Hodgkin (70) as the most probable explanation of the mechanism of depolarization which is the necessary, but not sufficient (if we accept the premise as posited

earlier that calcium is essential to ACh release) cause of synaptic transmission (i.e., ACh release). The resting potential of 50-90 millivolts as measured across the neuronal cell membrane has been shown to be due to the ratio of concentration of K^+ across the membrane plus the ratio of concentration of chloride as well. The exceedingly low intracellular concentration of sodium and conversely, i.e., high potassium concentration is maintained by the ATPase dependent Na^+-K^+ pump. It is constantly capturing sodium ions which have diffused intracellularly secondary to the favorable gradient across the membrane and pumping it back out extracellularly in exchange for extracellular K^+ ions. The latter are then constantly being pumped back into the cell in a fixed ratio relative to the sodium being pumped out. This pump requires energy, as it acts in a direction against those gradients of concentration and voltage which cause ions to enter. The pump must then be connected to an energy source which is derived ultimately from the cell.

When the resting potential across the cell is lowered through depolarization to the critical "firing level", a sudden change in the properties of the neural membrane occurs transiently which results in the electrical event which we call the action potential. Apparently these are self-regenerating changes which are nonlinear in character. They consist of a marked increase in sodium permeability initially with a sudden influx of that ion into the cell with a resultant excess of positive charge within but a fraction of a millisecond. This is represented graphically as the overshoot part of the

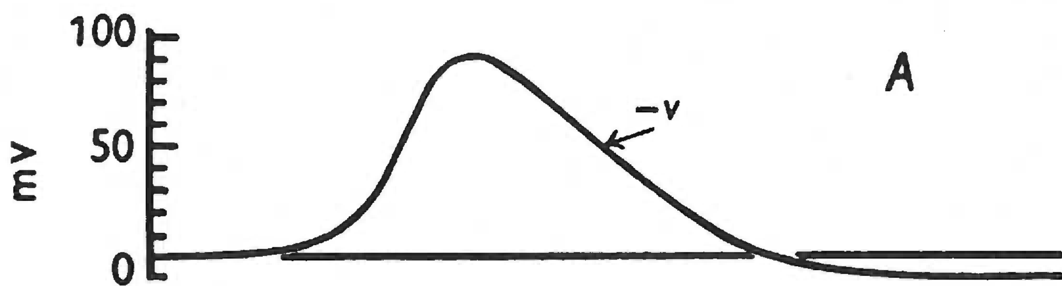
action potential. That this is indeed the case is supported by calculating the equilibrium potential for Na^{+} using the Nernst Equation which has a value then of 30-40 millivolts (the magnitude of the deflection observed). This event is soon terminated by a reversion to the old lack of effective permeability of the neural membrane and is referred to as *inactivation*. Then another augmented permeability occurs, i.e., K^{+} efflux is suddenly markedly increased with the net result being a rapid re-establishment of the earlier distribution of charge (i.e., a decrease in the positive charge of the inside of the cell relative to the outside of the cell is achieved). At the end of the action potential then, there has been a net effect of a slight increase in the intracellular sodium concentration and an equivalent decrease in the intracellular potassium concentration. As far as net voltage across the cell is concerned, because of a momentary persistence in the increased potassium permeability, there is a transient state of *hyperpolarization* or positive afterpotential (as though the system over-shot itself in correcting for the initial imbalance--the oscillatory pattern of self-correction so characteristic of biologic systems). The magnitude of the hyperpolarized state is much reduced when the preparation is such that a minimum of exposure of the nerve (i.e., separation of it from surrounding tissues) has occurred. Therefore, as an *in vivo* event this may be more artifactual than physiologic. Keynes successfully demonstrated that repetitive depolarization results in a quantitative increase in intracellular sodium concentration in agreement

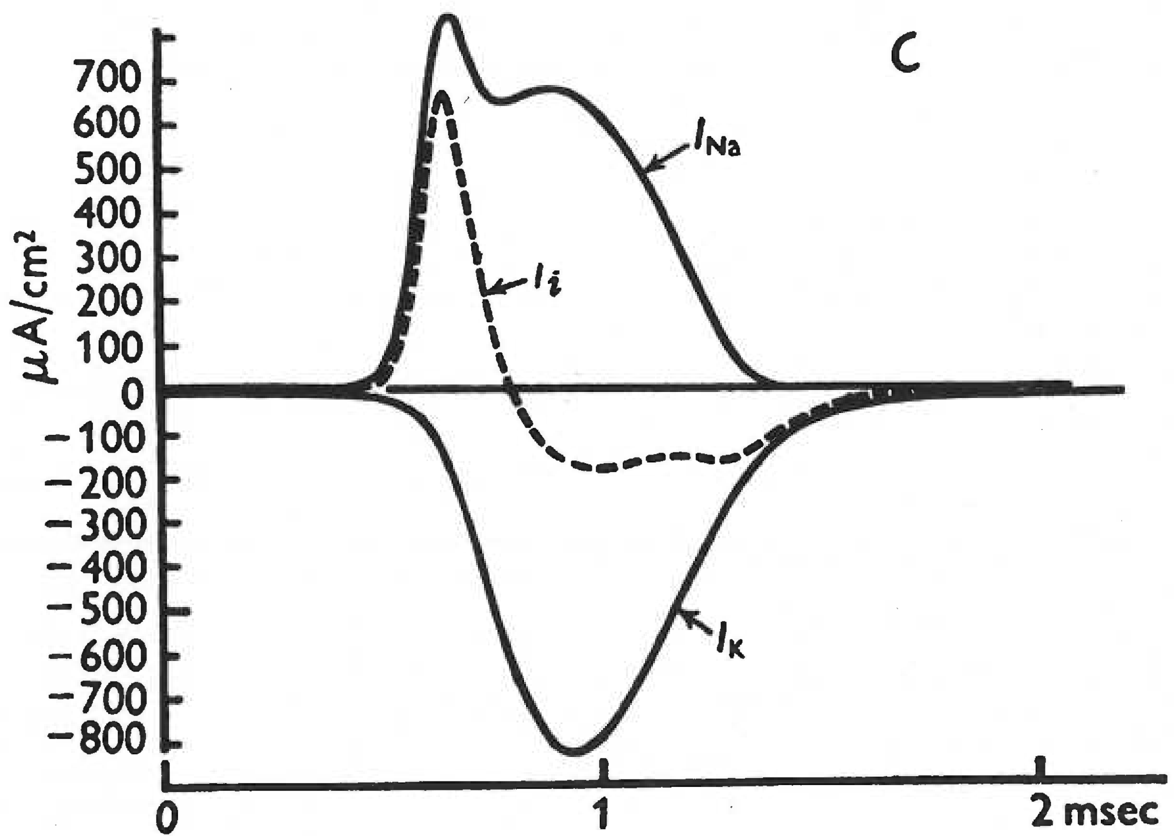
with that predicted by the sodium hypothesis. It has also been demonstrated that complete restitution of the normal ion concentrations occurs over a somewhat longer time period (i.e., restoration of normal low sodium and high potassium levels as existed prior to depolarization).

Another check on the validity of the hypothesis is the fact that the electrical conductivity and the movement of ions across the neural membrane have been demonstrated to match one another. Since current is mediated by ion movements, this is but the logical outcome and final confirmation of the correctness of the present view (Fig. 10).

A correlate to the sort of oscillatory rhythmicity inherent in the sodium hypothesis is the finding in clinical medicine of the tetanic muscle contraction of the metabolic state of hypocalcemia (e.g., Chvostek's Sign). With calcium concentrations lowered, the membrane responds to any stimulus by a long series of oscillations of repetitive depolarizations (the same event has been demonstrated to occur in squid giant axons bathed in a hypocalcemic media). Similarly, the events of <u>absolute</u> and <u>relative</u> refractoriness (periods when, in the former case, no amount of stimulation either as to duration or magnitude will result in propagation of an action potential; or periods when, in the latter case, an increased amount of stimulation is required to elicit an action potential) for neurons recently fired, can be explained upon the basis of the length of time required for recovery of the sodium inactivation process (naturally after more repetitive firing, the degree of disequilibrium

Fig. 10 Current fluxes occurring temporally during action potential according to the Hodgkin-Huxley equation. From Neuroscience, p. 309.





Current flow during action potential and the separation into sodium and potassium components according to the Hodgkin-Huxley equations.

would be greater and thus require a longer period for correction... indeed, this is what is observed).

From work done utilizing a preparation wherein squid giant axons' internal contents were extruded and replaced with known solutions, the selective permeability properties of the neural membrane and the changes undergone with depolarization can be scrutinized in a most exacting manner. This has lead to much quantitatively impressive data to support the view that there exist two types of pores or channels in these membranes: a fast channel through which the sodium ordinarily goes, but the number of which is ordinarily quite low; and a slow channel through which the potassium ordinarily moves and of which there exists in the resting state a large number. It is well to remember that this is a convenient model but not a literal statement of fact. By monitoring the fluxes of various ions, it has been possible to characterize these two entities as to permeability coefficients. Ammonium ion appears able to substitute for both potassium and sodium, being one-third as permeable as the appropriate ion for the appropriate channel. Chandler has shown that tetrodotoxin acts on the fast channel, not on sodium itself. In similar manner, tetraethylammonium appears to block the slow channel (71). The assignment of the names "fast" and "slow" relates to the kinetics of potassium and sodium fluxes during propagation of action potentials. When the alteration in permeability occurs, it is the sodium influx which occurs more rapidly and is in turn shortest-lived. On the other hand, potassium permeability rises to its maximum more gradually but is also more sustained. Thus, whatever

the molecular alterations may be, the transient opening of these pores or channels appears to explain reasonably well what is seen both electrically and ionically. The removal of calcium results in a generalized leakiness of the membrane to all substances not lipid soluble (72). After soaking squid giant axons in calcium-free media, bathing in calcium-free media, and internally perfusing with calcium-free media Rojas and Ehrenstein (73) were able to show that the membrane (utilizing voltage clamp techniques) had lost all of the time-and voltage-dependent properties responsible for the usual electrical activity. During the intermediate period when the membrane has yet to manifest leakiness, the resistance is unaltered, but Taylor (74) has found that the total number of slow channels has been decreased and replaced by channels with a conductance for potassium that is only about five times greater than that for sodium. Utilizing artificial membranes, Castillo, *et al*. (75) have demonstrated that an enzyme or antibody if positioned on one side of the membrane and an antigen or substrate added, a brief transient increase in conductance occurs. In these experiments the enzyme and substrate are on the same side of the membrane. Bean, *et al.* (76) have achieved a large reversible decrease in resistance utilizing artificial membranes made of purified phospholipid, tocopherol, and cholesterol and adding a "membrane-activating factor". Reversal of the effect is achieved by addition of the proteolytic enzyme chymotrypsin. In this model system the enzyme and substrate or activator need not be on the same side of the membrane. Whether there exists any analogy between these phenomena

and what occurs at the synapse is not known.

STATEMENT OF HYPOTHESIS

It is then postulated as follows: That the cholinergic synaptic vesicle possesses a large number of anionic binding sites which would be functional at a physiologic pH and would indeed bind newly synthesized ACh and store it in this physiologically protective manner. In turn, when the intracellular milieu just adjacent to the presynaptic membrane experienced a sudden transient increase in total inorganic cation concentration secondary to a depolarization or particularly when an action potential was propagated, into the bouton terminale region, such bound ACh would be either in part or completely displaced via competition for said anionic binding sites. Thus the described mechanism is best likened unto that possessed by an anion exchange resin now so commonly used in the organic or biochemical laboratory. So likewise, in testing such a hypothesis, it would be of value to compare, point for point, the behavior of such an anion exchange resin under identical conditions to that to which the synaptic vesicles might be subjected in an effort to demonstrate such properties. This then, is the purpose of the following series of experiments and their subsequent assessment. Whether or not this form of binding, should its existence be satisfactorily demonstrated, be the sole mechanism of release at cholinergic nerve endings--as previously observed--cannot as yet be answered and most probably it is not. Therefore, especially as regards the now well-publicized calcium-magnesium-barium phenomena, and perhaps then the actions of some pharmacologic agents

as well, the present hypothesis has nothing to offer if one presumes that these represent manifestations of a second stage or membrane-localized phenomenon.

MATERIALS AND METHODS

Materials:

Acetyl-1-^{14}C choline iodide on two separate occasions was purchased in 0.50 millicurie amounts from the New England Nuclear Corporation. Methyl-^{14}C iodide was purchased in a 0.25 millicurie amount from the New England Nuclear Corporation. Three-times crystallized trypsin was purchased from the Worthington Chemical Company. Phospholipase C derived from Clostridium welchii of a B grade was purchased from the Calbiochem Company. Phospholipase D derived from cabbage of a B grade was purchased from the Calbiochem Company. Amberlite CG-50 of a 200-400 mesh chromatographic grade was purchased from the Mallinckrodt Chemical Works. Sephadex G-10 for gel filtration was purchased from the Pharmacia Fine Chemicals Inc. 1.5 cm. dialysis tubing was purchased from the Food Products Division of the Union Carbide Corporation. Three hundred and twenty four New Zealand white rabbits ranging in weight from 2-4 lbs. were obtained through the University of Oregon Medical School Animal Care Department.

Methods:

All operations were conducted at 0-6°C, with analytical-grade reagents and freshly prepared glass-double-distilled water. Rabbit or rat forebrains were homogenized in 0.32 M sucrose + 1 x 10^{-6} M eserine sulfate in weight/volume ratio of approximately 1:2 (gm/ml)

and separated into fractions P_1, (nuclei, large myelin fragments, and tissue debris) by centrifugation at 1,100 x g for 10 minutes, and the supernatant (S_1) was separated into fractions P_2, (mitochondria, synaptosomes, small myelin fragments, and some microsomes), and S_2, (microsomes, some small mitochondria and synaptosomes) as described by Gray and Whittaker (77) by centrifugation at 10,000 x g, except that, to speed preparation and to diminish microsomal contamination, P_1 was not washed and P_2 was separated at 10,000 x g for twenty minutes instead of 17,000 x g for sixty minutes. In some experiments the P_2 was placed atop an interrupted sucrose gradient of 0.32 M, 0.6 M, and 0.8 M sucrose and spun down at 53,500 x g for two hours and a purified P_2 (the second opalescent layer) rich in synaptosomes utilized. On other occasions the P_2 fraction would be subjected to hyposmotic solutions (double-distilled H_2O, 5 cc.) and the supernatant of such ruptured synaptosomes placed atop on interrupted sucrose gradient consisting of 0.4 M, 0.6 M, 0.8 M, and 1.2 M sucrose spun down at 53,500 x g for two hours and the D fraction (0.4 M layer isolated for the purpose of labelling and cation exposure. Thus, both synaptosome and synaptic vesicle fractions were examined as seemed most convenient or appropriate.

A. Initial Experiments utilizing resin model.

Initial experiments were done utilizing Amberlite CG-50 200-400 mesh, weak carboxylic acid ion exchange resin (this preparation was cycled with NaOH and dilute HCl until $AgNO_3$ test

was negative and therefore resin was sodium free) placed in columns 7 x 0.7 cm. to which 1 ml. of double-distilled H_2O containing 1 x 10^{-3} mg. of ^{14}C acetyl-labelled AChI was added. After washing the columns continuously with water and collecting a number of 1 ml. samples, 1 ml. of variously: 10, 50, 100, 150 mM NaCl and KCl solutions were added with several washes in between additions. All such wash samples were then added to 19 ml. of a toluene-ethanol-PPO-POPOP mixture and the radioactivity determined with a Packard Tri-Carb Model 3003 Liquid Scintillation Counter.

B. Initial Experiments utilizing Nerve-ending particles:

Utilizing P_2 or nerve-ending particle fraction, incubation with ^{14}C-Choline-labelled-AChI 0.036 M was carried out in 5 ml. of 0.32 M sucrose with 1 x 10^{-6} M eserine sulfate. This was then followed by centrifugation at 10,000 x g for twenty minutes at 0° C, followed by repetitive washings in like manner with a like solution devoid of label (all such handling being carried out at a temperature of 6° C). Next, exposure to a similar solution of sucrose containing variously 10, 50, 100 mM NaCl or KCl for a period of fifteen minutes followed by twenty minutes centrifugation at 10,000 g at 0° C. followed by repetitive washings in like manner with a like solution devoid of organic salt. The radioactivity was determined as described above (mixed with 19 ml. of a toluene-ethanol-PPO-POPOP mixture and counted for twenty minutes on a Packard Tri-Carb 3003 Liquid Scintillation Counter).

C. Initial Experiments utilizing Synaptic Vesicles.

Utilizing the before mentioned modified technique of Whittaker, et al, (78); labelling was carried out prior to hypoosmotic shock utilizing 0.036 M ^{14}C-Choline-labelled-AChI incubating at 0° C. for thirty minutes. The separations of the organelles were then carried out as previously described above. 1 ml. samples were taken and counted from each of the five layers of the sucrose gradient as previously described.

D. Initial Experiments utilizing Synaptic Vesicles and Inorganic Salts.

All manipulations were carried out as described in C. save that the D fraction was isolated and divided into three 1.66 ml. aliquots which were then mixed variously with 3.33 ml. of 50, 100 and 150 mM NaCl or KCl and incubated for fifteen minutes at 6°C. This was then followed by centrifugation at 15,000 x g after diluting the mixture 2:1 with double-distilled H_2O for twenty minutes and repetition with washings utilizing double-distilled H_2O and subsequent counting as previously described.

E. Use of ^{14}C-labelled-Acetyl-1-ACh Iodide in Dialysis apparatus.

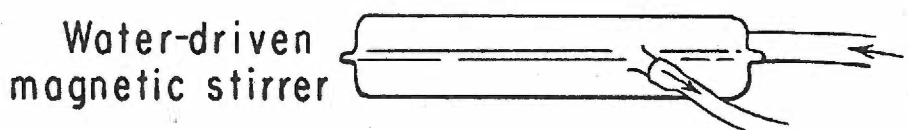
Due to problems of incomplete separation, low activities, and label leakage, presumably secondary to the lengthiness and abrasiveness of the separatory procedures, (repetitious centrifugation and resuspensions), this simple method was abandoned for the following:

1.5 x 10 cm. pyrex test tubes were fashioned with inlet and outlet ports such that they filled to an approximate volume of 5.0 ml. and then emptied. This volume requirement took into consideration the presence of a teflon-coated magnetic flea in the bottom of the test tube as well as double-over 1.0 cm. width dialysis tubing containing

0.5 ml. of volume. The arrangement was then that the dialysis tubing would serve as a compartment with maximum surface area to contain a suspension of the D or vesicle fraction which would then be dialyzed, specifically in regards to its heavy concentration of bound ^{14}C ACh label, and the dialysis wash solution collected in 5.0 ml. aliquots for counting. In this way the abrasiveness and leakage due to time delays would hopefully be avoided. In addition, the rate of decay of the phenomenon of binding under various conditions (variations in ionic strength, temperature, and pH) could be looked at more dynamically. Water-driven rotary stirrers connected in series were utilized, as the cold room lacked electrical outlet sufficient for the load of four separate magnetic stirrers and the advantage of synchrony of rate of agitation of the dialyzing medium in all cases. For purposes of washing, a central reservoir of sufficient capacity was suspended at an appropriate height above the dialysis apparatus and separate siphons run down to each in turn. By utilizing clamps and 21 gauge needle drips (see Fig.11) a uniform flow could be attained through careful monitoring.

Experiments utilizing both continuous and discontinuous flow of dialysis wash solution were tried, with the latter showing more uniformly reproducible results. The labelling of vesicles occurred as before and the D fraction separated into four aliquots of 0.5 ml. and washed for two or two and a half hours. At this time, leakage was reduced to a fairly constant amount from one sample collection to the next and a baseline could be established. Next, a 5.0 ml. bolus

Fig. 11 Apparatus for a dialysis washes of synaptic vesicles.


"Flow-thru"
dialysis bag c̄
synaptic
vesicles
Siphon
reservoir
21 gauge
needle
Graduated
sample
collector
Water-driven
magnetic stirrer

variously 50, 100 and 150 mM concentrations of NaCl or KCl would be introduced through the injecting port juxtaposed to the 21 gauge drip needle and allowed to displace completely the preceding 5.0 ml. of dialysis wash media. Then either incubation for five minutes with subsequent resumption of continuous or discontinuous flow washings would transpire. Second and third exposures to such solutions might then occur. Lastly, the suspension of synaptic vesicles would be collected at the termination of the experiment and boiled for five minutes in a steam bath and then placed in 19 ml. of the previously described scintillation solution and then counted.

F. Use of Sephadex G-25 and G-10 to Separate Labile Bound ACh from Synaptic Vesicles.

After the work of Marchbanks (79) the use of Sephadex to separate the macromolecular vesicles from any unbound or released ^{14}C ACh was attempted. The columns were 60 x 0.5 cm. and had a void volume of 6.4 ml. as determined using a blue dextran marker, and a flow rate of 2 ml./hr. Vesicle fractions were again divided four ways (one used as a control) and mixed with 1.0 ml. of 50, 100 and 150 mM concentrations

of NaCl and added to the tops of the columns. Fractions of 2.2 ml. fractions were collected and 1 ml. of this added to 19 ml. of scintillation solution and counted as before.

G. Use of ACh esterase to Separate Labile Bound ACh from Synaptic Vesicles.

After the work of Barker, Amaro, and Guth (80) suspensions of labelled synaptic vesicles (1.0 ml. of D fraction plus 1.0 ml. of double distilled H_2O, 1.0 ml. of 50, 100 and 150 mM concentrations of NaCl) were treated with 50 units of ACh esterase in 0.2 ml. of 0.32 M sucrose to each aliquot for 5, 10 and 15 minutes. Then 0.02 ml. of a 1 x 10^{-4} M solution of eserine sulfate was added in order to inactivate the AChE. 0.5 ml. of this solution was then added to 5.0 ml. of a 1:5 isoamyl alcohol:toluene solution and shaken vigorously in order to isolate any ^{14}C labelled acetic acid in the organic phase which may have resulted from the hydrolysis of ^{14}C ACh not bound or released from the labelled synaptic vesicles (vesicle binding protecting ACh from enzymic attack). Then 1.0 ml. of the organic layer in each instance was counted in the manner described previously.

H. Labelling with ^{14}C-Acetyl-1-Choline.

A solution of 5 cc. volume of 0.32 M sucrose + 1 x 10^{-6} M eserine sulfate + 1.02 x 10^{-2} millicuries of ^{14}C-1-Acetyl choline was used in which either the vesicles or synaptosomes, resulting from approximately eight grams of fresh tissue, these being incubated for 30 minutes at 0° C. prior to separation on the sucrose gradient,

and the instances of competitive inhibition of labelling, a similar concentration of label was used at 6° C for one hour with the purified forms of the vesicles being incubated in this manner.

I. Exposure to Cations.

Of the 5 cc D fraction obtained in each run, one cc was placed in one cc of an isosmolar solution containing one of the following concentration sof an appropriate cation (Na, K, Ca, Li, Rb, Cs, TMA, Ch, ACh, or Choline ethyl ether, chloride ion salts being used throughout): 0, 50, 100, and 150 mM (the osmotic difference being compensated for with an appropriate amount of sucrose). In addition, ratios of sodium and potassium ions of 25 mM/125 mM, 50 mM/100 mM, 75 mM/75mM using first one and then the other as the numerator were utilized as incubating media. The solutions were all kept at 0-6° C. save a duplicate of the salt free solution which was boiled for ten minutes and hence served as the "boiled control" representing all of the releasable acetylcholine available per cc. of the present batch of labelled vesicles.

Incubation or exposure was for ten minutes at the end of which time the solutions were diluted with double-distilled H_2O 1:25 and subjected to centrifugation at 48,500 x g at 0°C (for twenty minutes if synaptosomes and thirty minutes if synaptic vesicles). This afforded quite efficient and reproducible separation of the unbound from the bound label which were then counted and compared. A scintillation solution was used consisting of 925 ml of toluene, 525 ml of Triton-100X, and 75 ml of Liquiflor in 10 cc amounts per one cc of sample solution giving 68% efficiency when counting on a Beckmann CPM-100 Liquid Scintillation Counter.

All runs were done in duplicate or triplicate using the same batch of labelled vesicles and exactly the same conditions as to time and temperature. Any comparisons of the efficacy of cations as releasing agents occurred within runs and not between runs so as to minimize inaccuracy secondary to this factor of variation.

J. Treatment of Synaptic Vesicles with Enzymes.

In light of the hypothesis for anionic binding sites on the vesicles, the effects of treatment with enzymes specific for degradation of said sites on membranes was investigated. Based upon Whittaker, *et al*. (81) analysis of the vesicle fraction, the phospholipid, phosphatidyl choline with its phosphate group negatively charged over a wide pH range was one such candidate. The group of compounds known as the gangliosides with their ceramide group linked through two hexose groups and an N-acetyl-galactosomine to an N-acetylneuraminic acid group - it is the latter's carboxylic acid which might serve as an anionic binding site within the known physiologic pH range - was chosen as the other most likely candidate. Thus, a purified neuraminidase from Vibrio cholerae was obtained from the Behringwerke Company of a strength of 500 units/ml. Enough of this enzyme to cleave 20 moles of NANA/hr. was placed in two test tubes containing 1 cc each of labelled synaptic vesicles and incubated one half hour and one hour at 37°C. A third and fourth test tube with labelled synaptic vesicles in it was also incubated for one half hour and one hour to act as controls. Then the samples were diluted up to 25cc with double distilled water and centrifuged for thirty minutes

at 48,500xg for thirty minutes at $0^{o}C$. Then one cc of supernatant plus a one cc solution of 0.6 N HCl containing the entire pellicle of labelled vesicles were placed in 10 cc of the scintillation solution and counted. The presence of released NANA in the supernatant of these samples was measured by the Svennerholm Method (82).

In the case of phosphatidyl choline, phospholipase C of a strength of 1.6 units/mg was used. Enough of this enzyme to cleave 0.64 umoles of phosphatidyl choline/hr. was dissolved in 1.0 ml of 0.1 M trismaleate buffer at pH 6.0 plus 0.1 ml of 0.05 M $CaCl_2$ solution and then placed in two test tubes containing 1 cc each of labelled synaptic vesicles and incubated for one half and one hour at $37^{o}C$. Controls, techniques of separation and counting were carried out as before. The presence of released phosphate in the supernatant of these samples was determined by a modification of the method of McFarlane and Knight (83).

Next, as the vesicles had been demonstrated to previously contain a good deal of protein (84), three times crystallized trypsin in the amount of 2.8 gm was dissolved in 1.0 ml of 0.1 M trismaleate buffer at pH 6.0 and then placed in two test tubes containing 1 cc each of labelled synaptic vesicles and incubated for one half and one hour at 37° C. Controls, techniques of separation and counting were as before.

Finally, vesicles were treated in an identical manner to that described in the previous paragraph; the trypsin inhibited by an appropriate amount of soybean inhibitor and then the vesicles treated with phospholipase C, separated and counted in a manner identical to that previously described.

K. Electrophoresis.

In keeping with the concept that these are highly negatively charged

particles, there were attempts to electrophorese the "D" or vesicle fraction in a Spinco Model H microelectrophoresis apparatus. Initially, Na_2HPO_4 /NaH_2PO_4 buffers of 0.01 N and 0.1 N at pH 7.0 were employed in separate runs. Then a glycine/HCl buffer pH 3.5 of 0.1 N ionic strength was employed to examine pH effects. In all cases a current of 16 milliamperes, a temperature of $0^{o}C.$, and a total electrophoresis time of approximately 2.5 hours was employed. Electrophoretic mobility was calculated, utilizing the following formulae:

$$\text{MOBILITY} = h/t \times kA/i$$

where h = the distance migrated in centimeters, t is the time interval for migration in seconds, k is a constant of specific conductance derived using the equation:

$$\frac{1}{\text{R Standard (10,000 ohm)}} + \frac{1}{\text{R Cell}} = \frac{1}{\text{R Total}}, \text{ Solve for R Cell}$$

where A = the area of the cell in centimeters squared, i is the current in amperes and R's were the resistances which were measured utilizing a wheatstone bridge, thus R total was measured in this manner--R Standard is a 10,000 ohm resistance included in the circuit.

The effects of enzymic treatment of the vesicles on their electrophoretic mobility was investigated. A purified neuraminidase derived from _Vibrio cholerae_ of a strength of 500 units/ml. was used as before. A full 500 units was added to a full 5 ml. D Fraction and incubated at $37^{o}C.$ for one hour. Subsequently, the mixture was diluted up to 100 ml. with double distilled H_2O at $0^{o}C.$ and centrifuged at 48,500 x g using the Sorvall RC2-B High-speed Centrifuge for 20 minutes at $0^{o}C.$ This resulted in an adequate separation of vesicles into a pellicle while the water

soluble enzyme and inorganic salts remained in suspension (as subsequent assay showed). Next, the pellicle was resuspended in 11 ml. of the previously described sodium phosphate buffer at pH 7.4 and ionic strength of 1.0. This mixture was then added to the cell of the Model H Spinco apparatus and electrophoresed for some three hours using a current of 16 milliamperes, a temperature of 0°C.

Phospholipase D derived from cabbage leaves was utilized as before. A total of 0.75 mg. or 0.375 international units was incubated with 5 ml. of Fraction D plus 10 ml. of a tris-maleate buffer at an ionic strength of 0.1M, and pH of 6.0 for a period of one hour at 26°C. This was followed by an identical separation procedure as that described for the neuraminidase-vesicle mixture. Finally, the resultant pellicle was handled in like manner as regards the carrying-out of electrophoresis.

A three times crystallized trypsin was utilized as before. A total of 5.4 mg.was incubated with 5 ml. of Fraction D plus 10 ml. of tris-maleate buffer at an ionic strength of 0.1M, and adjusted to a pH of 6.0 for a period of one hour at 37°C. This was followed by an identical separation procedure to that described for the neuraminidase-vesicle mixture. Finally, the resultant pellicle was handled in like manner as regards the carrying-out of electrophoresis.

Finally, 5 ml. of Fraction D was incubated for one hour with exactly the same amount of trypsin previously described under exactly the same conditions. This then was followed by inactivation of the proteolytic enzyme with an appropriate amount of soyabean inhibitor (arrived at through empirical preliminary experimentation to determine the amount necessary to terminate tryptic activity). Then the inactivated trypsin-soyabean inhibitor-vesicle preparation was incubated with

exactly the same amount of phospholipase D as described previously and under the exact same conditions. After this, separation and electrophoresis were carried out in the exact same manner as described previously for the neuraminidase-mixture.

L. Preparation and Labelling Utilizing Tetramethylammonium Iodide:

11.9 mg. of methyl-^{14}C iodide (0.25 mc.) was dissolved in 10 ml. of diethyl ether and then combined with freshly distilled trimethylamine to form methyl-^{14}C-tetramethylammonium iodide. Labelling procedure was then identical to that described for acetyl-1-^{14}C-choline with 1 mg. amounts being employed as before since the specific activity was equivalent.

RESULTS

Preliminary Comments:

As previously noted, the use of the weak carboxylic acid resin (Table 1) and the use of crude nerve-ending particle preparations (Table 2) yielded results indicative that binding and release of acetylcholine was, indeed, influenced in a manner akin to that predicted if binding was of an ionic nature. However, as also previously stated, neither these nor the subsequent manipulations with labelled synaptic vesicle fractions yielded data of sufficient precision and reproducibility, and thus other approaches to the problem were explored.

Dialysis Apparatus:

The utilization of the previously described dialysis apparatus initially appeared to be yielding ideal results (Fig. 12 and 13). It was not until the use of the labelling solution containing the usual dose of acetyl-1-^{14}C choline in the apparatus and the resultant data showing release of label in identical manner to that previously observed using vesicles that it was realized that the phenomenon being monitored related more to the properties of the apparatus than to the vesicles. After some thought, it has been concluded that what was being observed was the ion exchange properties of the dialysis membrane. Presumably, the label became adherent to the dialysis membrane (which most probably contains anionic sites) and was subsequently being displaced in the manner proposed for the vesicles.

TABLE 1

TABLE OF CG-50 RESIN LABELLING AND RELEASE

(Data given in average counts per minute/ml.)

Salts	Concentrations				
	Back-ground	10 mM	50 mM	100 mM	150 mM
NaCl	32	39	31	10,183	7,701
KCl	30	49	129	15,478	935

TABLE 2

TABLE OF NERVE-ENDING PARTICLE LABELLING AND RELEASE

(Data given in average counts per minute/ml.)

Salt	Concentrations			
	Control	50 mM	100 mM	150 mM
NaCl	50	52	74	61

Fig. 12 The bar graph on the left shows acetylcholine release with repeated washings utilizing dialysis apparatus in response to injections of NaCl solutions of varying concentrations.

The bar graph on the right shows residual bound ACh from each of the appropriate dialysis bags.

white = control

red = 50 mM

blue = 100 mM

green = 150 mM

CPM /ml

1500
1300
1100
900
700
500
300
100

Wash # 8
9
10 NaCl
11 Post NaCl$_1$
12 Post NaCl$_2$

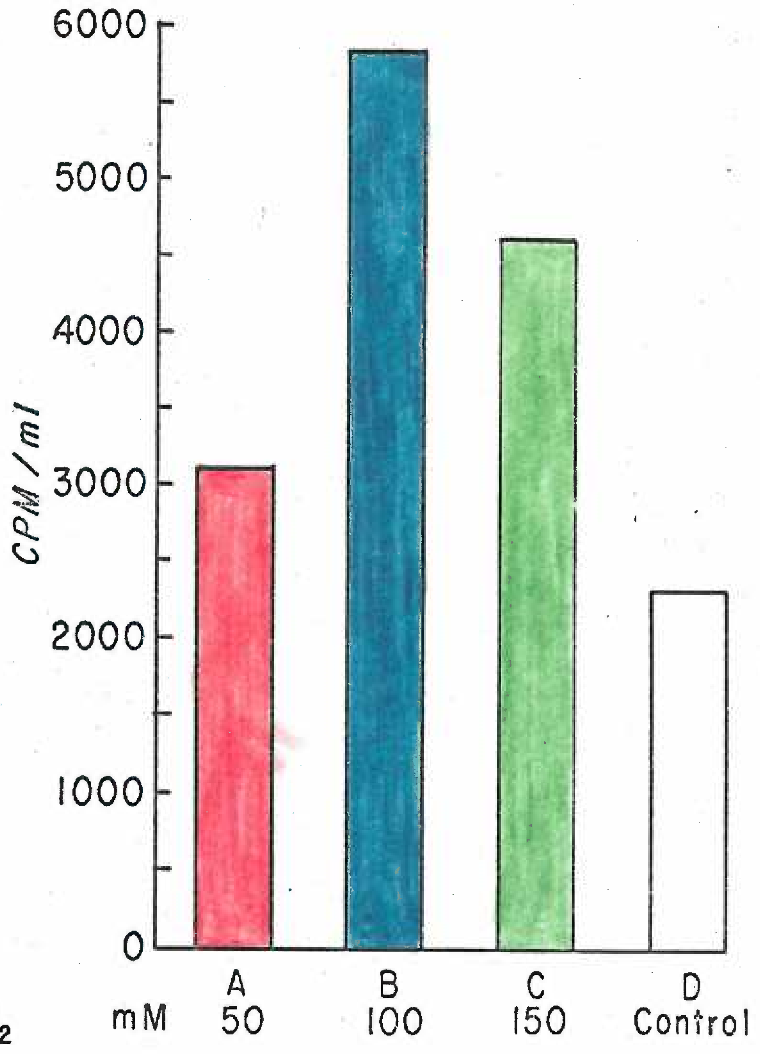


Fig. 13 The bar graph on the left shows acetylcholine release with repeated washings utilizing dyalysis apparatus in response to injections of KCl solutions of varying concentrations.

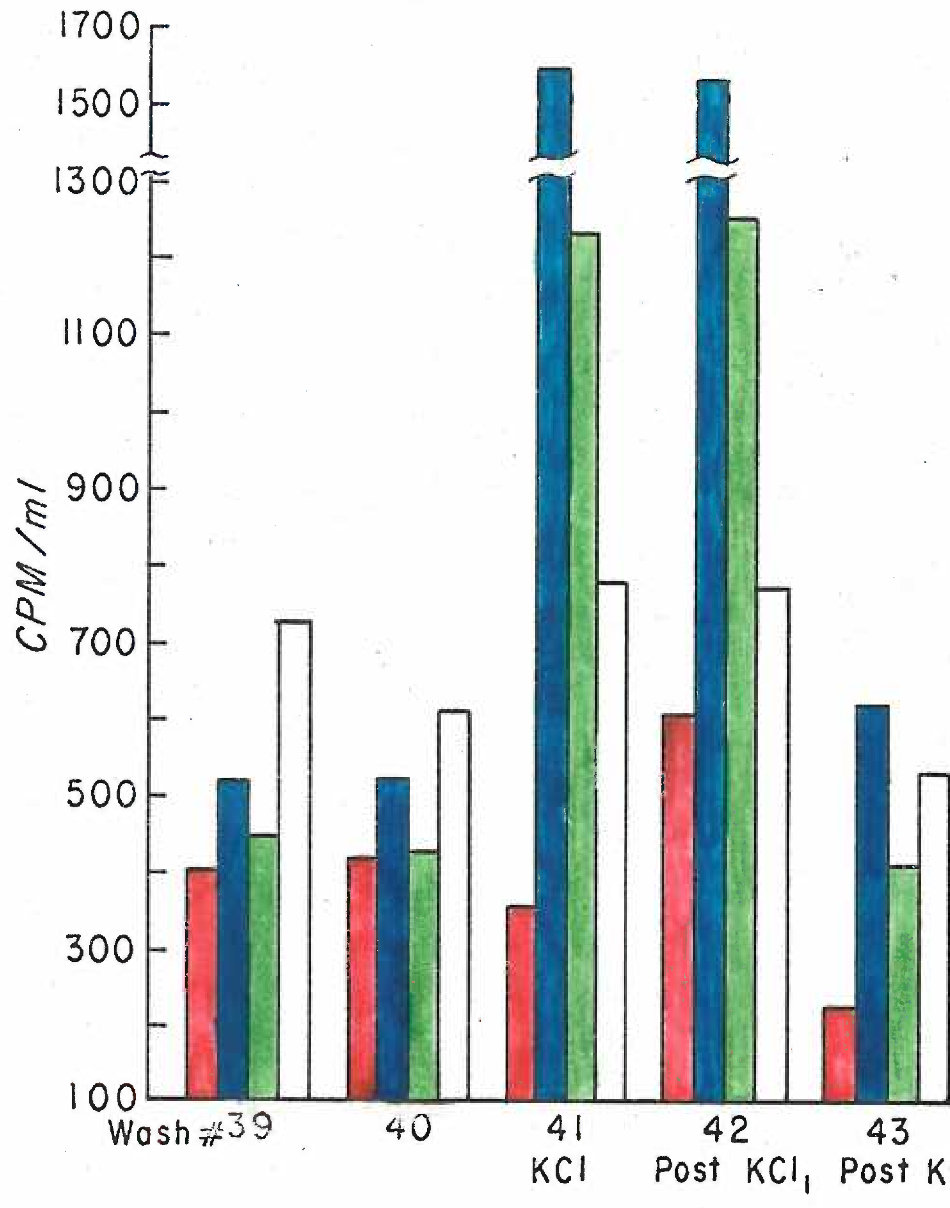
The bar graph on the right shows residual bound ACh from each of the appropriate dialysis bags.

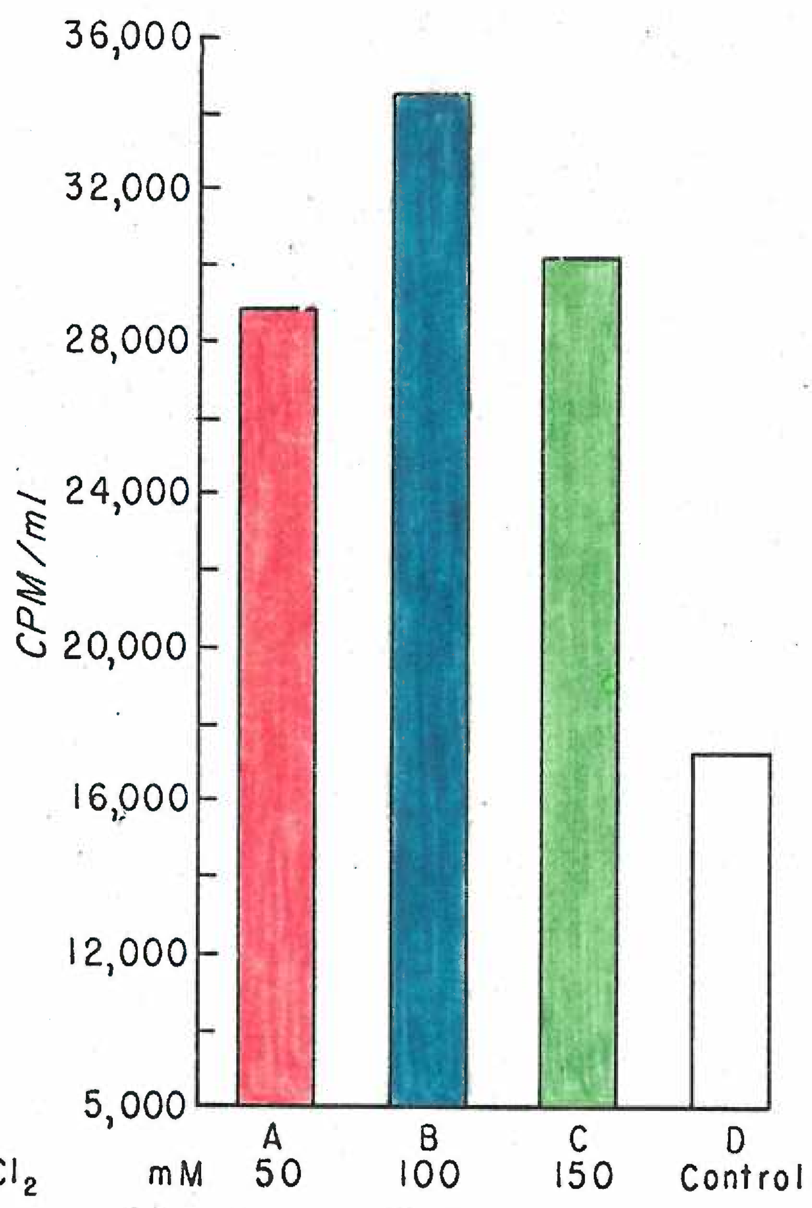
white = control

red = 50 mM

blue = 100 mM

green = 150 mM


CPM/ml
1700
1500
1300
1100
900
700
500
300
100
Wash #39
40
41
KCl
42
Post KCl1
43
Post KCl2


CPM/ml
36,000
32,000
28,000
24,000
20,000
16,000
12,000
5,000
mM
A
50
B
100
C
150
D
Control

Sephadex Separation Technique:

The utilization of Sephadex G-10 was inspired primarily by the favorable reports of a similar application of this molecular seive to compartmentation in the synaptosome (85). From the table (Table 3) it can be seen that a clean separation could not be achieved. The only constant finding appears to be that the application of increasing concentrations of cation resulted in increasing recovery of total counts. This led to the proposition that once again a substance not generally regarded as possessing ion exchange properties was clearly showing such properties and thus disqualifying itself as a useful tool in these investigations where the magnitude of the fluxes being monitored is really of such modest proportions as to be totally masked by these extenuating conditions.

Acetylcholine Esterase-Acetic Acid Extraction Technique:

The utilization of acetylcholine esterase to delineate bound from unbound ACh in the vesicle preparations was guided by the reports of Barker, Amaro, and Guth (86) of a similar application of the principle. These investigators used only the guinea pig ileum bioassay technique to detect the presence of ACh which survived the esterase treatment while in the present experiments, the more sensitive radioactive-labelling technique was employed. A second difference lies in the use of an isoamyl alcohol-toluene extraction technique to pick up the ^{14}C acetic acid that resulted from hydrolysis of the unbound ACh, i.e., it was the measurement of the unbound

TABLE 3

TABLE OF SEPHADEX G-10 SEPARATION OF BOUND FROM UNBOUND ACETYLCHOLINE

EXPERIMENT # 1 *

	50 mM NaCl	100 mM NaCl	150 mM NaCl	Control
1.	18	31	19	22
2.	788	1,672	717	67
3.	432	160	264	15
4.	12	25	12	14
5.	14	13	14	15
6.	14	13	13	12
7.	433	65	13	12
8.	13	25	13	13
9.	26	16	24	15
10.	14	15	64	15
Total	1,764	1,935	1,353	200

EXPERIMENT # 2 *

	50 mM NaCl	100 mM NaCl	150 mM NaCl	Control
1.	30	33	27	43
2.	55	63	1,729	36
3.	2,963	1,087	1,645	14
4.	32	20	24	38
5.	18	19	16	18
6.	32	27	14	14
7.	23	16	15	15
8.	17	14	13	16
9.	19	11	15	16
10.	16	15	16	18
Total	3,205	1,315	3,514	228

* (2 ml. samples used. Data given in counts per minute/ml.)

or released ACh which was carried out in the present experiments and not measurement of what was left behind. Ultimately, it was this last aspect of the technique evolved plus its lack of reproducibility that lead to its abandonment. The inability to monitor both bound and unbound stores simultaneously was felt to be a serious liability in the face of the seemingly recurrent problem of a lack of reproducibility for no apparent reason. When compared simultaneously with the high speed centrifugation technique that served as the ultimate tool utilized in exploring the nuances of the binding and release phenomena, the inefficiency in regard to counts lost utilizing the esterase-treatment-acetic-acid extraction technique became more readily apparent (Table 4). So, likewise, the latter's superior reproducibility was demonstrated also.

Release:

Utilizing the high speed centrifugation technique as described on page 30, the following results were obtained. When either synaptic vesicles or synaptosomes were isolated and labelled and placed in various concentrations of cations, release of approximately 40 percent of the releasable bound ACh was achieved when concentration was either 100 mM or 150 mM. Between 0-100 mM concentration a general linear progression of quantity released under concentration is observed, while above this concentration no further label is displaced as seen in Table 5 and 6. In addition, ratios of sodium and potassium ions of 25mM/125mM, 50mM/100mM, 75mM/75mM using first one and then the other as the numerator were utilized as

TABLE 4

TABLE OF ACETYLCHOLINE ESTERASE SEPARATION OF BOUND FROM UNBOUND ACETYLCHOLINE

(Data given in range of average counts per minute/ml.)

NaCl

Binding	Concentrations				
	Control	50 mM	100 mM	150 mM	Boiled control
Unbound	930	1,659	1,669	1,537	
Bound					2,836

KCl

Binding	Concentrations				
	Control	50 mM	100 mM	150 mM	Boiled control
Unbound	2,110	1,800	1,270	1,900	
Bound					2,120

TABLE 5

TABLE OF RELATIVE RELEASING CAPACITY OF VARIOUS INORGANIC CATIONS

Data given in

$$\frac{\text{Average DPM/ml.* Control-Average DPM/ml. Retained}}{\text{Average DPM/ml. Control}}$$

Salts	Concentrations		
	50 mM	100 mM	150 mM
NaCl	162/666 = 0.243	196/666 = 0.295	208/666 = 0.319
KCl	58/1,139 = 0.051	173/1,139 = 0.152	140/1,139 = 0.123
LiCl	189/840 = 0.225	161/840 = 0.225	241/840 = 0.287
RbCl	60/1,262 = 0.048	132/1,262 = 0.104	542/1,262 = 0.238
CsCl	114/1,142 = 0.098	31/1,142 = 0.026	30/1,142 = 0.026
$CaCl_2$	660/586 = 0.113	106/586 = 0.181	121/586 = 0.262

Conditions: All incubations were carried out at 4° C., in solutions of sucrose such that the osmolarity was always 320 mOsmols, pH 7.4. Incubation times for release were always 15 minutes, the synaptosomes coming from a common previously-labelled pool.

* Discharges per minute/ml.

TABLE 6

TABLE OF RELATIVE RELEASING CAPACITY OF VARIOUS ORGANIC CATIONS

Data given in

$$\frac{\text{Average DPM/ml. Control} - \text{Average DPM/ml. Retained}}{\text{Average DPM/ml. Control}}$$

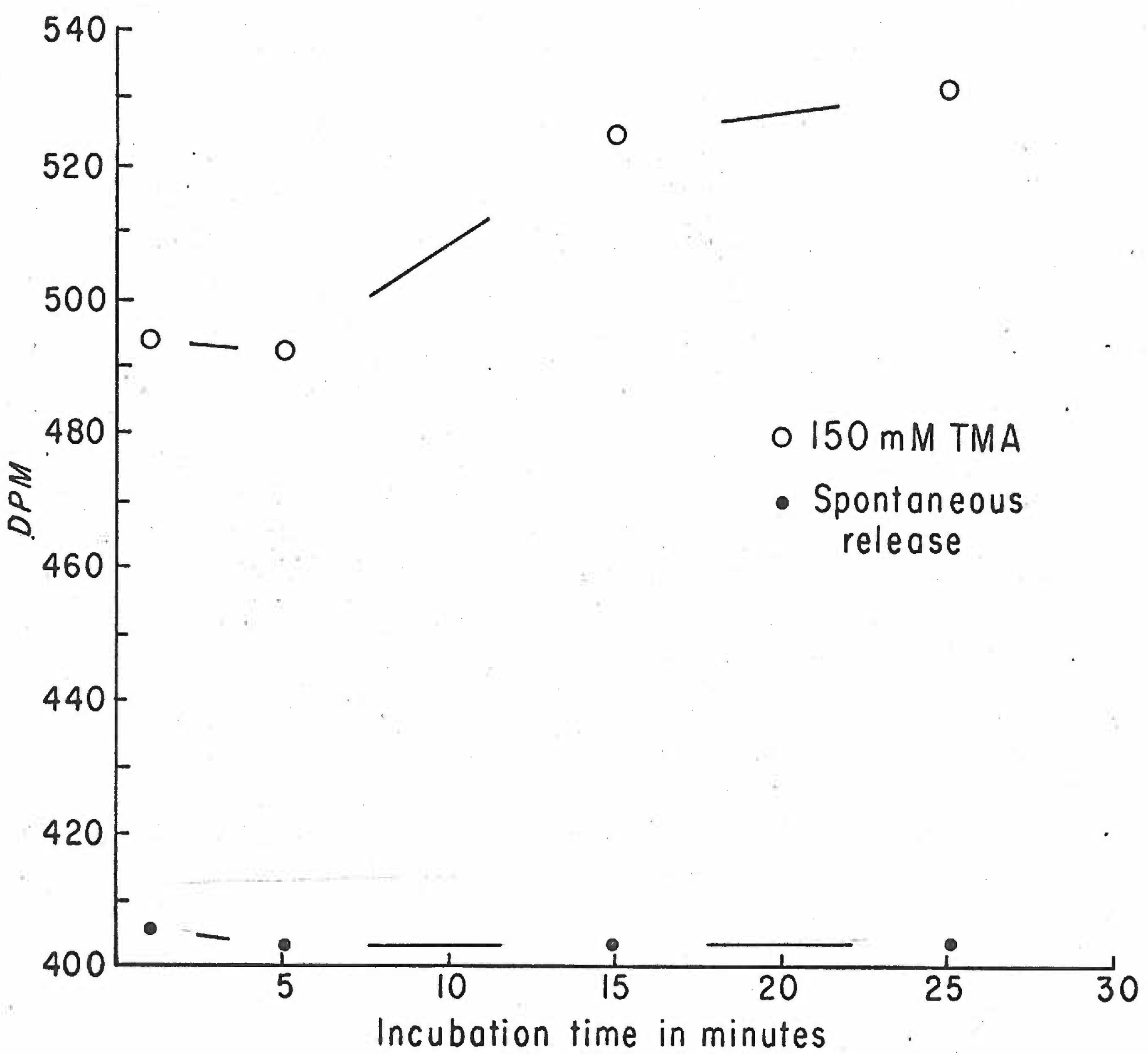
Salts	Concentrations		
	50 mM	100 mM	150 mM
Choline Cl	180/1,339 = 0.135	220/1,339 = 0.163	244/1,339 = 0.173
ACh Cl	79/574 = 0.138	92/574 = 0.160	98/574 = 0.171
Ch ethyl ether	107/1,237 = 0.087	251/1,237 = 0.203	429/1,237 = 0.235
TMA Br	217/1,409 = 0.154	238/1,409 = 0.169	270/1,409 = 0.192
Guanidinium HCl	110/675 = 0.163	343/675 = 0.508	219/675 = 0.324

Conditions: All incubations were carried out at 4°C., in solutions of sucrose such that the osmolarity was always 320 mOsmols, and with no pH adjustment. Incubation times were always 15 minutes. The synaptosomes utilized were contained within 1 cc. aliquots that came from a common previously-labelled pool.

Fig. 14 Release of acetylcholine from synaptic vesicles over time spontaneously and then incubated with 150 mM TMA.*

* Spontaneous release is represented by a solid circle

TMA stimulated release is represented by an open circle.



540
520
500
480
460
440
420
400
DPM
5
10
15
20
25
30
Incubation time in minutes
150 mM TMA
Spontaneous release

Fig. 15 Release of acetylcholine from synaptic vesicles in relation to changes in temperature both spontaneously and when incubated with 150 mM TMA.*

* Open circle represents acetylcholine

Closed circle represents bound acetylcholine

Triangles represents boiled control

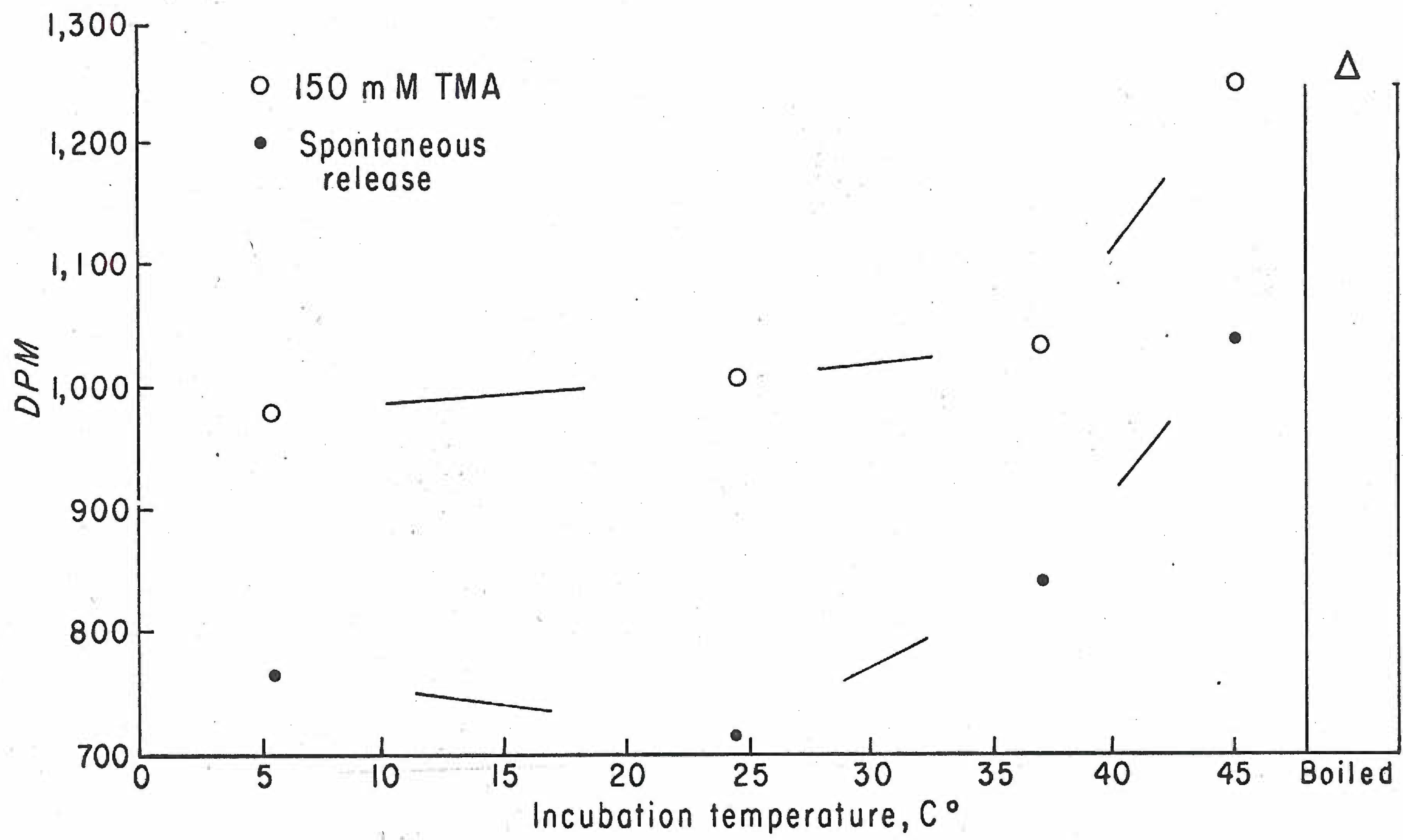
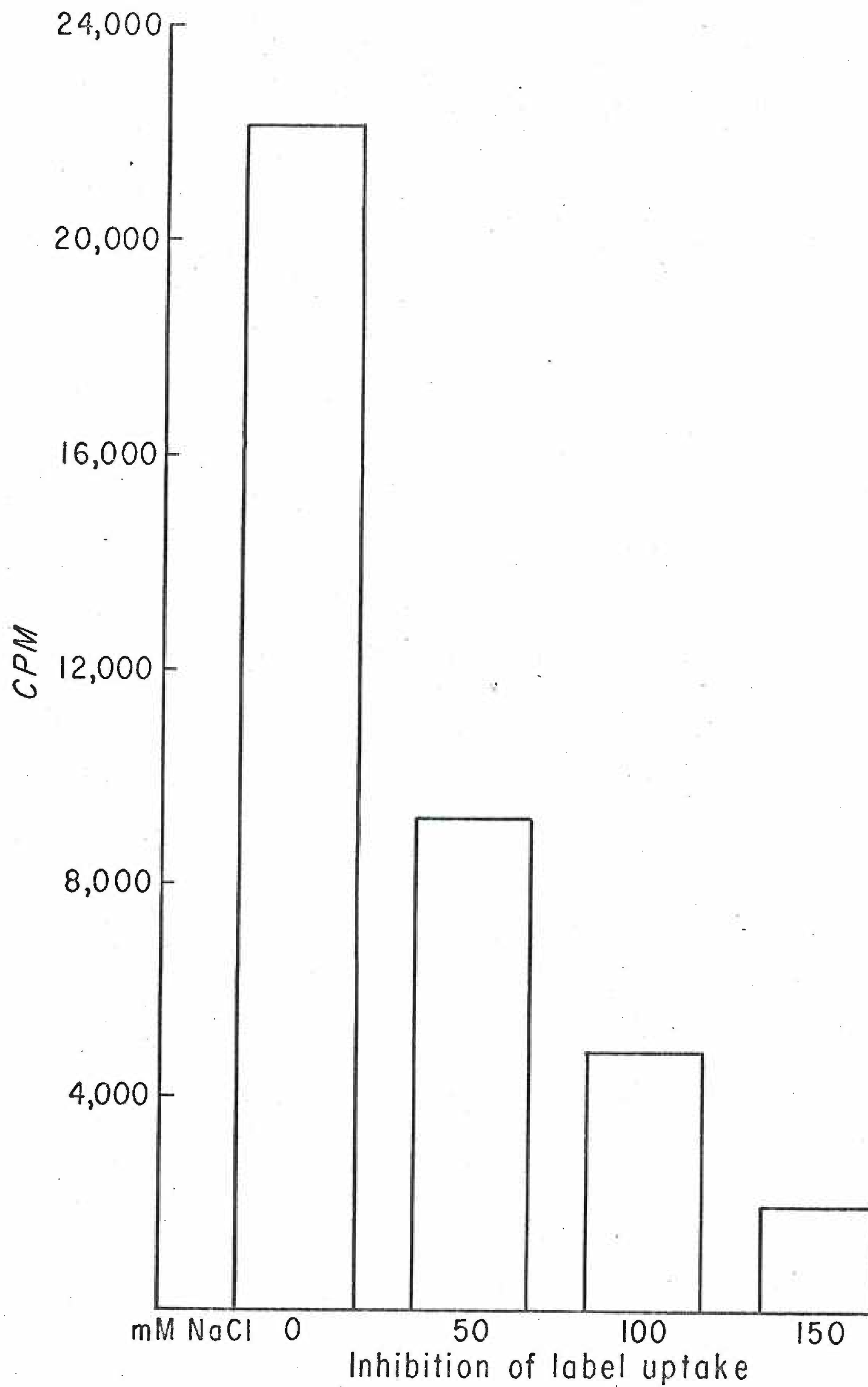

DPM
1,300
1,200
1,100
1,000
900
800
700
0
5
10
15
20
25
30
35
40
45
Boiled
Incubation temperature, C°
150 m M TMA
Spontaneous release
Δ

Fig. 16 Inhibition of labelling with ^{14}C-acetylcholine Iodide by rising cation concentration.



Inhibition of label uptake

incubating media. It was found that the magnitude of release was essentially unchanged either in terms of total counts or relative to the concentrations utilized (i.e., all appear to have released essentially equivalent amounts of label). Once again, reaffirming the nonspecificity of the ionic binding character of the vesicles as manifested in the case of the other ions employed.

Kinetics of the Binding:

Synaptic vesicles when isolated and labelled and placed into solutions of either 0 or 150 mM TMA and incubated: (a) over-time periods of 1, 5, 15, and 25 minutes, (b) for 10 minutes at 6°, 25°, 37°, and 45°C. with both 0 and 150 mM TMA. From Fig.14, it is evident that there is a linear progression over time of the release of label. On the other hand, there is an unexplained stability of the binding up to 37°C. (Fig.15). (An observation made before under somewhat different experimental conditions by Hosein and Levy.) (87)

Inhibition of Labelling:

The D function has been taken and exposed for 30 minutes (at 6°C.) to solutions containing 1.02 x 10^{-2} millicuries acetyl-1-^{14}C choline plus as follows: 0, 50, 100, or 150 mM of K^+ or Na^+ ion and an appropriate amount of sucrose to form an isosmolar solution. The results as indicated in Fig. 16, show inhibition of labelling in direct proportion to the concentration of either ion species.

Labelling and Release with Tetramethylammonium Ion:

There was no difficulty encountered in either the synthesis of an appropriately labelled tetramethylammonium compound or in getting synaptic vesicle fractions to bind the quaternary ammonium group preferentially. As will be later noted during the discussion, the symmetry of this particular quaternary group was the reason for its application. The results indicate a more typically linear pattern of release in relationship to concentration of displacing cation (the sort of effect predicted), however, the magnitude of this trend towards linearity both in considering the raw data (Table 7) and subsequently statistically raises serious question as to its genuine significance.

Enzyme Treatment of Synaptic Vesicles:

The treatment of labelled synaptic vesicles with neuraminidase shows no detectable change in label and no detectable release of neuraminic acid by the Svennerholm Reaction. In contrast, there is a demonstrable decrease in label following treatment of labelled synaptic vesicles with phospholipase C (see Table 8). Furthermore, the increase in inorganic phosphorus, presumably arising from the synaptic vesicles secondary to the phospholipase with treatment is nicely demonstrable utilizing the modified method of McFarlane and Knight (88).

Electrophoresis of Synaptic Vesicles:

The experiments involving the measurements of electrophore-

TABLE 7

TABLE OF ^{14}C-TMA LABELLING

Data given in

$$\frac{\text{Average DPM/ml. Control} - \text{Average DPM/ml. Retained}}{\text{Average DPM/ml. Control}}$$

Salts	Concentrations		
	50 mM	100 mM	150 mM
TMA Br	7,520/14,950 = 0.503	8,775/14,950 = 0.585	9,427/14,950 = 0.630
NaCl	8,615/14,950 = 0.576	8,410/14,950 = 0.563	10,415/14,950 = 0.699

Competitive Inhibition of Labelling with TMA

Salts	Concentrations		
	Cold 50 mM	Cold 100 mM	Cold 150 mM
TMA Br	404/1,417 = 0.285	1,064/1,417 = 0.752	1,009/1,417 = 0.713
NaCl	-122/1,417 = -0.086	179/1,417 = 0.127	883/1,417 = 0.624

Conditions: All incubations were carried out at 4°C., in solutions of sucrose such that the osmolarity was always 320 mOsmols, pH 7.4. Incubation times for release were always 15 minutes with the synaptosomes coming from a common previously-labelled pool. In the inhibition experiments, 1.275 microcuries of ^{14}C-TMA Br was placed in each solution of sucrose plus the stated cation concentration with an osmolarity of 320 mOsmols. This solution was then combined with the synaptosomes (unlabelled) from the common pool and incubation time was one hour at 4°C. and pH 7.4.

TABLE 8

TABLE OF ENZYME EFFECTS ON LABELLING

Conditions	Results (Average DPM/ml. Control-Average DPM/ml. Retained) / (Average DPM/ml. Control)
Phospholipase C 1/2 hr.	106/534 = +.198
Phospholipase C 1 hr.	118/450 = +.262
Trypsin 1/2 hr.	-1161/1078 = -1.077
Trypsin 1 hr.	-587/1311 = -.447
Trypsin + Phospholipase C 1/2 hr.	6,991/8,190 = +.853
Trypsin + Phospholipase C 1 hr.	4,604/5,574 = +.826
Neuraminidase 1/2 hr.	240/2,519 = +0.095
Neuraminidase 1 hr.	49/1,832 = +0.027

Conditions: The phospholipase C (0.4 mg.) was incubated with 1 cc. of synaptic vesicles labelled with Acetyl-1-^{14}C-choline Cl in 1 cc. Tris-Maleate buffer pH 7.3, Temperature 37° C with 0.1 ml. of a 0.05 M $CaCl_2$; the Trypsin (1.4 mg) was incubated with 1 cc. of synaptic vesicles labelled with acetyl-1-^{14}C-choline Cl in 1 cc. Tris-Taleate buffer pH 7.3, Temperature 37° C. the neuraminidase (500 units of activity, derived from Vibrio Cholerae) was incubated with 1 cc. 0.05 M NaAc buffer with 1 mg $CaCl_2$ + 9 mg. NaCl at pH 5.5

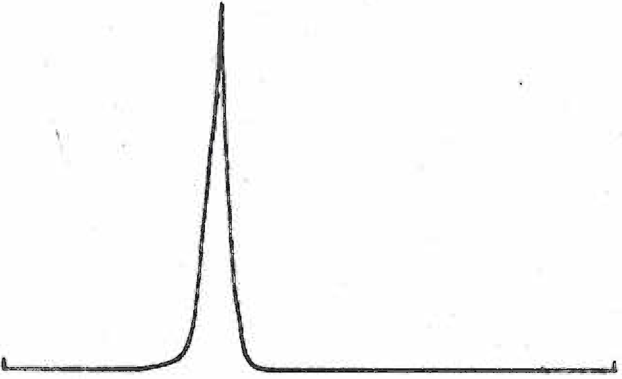
Fig. 17 A represents the boundary pattern as seen via a schlieren system of the synaptic vesicle fraction in 0.1 M NaH_2PO_4/Na_2HPO_4 buffer at pH 7.4.

B represents the resultant pattern at two and one half hours using a 16 milliamperiere current at 0° C.

Conditions	Result
(all run at pH 8.0, 0° C. in 0.1 M NaH_2PO_4/Na_2HPO_4 buffer)*	
pH 7.4, 0.01 M buffer	0
pH 7.4, 0.01 M buffer	0
pH 3.5	-1.2×10^{-5}
pH 8.0	0
Phospholipase D	-2.76×10^{-5}
Trypsin	-5.29×10^{-5}
Trypsin then Phospholipase D	-7.45×10^{-5}
Neuraminidase	0

* unless otherwise stated

A



B

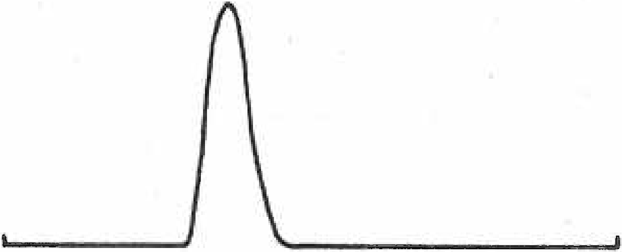
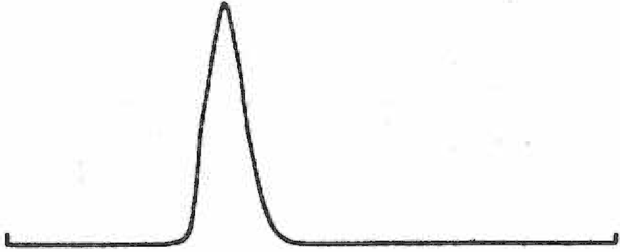


Fig 18 A represents the boundary pattern as seen via a schlieren system of the synaptic vesicle fraction in 0.01 M NaH_2PO_4/Na_2HPO_4 buffer at pH 7.4.

B represents the resultant pattern at two and one half hours using a 16 milliamperiere current at 0^oC.

A



B

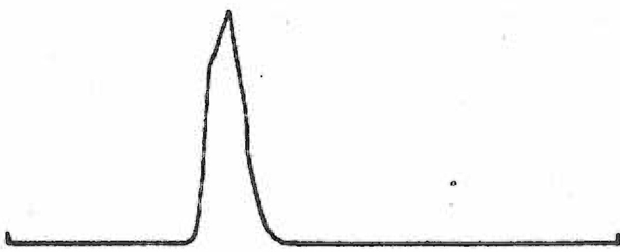
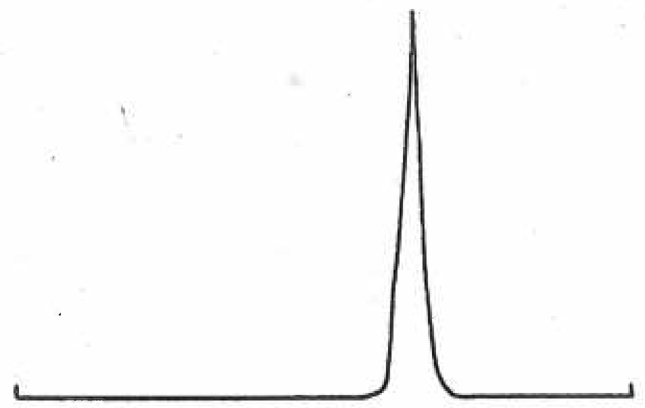


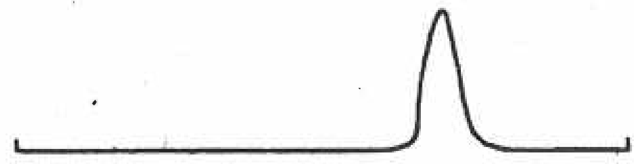
Fig. 19 A represents the boundary pattern as seen via a schlieren system of the synaptic vesicle fraction in 0.1 M NaH_2PO_4/Na_2HPO_4 buffer at pH 3.5.

B represents the resultant pattern at two and one half hours using a 16 milliamperiere current at 0° C.

A



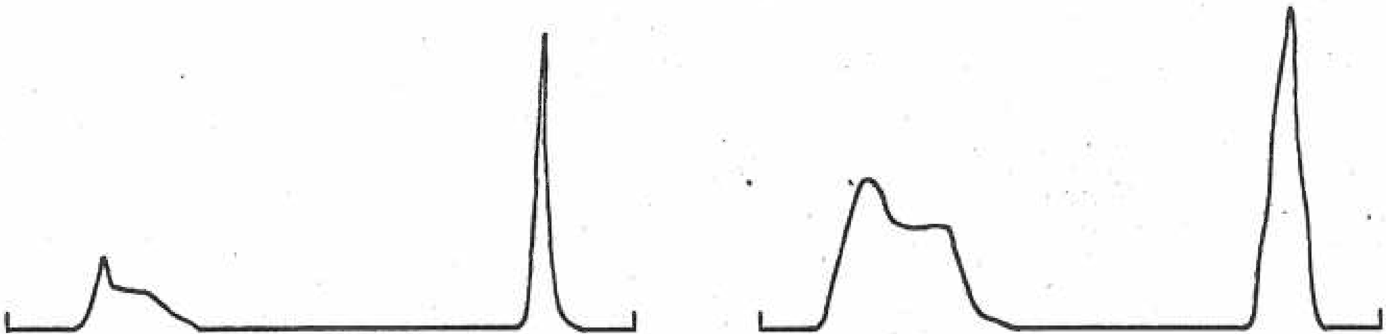
B



.1M pH 3.5

Fig. 20 A represents the boundary pattern as seen via a schlieren system of the synaptic vesicle fraction when pretreated with trypsin buffer at 8.0

B represents the resultant pattern at two and one half hours using a 16 milliamperiere current at 0° C.


a
b

tic mobilities both at varying pH and ionic strengths as well as following enzyme treatment proved perhaps the most informative of our studies or at least the most provocative.

The electrophoretic mobility at pH 7.4 at both 1.0 and 0.1 normal ionic strength was essentially zero using the Model H Spinco apparatus as measured at one-half, one, one and one-half, two, and two and one-half hour intervals at zero degrees Centigrade in a NaH_2PO_4/Na_2HPO_4 buffer (see Fig. 17 and 18).

The electrophoretic mobility at pH 3.5 at 1.0 N ionic strength, using the equation: (Fig. 19)

$$\text{MOBILITY} = h/t \times kA/i$$

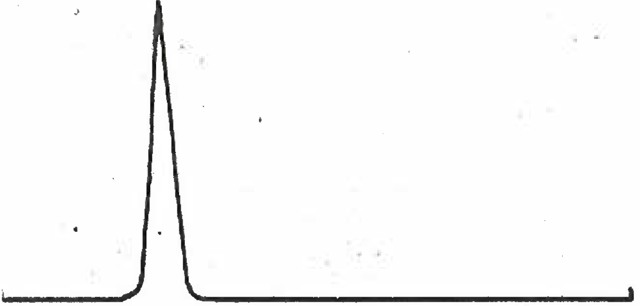
where h = the distance migrated in centimeters, t = time in seconds, k = the calculated specific conductance of the solution, A = the surface area of the electrophoretic cell in square centimeters, i = the current in amperes, calculates out to -1.20×10^{-5} $cm.^2$ $Volt^{-1}$ $seconds^{-1}$. This value proves to be quite small when one considers that the normal electrophoretic mobility of the red blood cell is -1×10^{-4} $cm.^2$ $Volt^{-1}$ $seconds^{-1}$, i.e., a whole power of ten greater.

Following incubation with a pure lyophilized preparation of trypsin the electrophoretic mobility at pH 7.4 became -5.28×10^{-5} $cm.^2$ $V.^{-1}$ $sec.^{-1}$ based upon measurements at one and one-half and three hours, 0 degrees C. in NaH_2PO_4/Na_2HPO_4 buffer as before (Fig. 20). Incubation with a preparation derived from cabbage leaves of phospholipase D produced an electrophoretic mobility of

Fig. 21 A represents the boundary pattern as seen via a schlieren system of the synaptic vesicle fraction when pretreated with phospholipase D buffer at pH 8.0

B represents the resultant pattern at two and one half hours using a 16 milliamperiere current at 0° C.

A



B

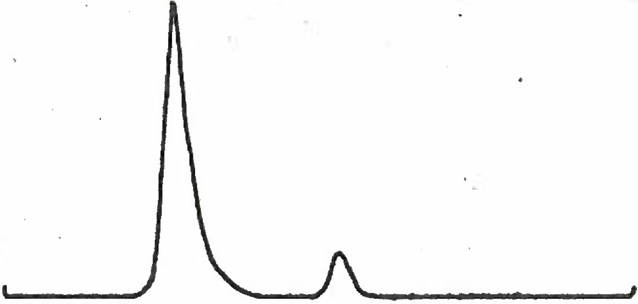
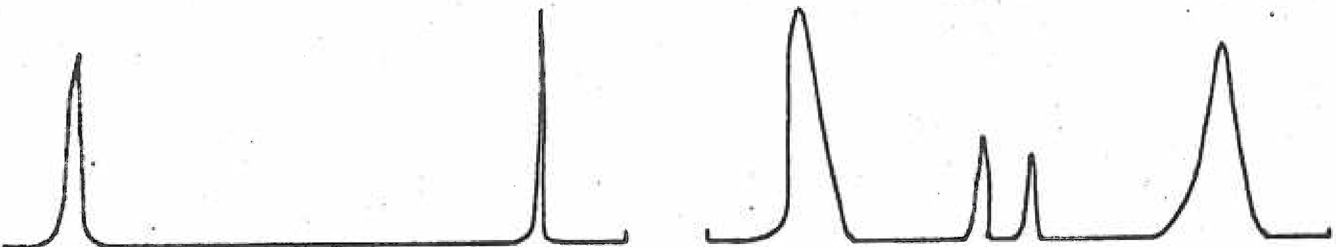


Fig. 22 A represents the boundary pattern as seen via a schlieren system of the synaptic vesicle fraction when pretreated with trypsin and phospholipase D at pH 8.0.

B represents the resultant pattern at two and one half hours using a 16 milliamperiere current at 0° C.


A
B

-2.76×10^{-5} $cm.^{2}$ $V.^{-1}$ in like buffer at like pH and temperature (Fig. 21). When treatment with trypsin was followed by inhibition with soyabean inhibitor and then followed by treatment with phospholipase D, the resultant electrophoretic mobility was -7.45×10^{-5} $cm.^{2}$ $V.^{-1}$ (Fig. 22). Finally, when incubation was carried out utilizing a purified neuraminidase from *Vibrio cholerae* (as described previously) the electrophoretic mobility remained essentially immeasurable at pH 7.4.

DISCUSSION

Perhaps, the main prerequisite for release of ACh from nerve endings *in vivo* (and, therefore, the least likely to be artifactual) is the physiologic event of depolarization. The work of Fatt and Katz (89), suggests that it is not essential that this be a full-blown action potential, as both frequency and amplitude of m.e.p.p.'s are increased by subthreshold degrees of depolarization. An increase in total intracellular cations appears to trigger release. The observations of Katz and Miledi's (90) that the uptake of calcium ions but not of sodium ions is essential for the release of ACh at nerve endings was based upon observations using tetrodotoxin (91), supposedly a specific inhibitor of membrane transport of sodium. Subsequently the lack of exact knowledge of the precise mechanism of action of tetrodotoxin has become apparent. This has raised doubts as to the validity of the interpretation placed upon their results. However, these phenomena may not be simply explicable in terms of "key" or essential ions. The highly quantifiable electrical measurements invariably made can only reflect a relative increase in intracellular positivity or decreased negativity, since the known transport phenomena so far documented implicate only cations with passive following of the anions, it would seem safe to conclude that increase in cellular positivity and not the decreased negativity was the actual state of things.

Birks work (92) dealing with varying the level of sodium in the perfusate and its apparent direct relationship to increased release is quite in accord with the summarical statement found in the preceding paragraph. So, likewise, is the long-standing compatible observation that marked elevation of the potassium concentration extracellularly eventually results in spontaneous release of ACh. This unique property of spontaneous triggering seems best explained upon the basis that, in the resting state, the cell membrane is freely permeable in an inward direction to potassium and therefore susceptible to intracellular cation elevation while the perpetually homeostatic activity of the Na^{+}-K^{+} pump precludes a similar action by Na^{+}. Once depolarization transiently alters membrane permeability, as in Birks' experiments, then Na^{+} should be and indeed is equally as effective in precipitating release.

At this juncture it seems appropriate to draw a distinction between events occurring at the cell membrane surface and in the intracellular milieu just beyond where the synaptic vesicles, and hence the ACh, reside. Even if one does not subscribe to the various possible schemes as conjured up by Burton (93) and Whittaker (94), clearly some mode of exodus of the ACh molecules through the membrane or cell wall must be afforded. Therefore, it is at least a very real possibility at this point that release from vesicles is a separate process from transport across the membrane (of course, the previously described hypotheses regard the two events as eventually synonymous). Thus, the role of calcium in release might be best understood as being focused in the character of membrane porosity

(a contention for which work with EDTA, for example, lends support) (95). This suggestion, at least in part, finds its genesis in the observation calcium fits in absolutely nowhere in the Hodgkin-Huxley schema for depolarization and events associated with the action potential save in terms of conformational changes in membrane protein, i.e. porosity. So, likewise, the parallel properties of barium and equal but antagonistic properties of magnesium ions might be so then explained.

ACh has a quaternary ammonium group which would lend itself, at least, to a good deal of nonspecific binding with randomly available anionic sites on structural protein, could such a property suffice to bind it protectively to a synaptic vesicle? Could this sort of binding be appropriately modulated by the action potential or intermediate grades of depolarization so as to explain m.e.p.p.'s and synaptic transmission? First of all, one can recall that the choline esterase has been shown to have an anionic site as well as an esteratic site. If the quaternary ammonium group were unavailable to bind with this site, obviously the lytic process could not occur. Secondly, the proposed anionic binding site on the vesicle most certainly would be subject to the normal competition of other cations in higher concentrations than might be present at the physiologic resting level. Such a higher concentration of inorganic cations would exist very transiently (a mere few microseconds) during an action potential resulting in a competitive displacement of an appropriate amount of previously bound ACh-ergo

release. Such a mechanism, in reality could prove to be quite a precise and sensitive "on-off" switch for transmitter release. It is not unrealistic to speculate that a similar sort of binding might exist for a number of so-called biogenic amines (including the catecholamines, serotonin, histamine and gamma aminobutyric acid). Perhaps the primary release of ACh at adrenergic nerve endings, might be explained upon the basis of a two-step release mechanism in which ACh would be released in the manner as described before. ACh in turn served to release the various biogenic amines then from their binding sites, as demonstrated by Burn and Rand (96). (Both or only the biogenic amines might act simultaneously at the post-synaptic membrane) (97).

Briefly, in concluding then these opening remarks, it might be of some interest to discuss how the ion exchange hypothesis suggested itself initially. Early on, there were the reports of Burton as regards the finding of an unusually high concentration of gangliosides in the synaptic vesicle fraction and the subsequent considerably more thorough-going analysis of synaptic vesicle composition by Whittaker, et al in which a relatively reduced concentration of ganglioside but an elevated concentration of the phosphatides of ethanolamine and choline were reported for the vesicle fraction. It seemed that the reported presence of such substances in such high concentrations in vesicles could only imply some physiologically significant function--obviously this was also Burton's conclusion. At about the same time as these papers were being read, this neurochemistry laboratory and this investigator had embarked upon some rather ambitious experiments involving the use of MacIntosh and

Birks' superior cervical sympathetic ganglion of cat perfusion preparation (98). These experiments with the cat ganglion were designed to explore the hypothesis that pharmacologic compounds such as tetraethyl and tetramethyl ammonium salts (the so-called ganglionic blockers), although classically described as acting post-synaptically by blocking the anionic receptor portion of the cholinergic receptor site (99), might, at least in part, act pre-synaptically by precipitating the release of a bolus of ACh thus depleting the existing stores of the transmitter substance as well as preventing any further synthesis of physiologically active transmitter. This might be demonstrated as follows: By perfusing the ganglion with a solution of Ringer's Locke at physiologic pH and temperature and then altering the concentrations of TMA ions until a blocking concentration could be achieved as demonstrable by monitoring a nicitating membrane preparation's response post-synaptically; all during these proceedings the perfusates would be serially collected, stored and assayed for ACh released by a newly-developed and chemically highly specific photofluorometric method for micro-assay of ACh. If the hypothesis were correct, a gradual increase in concentrations of ACh in the perfusates would be seen commensurate with the increases in TMA ion concentration until the blocking level was achieved. At this point, a marked increase in the amount of ACh released would be seen followed by a cessation of ACh release into the perfusate during the period of sustained ganglionic blockade in spite of continued presynaptic electrical stimulation. A great deal of time and effort was subsequently expended in almost all phases of the endeavour,

but especially in regards to achieving a ganglion perfusion preparation with minimal vascular bed leakage.

Once the above mentioned manipulations had been performed and the hypothesis been confirmed, subsequent experiments were done on cerebral cortical brain slices (incubated in varying concentrations of TMA with subsequent assay for ACh content) (100) (101), the ion exchange resin analogy began, at this point, to become extraordinarily appealing. Robinson, et al.,in their article of 1965 (102) discuss the same conception of cationic exchange resin-like properties for nerve-ending particles as to their capacity to bind 5-HT. They utilized a technique of titration which revealed 3mEq of H^+/gm protein were bound between pH 7 and 8. An affinity of lipids for amines was noted by Green (103) and later was underscored by the observation that norepinephrine was a contaminant in ganglioside preparations (Robinson et al.) (104), and of acetylcholine in highly purified preparations of cerebroside sulfate (Green et al.(105). Finally, von Euler and Lishako (106) noted a direct relationship between increase of pH and increased uptake of norepinephrine by adrenergic nerve granules. Thus the possibility that the various types of synaptic vesicles which store the different neurotransmitters may operate upon the same principle is not without basis at this time. Perhaps recent reports of more than one morphologic type of vesicle at a given synapse is related to a mechanism whereby there is an initial release of one transmitter agent which, in turn, triggers the release of the second more predominant transmitter for that particular synapse--in light of work done on adrenergic synapses to date, this does, indeed, seem to be the case (107).

Thus, with the obviously implied abundant availability of the necessary anionic binding sites at physiologic pH, i.e., either the carboxylic group of the neuraminic acid moiety of the gangliosides or the phosphate group of a phosphatide, lead to the conclusion that here was a model worth investigating. That such was indeed pursuable at a subcellular level was also appreciated at this point, due to the fact that some two years previous this investigator had been taught the methods of Whittaker for isolation of synaptosomes and synaptic vesicles and these had been utilized sporadically in this laboratory ever since. It was also known, through the work of Burton that both of these particles had been successfully labelled with radioactive ACh and choline and thus minute changes in the amounts bound to these particles under varying conditions would seem to be readily detectable. It is, interesting that Burton, et al in their paper of 1967 (108), observe an apparent release phenomenon as relates to ^{14}C labelled choline vesicles when tonicity of solution was raised and commented briefly as that this most likely represented a nonspecific ion exchange effect. Thus, the scene was set ...

Now, in considering the results obtained, it is apparent that a wide number of cations, both organic and inorganic, are able to release labelled acetylcholine in part from synaptosomes or synaptic vesicles.

As the fundamental design of the experiments dealing with release was one which lent itself especially well to the statistical analysis

known as an analysis of variance for two factors with repeated measurements, it was elected to treat the core data in this relatively simple and straightforward manner. The two factors or variables being controlled in the bulk of these experiments were those of salt concentration and type of salt. In addition, some experiments were performed in which the two variables were the type of enzyme being utilized in treatment of the vesicles and the time of incubation. The usual calculations were performed for obtaining the sum of squares utilizing the general formula:

$$\text{SUM OF SQUARES X} = \Sigma X^2 - (\Sigma X)^2/N$$

where N = the number of measurements of X, the item being measured. The degrees of freedom being defined as N-1 in this particular case)groups being>30). In turn, the expression of Mean Squares was calculated utilizing the general formula:

MEAN SQUARES X = Sum of Squares/ degrees of freedom

Finally, the F test was applied as follows:

MEAN SQUARES X/ MEAN SQUARES Y = F

where either the mean squares salt or mean squares concentration (or in the instance of the enzymes, the mean squares enzyme or mean squares time) would be compared with the mean square interaction, a term arrived at by utilizing the following formula:

MEAN SQUARES INTERACTION = (Sum of Squares between groups - Sum of Squares concentration - Sum of Squares salt)/(degrees of freedom between groups - degrees of freedom concentration - degrees of freedom salt).

Table 9 indicates the appropriate p values. In the instances of the inorganic salts it is seen that the p value is < 0.01 for both comparisons between concentrations and types of salts. Similarly, in the case of the organic salts (Table 10) it is seen that the p value for salts is < 0.05 and in the case of the concentrations, the p value is < 0.01. That these values indicate an absence of any significant interaction requiring a further breakdown or grouping of the data should be obvious. Thus, as a consequence, it is possible to examine the normalized data with a statistical confidence of meaningfulness of the relationships that occur which might otherwise not be justifiable.

It is not the intent of the author to draw any further comparisons from this data than that discussed herein. In such an exceedingly fragile and highly variable biologic preparation as the one being scrutinized, that this much reproducibility could be demonstrated is remarkable.

The p values for the vesicle treated enzyme experiments also fall below the 0.01 level, while for incubation time, there is only a p value below the 0.01 level which would be predictable from a mere perusal of the raw data (Table 11). This decrease in significance according to chance may be, perhaps, understandable upon the basis that substrate concentrations are probably exceedingly low.

Finally, the marked drop of the F ratios for the ^{14}C TMA labelled vesicle experiments below the 0.1 p value both for concentration and salt type (Table 12) may be due primarily to the

TABLE 9

TABLE OF ANALYSIS OF VARIANCE FOR INORGANIC SALTS

Source	Sum of Squares	Degrees of Freedom	Mean Squares	F Ratios	P Values
Salts	0.215	5	0.043	12.3	<.01
Conc.	0.041	2	0.021	15.0	<.01
Inter-action	0.074	10	0.007		
Total	0.370	17	0.021		

TABLE 10

TABLE OF ANALYSIS OF VARIANCE FOR ORGANIC SALTS

Source	Sum of Squares	Degrees of Freedom	Mean Squares	F Ratios	P Values
Salts	0.136	4	0.034	6.8	<.05
Conc.	0.060	2	0.030	12.0	<.01
Inter-action	0.082	8	0.010		
Total	4.770	14	0.341		

TABLE 11

TABLE OF ANALYSIS OF VARIANCE FOR ENZYMES

Source	Sum of Squares	Degrees of Freedom	Mean Square	F Ratios	P Values
Enzymes	5.395	3	1.798	36.8	$<.01$
Time	0.090	1	0.090	5.5	.10
Interaction	1.147	3	0.049		
Total	5.651	7	0.807		

TABLE 12

TABLE OF ANALYSIS OF VARIANCE FOR TMA-LABELLED SALTS

Source	Sum of Squares	Degrees of Freedom	Mean Square	F Ratios	P Values
Salts	0.016	1	0.016	6.4	$<.25>.10$
Conc.	0.052	2	0.026	5.2	$<.25>.10$
Interaction	0.010	2	0.005		
Total	0.011	5	0.002		

small number of measurements and hence the lessened chance of the sample being truly representative of the population under consideration. Thus, it must be recognized that statistically very little can be made of this increased linearity in relationship to changes in salt concentration.

The pattern which does emerge, however, as seen from Tables 5 and 6, is, that while the quantity released over the range of 0-100 mM concentration is relatively linear; that above this concentration there is no increase in amount released in spite of some 35-40% of label being retained. Thus, the character of the binding can hardly be said to be purely ionic--unless there is a good deal of nonspecific binding to non-vesicle constituents in the synaptosome, hence the necessity of dealing with synaptic vesicles in later experiments as indicated in the text of this paper. They asymmetric nature of the acetyl-choline molecule early on suggested the possibility that Van der Waals forces might be operative and account for the non-ionic characteristics of the data. The lack of dependence of binding on temperature would appear to argue against the Keesom Effect of permanent dipole interactions and suggest, perhaps, that the induced dipoles (London Dispersion Forces) may be the other prime component. The efforts to test this hypothesis using ^{14}C-methyl-tetramethylammonium iodide as label suggested but did not conclusively confirm this contention when synaptosomes were employed as previously noted. The manner in which cations can clearly inhibit labelling in a semi-linear fashion with progressively increasing concentrations clearly implies a strong io-

nic character to the bond as well as being by no means contradictory of the above hypothesis of dipolar forces when one considers range of interaction for ionic as opposed to these relatively short range forces.

The enzymic degradation treatment using phospholipase C with concommitant reduction in binding appears to strongly support the role of phosphatidyl choline as the probable binding site. Similarly, the initial electrophoretic characterization of the untreated synaptic vesicles as apparently a single negatively charged species over a pH range of 3.5-7.0 is supportive of the view that the vesicles act analogously to anionic resin beads. The later findings of markedly altered character of charge following treatment with phospholipase D is again strongly supportive evidence for phosphatidyl choline as being predominantly responsible for this anionic character.

In a more speculative vein of thought, the magnitudes and seeming interactions between the two enzyme effects may or may not carry with them meaningful information as to the vesicle's structure. It is clear that the cleavage of the choline from the phosphatidyl choline unit does expose numerous anionic sites. It is clear that the nonspecific proteolytic activity of trypsin leads to a similar increase in the number of exposed anionic sites of even greater magnitude. That the latter process bears no relationship particularly to the submitted hypothesis is at once appreciated. What then is the significance of either of these observations? Simply, that there appears to be some phosphatidyl choline available for enzymic

degradation and similarly some nonspecific protein available for enzymic degradation.

The original intent of the use of the trypsin was to consider two possible situations: (1) that there was a sort of protein matrix or outer shell surrounding and therefore, shielding, phospholipid which might be thought of as an inner shell or core to the vesicle; (2) that the binding that we see to the vesicle of labelled ACh and the subsequent release in the face of rising ionic strengths might relate more to the structure of this protein matrix than to phospholipids or gangliosides (if it relates to the latter at all). The manner of examining these two propositions was to observe the effect of using first the trypsin and then the phospholipase in both labelling uptake and electrophoresis experiments. For thoroughness, it must be observed that a reverse order of sequence as well as the use of a trypsin-neuraminidase combination would surely have been in order had time allowed. A final comment along the same lines would be that incubation with the enzymes for varying periods of time should have been done as well in all instances.

In light of the lack of thoroughness in approach to examining the interactions of the two or three enzymes used, it would seem untenable to suggest much in regards to an interpretation as to the import of the results. It could be that there exists an outer layer of protein shielding the phospholipids; it could be that protein and not phospholipid is the source of the anionic sites detected through these two different approaches; or it could be

that the additive effect is no demonstration of interaction at all, but that the devastating effects of the combination of the two enzymes upon the integrity of the vesicle's structure are so immense that to attach any significance to the observation would be folly - in this regard, the lack of electron micrographs of enzyme treated samples is again a regrettable omission related more to circumstance than time.

The picture seen with electrophoresis of enzyme-treated preparations shows invariably two peaks where there had originally been only one relatively homogeneous peak in the non-treated specimens. This may imply a heterogeneous population of vesicles relative to morphology and function or an incomplete attack of the substrate by the enzyme for one of a variety of reasons. The latter would seem less likely, as there appears to be no intermediate peaks (save in the case of the trypsin) but only the immobile predominant peak and the smaller mobile one upon which the calculations for the mobilities were made. Nonetheless, the use of time variation of incubation would have been of decided utility at this juncture in resolving this question at least partially.

Only as an aside, the decided efficacy of guanidinium hydrochloride as a releasing agent may hold some significance in elucidating the pharmacologic efficacy of this substance in treating the myasthenic like symptoms of Eaton-Lambert Syndrome (which has been shown to be a defect in presynaptic release and not a defect at the receptor site as is the case in its clinically indistinguishable twin myasthenia gravis (109). To make any truly meaningful statement, again, the use of the brain slice and ganglion perfusion preparations to test

for similar response would seem in order. The most likely explanation for this organic salts' seeming greater relative potency as a releasing agent would appear to be its obvious acidity (in most instances a pH effect could hopefully be discounted, but here the magnitude of a pH shift would be too great to overlook).

Since embarking upon these investigations, a number of papers of interest have appeared in publications which deserve some consideration. Marchbanks, working in the laboratories of Whittaker, has reported several observations that run counter to those recorded herein. In particular, in his Biochemical Journal article (110), he describes a method of utilizing a Sephadex resin column to separate unbound from bound ^{14}C labelled ACh from either synaptosomes or synaptic vesicles. In view of the preponderance of evidence offered, the defect in this method should be predictable, but the attempts described earlier to duplicate his work confirm nicely this suspicion, i.e., sephadex is not lacking in ion exchange properties and tends to bind synaptic vesicles but not synaptosomes, which can, in turn, be flushed from the resin by the adding of solutions of various concentrations of inorganic cations (see Table 3). This would probably explain the other point of disagreement between his data and that reported herein, i.e., that while quite adequate binding of ^{14}C labelled ACh was measured by his method for synaptosomes, he found the vesicles failed to be labelled save only slightly (it being postulated that most of the counts, i.e. synaptic vesicles and their complement of label were held up on the columns due to their heavy anionic charge and were not collected save for some leakage or column overload amounts).

The important point to be made from the misfortunes plaguing the present investigation as regards unsuspected ionic properties of membranes and resins, is that future experimenters must always be wary of the possibility of an ion exchange effect with various apparatus they may bring synaptic vesicles into contact with and consequently derive erroneous data.

Next, as briefly mentioned in the introduction, and borne out by our own work, the evidence for significant levels of gangliosides being found in the vesicle fraction is almost nil outside of the initial reports by Burton. This, however, leads into a discussion of two more recent, and on the surface more promising, papers by Vos, Kuriyoma and Roberts (111) (112). They have reported upon their findings concerning the effects of pH and neuraminidase treatment upon both radioactive labelling and electrophoretic mobility of vesicles. The espoused goal in mind being that the technique of electrophoresis might in some manner enable the separation of what is now recognized as being a heterogeneous population of vesicles which is attained via the Whittaker or De Robertis technique of isolation into various subgroups of more nearly homogeneous species of vesicles in terms of the types of transmitters stored. Labelling with ^{14}C epinephrine, ^{14}C GABA, and ^{14}C ACh simultaneously was performed followed by electrophoresis of the tri-labelled preparations on a curious continuous gradient running from 0.32 to 1.0 Molar Sucrose. Such would appear most inappropriate for this purpose, as it has already been described how vesicles become immobile in the 0.4 Molar Sucrose layer under a x g force of 52,500. The calculations described as the means by which

the various electrophoretic mobilities were determined would appear to be inapplicable. An estimate of the true mobility from a density gradient electrophoresis study requires a fairly sophisticated evaluation of a mobility integral and the method is therefore inappropriate for measurement of electrophoretic mobilities (113).

With the above defects in basic experimental design and data handling in mind, their results must now be considered. First of all, they describe a baseline value of 0.042 micromoles/mg. for vesicle preparations and 0.052 for synaptosome of protein of neuraminic acid release from the various fractions (recent work shows that synaptosomes are much richer in gangliosides than vesicles, presumably due to the richer concentration in cell wall at the bouton terminale and post-synaptically). Other investigators (especially Whittaker, et al. and this author) fail to detect such large amounts. Next, it was found that there existed a pH effect quite unlike the one described in the present work. They assign mobilities at pH 7.0 and approximate isoelectric points respectively, as follows: synaptic vesicles, -0.96×10^{-6} seconds^{-1} Volt^{-1} cm.^{2} and pH 4.0; synaptosomes, -1.68×10^{-6} seconds^{2} Volt^{-1} cm.^{2} and pH 3.7; brain mitochondria, -1.58×10^{-6} seconds^{-1} Volt^{-1} cm.^{2} and pH 4.2. Treatment with neuraminidase resulted in no significant alteration in these measurements, however, it is reported that the enzymic treatment did significantly reduce synaptic vesicle binding capacity. The latter, of course, is in direct contradistinction to the findings reported in the present work. Finally, just as Burton (114) had incidentally noted that increasing the tonicity

of a solution of synaptic vesicles decreased labelling capacity (and speculated that it might prove to be some sort of nonspecific ion-exchange effect), these investigators noted an inhibition of binding or labelling of ACh and norepinephrine, but what appeared to be an actual sodium ion dependence phenomena as regards GABA binding (i.e., that Na appears to facilitate binding in the final instance).

As far as attempting an explanation for such discrepancies as the assay they report for sialic acid content (hence ganglioside content), one can only be referred to the more recent analysis of vesicle composition available in the literature.

Very recently, two other pertinent papers have appeared in the literature. The first, by Takeno (115) deals with the distribution of bound ACh in nerve ending particles utilizing the French Press technique for rupture and release of synaptic vesicles. They found that a certain fraction of the bound ACh is sequestered or attached to the presynaptic membrane itself in contradistinction to that which is bound in the vesicles. By incubating vesicle fractions in solutions of varying tonicity and ionic species (Na^+, K^+, Ca^{++}, and Mg^{++}) it was observed that acetylcholine was released equally by all the cations in a roughly linear fashion with ionic strength. A similar ionic effect was not observed for that fraction somehow bound to the presynaptic membrane. The ionic strengths employed in these experiments were much higher than the ones chosen for our experiments (i.e., they exceeded normal physiological levels considerably) with a maximum of 320 mM. Furthermore, the ACh release was measured by the frog rectus abdominus bioassay technique which would leave much to be desired in

terms of sensitivity and specificity. Nonetheless, the fundamental observations made in this paper serve to support the work described herein.

The second paper, was by Sellinger, et al. (116). This paper affords little further food for discussion above and beyond that focused on the work of Kuriyama and Roberts (117), as they too employed zonal density electrophoresis. They did, however, examine the relative sialic acid content hence ganglioside content of synaptic vesicle versus presynaptic membrane. Here they found that the presynaptic membrane (nerve ending particles) were indeed rich in sialic acid and that treatment with neuraminidase did markedly alter the electrophoretic mobility of such particles. This is, as they noted in their discussion, in sharp disagreement with the findings of no effect on electrophoretic mobility following such treatment for either vesicles or nerve ending particles by Robert's group. This coupled with Whittaker (118) and Lapetina, Soto and De Robertis' (119) finding of the bulk of the sialic acid only in the presynaptic membrane, can only serve to raise even greater doubts as to whether the observations made by Robert's group can be regarded as valid. In a sense, it seems surprising to report high levels of sialic acid release from vesicles and then turn around and state that it in no way alters the physical properties of said particles as a result. The object of the work by Sellinger's group, it should be noted, did not in any way deal with synaptic vesicles; rather the focus was upon the relationship of the sialic acid content of the presynaptic membrane to the binding of acetylcholine esterase.

Finally, while this work attempts to demonstrate ion-exchange properties for synaptic vesicles, it cannot be said that any evidence that this phenomenon plays any meaningful role in the physiologic *in vivo* state has been offered. Only the weak chain of the similarity of action of TMA^+ in a relatively intact (cat ganglion perfusion) preparation a less intact (brain slice) preparation, an even less intact (nerve-ending particle) preparation, and a decidedly non intact (synaptic vesicle) preparation can be offered as support for relevance of any kind. As stated earlier, it may well be that release occurs in two steps and a membrane alteration is equal or greater in overall significance to the *in vivo* state. The alternative should be obvious: ion exchange properties may be quite nonspecific and occur in any subcellular preparation and thus these properties are of no significance save in relationship to the clinical states of extreme acidosis and alkalosis as a sort of epi-phenomenon. That much was not done to tighten-up arguments, that all alternative explanations were not examined thoroughly, in sum that this has been a sufficiently complete investigation of the possible role of ion exchange in storage and release of acetylcholine from the cholinergic synapse is patently false. That too much was ventured with too little scientific rigor, i.e., a presumptive, hasty sloppiness is indeed a fair criticism of the work as a whole. That not one single electron micrograph demonstrating the purity of the samples used was ever obtained is, however, a criticism that this author must disclaim the dubious honor of accepting, the blame in this regard clearly lies elsewhere. It is a lack of this sort of quality control as well as a medium which would prove more

physiologic than sucrose (both from the pH stabilization standpoint and the overall ionic strength as relates to the true condition *in vivo*) that seem to be, to the author, two of the most glaring defects of universal applicability to all the work that was attempted.

Thus, it might best be said that the work contained herein indicates that an ion-exchange hypothesis is a *possibility*, but fails to demonstrate it as being either a *probability* or *certainty*. It is compatible with the questions asked by way of experimentation, no more no less. Perhaps the true value of this work lies in the application of a variety of relatively esoteric preparations in fairly imaginative manner, thus perhaps suggesting to some more sophisticated and better equipped researcher the utility of such preparations in examining more reasonably circumscribed problems. Similarly, the tracing of a phenomenon through progressively more isolated (less *in vivo*, more *in vitro*) preparations would seem to be a paradigm with some intrinsic merit.

ABSTRACT:

The synaptic vesicle, a subcellular particle found in electron micrographs only at axon terminals, is felt to be the site of storage and release of the neurotransmitter substance acetylcholine. The basis for this view rests primarily upon the demonstration of miniature end-plate potentials (discrete small-number multiples of basic subunit of depolarization seen as the pattern of "noise" at the synapse between episodes of transmission) by Fatt and Katz and the findings by DeRobertis *et al.*, and Whittaker *et al*. that the acetylcholine-rich fraction and the synaptic vesicle-rich fractions obtained from subcellular fractionation of brain cortex homogenates coincide. Furthermore, this associated acetylcholine was demonstrated to be bound in such a manner that it was protected from hydrolysis by acetylcholine esterase and could be released from this bound state by heat, lowering pH, and ultrasonication.

SUMMARY:

It is proposed in this thesis then, that the nature of this binding of acetylcholine to the synaptic vesicle is one which is analogous to that of a cation being bound to an anionic site on an ion exchange resin bead. That furthermore, the minute fluctuations of ionic strength intracellularly which accompany the event of depolarization, result in a displacement of acetylcholine from these anionic sites by the influxing sodium ions and hence their release.

In an effort to examine the merits of this hypothesis then,

initial experiments utilizing the carboxylic type ion exchange resin CG-50 were carried out. The resin was washed with solution containing radioactive acetyl-1-^{14}C-choline iodide and then washed until there was no longer any activity in the collected samples. Next the resin was washed with 10, 50, 100 mM NaCl or KCl and samples collected and measured for evidence of radioactivity (i.e. acetyl-1-^{14}C-choline released or displacement).

Using this preparation then as the model, the subcellular fractionation technique of Whittaker _et al_. was used to obtain a relatively pure specimen of synaptic vesicles. Prior to ultimate separation by ultracentrifugation through an interrupted sucrose gradient, nerve-ending particles were incubated with radioactive acetyl-1-^{14}C-choline iodide. It was found that the synaptic vesicle rich fraction coincided with the radioactive acetyl-1-^{14}C-choline iodide rich fraction. Subsequently, similar preparations of labelled synaptic vesicles were incubated in solutions of 50, 100, 150 mM NaCl, KCl, LiCl, RbCl, CsCl, TMABr, AChI, and choline ethyl ether. In all instances the incubating solutions were adjusted to a constant osmolarity of 320 milliosmol. Separation of labelled vesicles and free or unbound radioactive acetylcholine was accomplished by high speed centrifugation. Then both vesicle residues and supernatants were measured for level of radioactivity.

Other techniques of separating bound from unbound acetylcholine were examined. The use of small dialysis bags with a maximum of surface area and a highly uniform flow of bathing medium was utilized. Such techniques afforded the advantages of markedly decreased handling and hence trauma as well as a better means of following the kinetics

of the phenomenon over time. The demonstration of ion exchange properties for the dialysis membrane, however, forced abandonment of the technique as the phenomenon being studied could not be delineated from that of the membrane.

Next, the use of a molecular seive, Sephadex G-10, was examined as a possible means of separating vesicles and their bound complement of labelled acetylcholine from the much smaller and therefore presumably much more rapid moving free labelled acetylcholine. Again, the belated discovery of ion exchange properties for the Sephadex lead to its subsequent abandonment.

Finally, the use of acetylcholine esterase to attack the unbound labelled acetylcholine and reduce it to radioactive acetic acid and choline with a subsequent solvent separation of the latter was examined. Again, due to apparently the extremely low concentration of substrate, the results yielded by this technique lacked sufficient reproducibility to make it superior or even equivalent to high speed separation.

The treatment of vesicles with specific enzymes and the effects this had upon subsequent efforts at labelling with radioactive acetyl-1-^{14}C-choline iodide was examined. The choice of enzymes, and for that matter the ultimate purpose of such manipulations was dictated by theoretical considerations of the possible molecular character of the proposed anionic binding sites. Based upon studies of chemical composition of synaptic vesicles by Burton and Whittaker, the neuraminic acid group of gangliosides with its available anionically charged carboxyl radical available at physiologic pH, similarly

the phosphate group of phosphatidyl choline and ethanolamine and lastly the anionic sites upon proteins of unknown structure were all considered likely candidates. Thus, neuraminidase from *Vibrio cholerae*, (which cleaves and thus solublized the the Sialic acid), phospholipase C from cabbage (which cleaves the choline phosphate and thus solublizes that anionic entity), and trypsin with its obvious proteolytic properties were all incubated with vesicles prior to labelling and the effect upon the latter measured as previously described.

Finally, the electrophoretic mobility of synaptic vesicles utilizing free boundary electrophoresis and a schlieren optical system to visualize, was measured. Subsequent to the finding that the vesicle fraction contained a relatively homogeneously anionically charged species over a wide range of pH, the effect of incubation with the enzymes neuraminidase, trypsin, and phospholipase D (the latter cleaves the choline group from the phosphatidyl choline and the ethanolamine group from phosphatidyl ethanolamine thus exposing additional anionic groups on the phosphate) upon electrophoretic mobility (in light of the effects upon vesicle binding capacity) was examined.

Synaptic vesicles bind and release acetylcholine in an analogous manner to the resin model. Increasing concentrations of cations decreases ACh binding by vesicles. Release of ACh increases as cation concentration increases although in a less linear fashion. Trypsin and phospholipase C both enhance the binding capacity of vesicles. Neuraminidase had no effect and no evidence of

NANA release by the Svennerholm assay technique could be demonstrated. The negative electrophoretic mobility of synaptic vesicles is increased by prior treatment with phospholipase D or trypsin, but remains unaltered by treatment with neuraminidase.

REFERENCES

1. Del Castillo, J. and Katz, B. Biophysical aspects of neruo-muscular transmission. Prog. Biophysics. 1956, 6, 121-170.

2. Eccles, J.C., Eccles, R.M. and Fatt, P. Pharmacological investigations on a central synapse operated by acetylcholine. J. Physiol. (London), 1956, 131, 154-169.

3. Hebb, C.O. Biochemical evidence for the neural function of acetylcholine. Physiol. Rev., 1957, 37, 196-220.

4. Whittaker, V.P. and Sheridan, M.N. The Morphology and acetylcholine content of isolated cerebral cortical synaptic vesicles. J. of Neurochem., 1965, 12, 363-372.

5. Whittaker, V.P. and Sheridan, M.N. The Morphology and acetylcholine content of isolated cerebral cortical synaptic vesicles. J. of Neurochem., 1965, 12, 363-372.

6. Whittaker, V.P. and Sheridan, M.N. The Morphology and acetylcholine content of isolated cerebral cortical synaptic vesicles. J. of Neurochem., 1965, 12, 363-372.

7. Whittaker, V.P. and Sheridan, M.N. The morphology and acetylcholine content of isolated cerebral cortical synaptic vesicles. J. of Neurochem., 1965, 12, 363-372.

8. Lund, R.D. and Westrum, L.E. Synaptic vesicle differences after primary formalin fixation, J. Physiol. (London), 1966, 185, 7P-9P.

9. Fatt, P. and Katz, B. Spontaneous subthreshold activity at motor nerve endings. J. Physiol. (London), 1952, 117, 109-128.

10. Birks, R., Huxley, H., and Katz, B. The fine structure of the neuromuscular junction of the frog. J. Physiol. (London), 1960, 150, 134-144.

11. Whittaker, V.P. The isolation and Characterization of acetylcholine containing particles from brain. Biochem. J., 1959, 72, 694-706.

12. Whittaker, V.P. Pharmacological studies with isolated cell components. Biochem. Pharm., 1962, 9, 61-69.

13. De Robertis, E.D.P. and Franchi, C.M. Electron microscope observations on synaptic vesicles in synapses of the retinal rods and cones. J. Biophys. Biochem. Cytol., 1956, 2, 307-318.

14. De Robertis, E.D.P., Pelligrino De Iraldi, A., Rodriguez De Lores Arnaiz, G., and Salganicoff, L. Cholinergic and non-cholinergic nerve endings in rat brain. I. Isolated and subcellular distribution of acetylcholine and acetylcholine esterase. J. Neurochem., 1962, 9, 23-35.

15. Whittaker, V.P., Michaelson, I.A. and Kirkland, R.J., The separation of synaptic vesicles from nerve-ending particles ("synaptosomes"). Biochem. J., 1964, 90, 293-305.

16. Whittaker, V.P., Michaelson, I.A. and Kirkland, R.J. The separation of synaptic vesicles from nerve-ending particles ("synaptosomes"). Biochem. J., 1964, 90, 293-305.

17. Burton, R.M., Gangliosides and acetylcholine of the central nervous system. III. The binding of radioactive acetylcholine by subcellular particles of the brain. Int. J. Neuropharmacol., 1964, 3, 13-21.

18. Whittaker, V.P. Some properties of synaptic membranes isolated from the central nervous system. Ann. N.Y. Acad. of Sci., 1966, 137, 982-998,

19. Burton, R.M. and Howard, R.E. Gangliosides and acetylcholine of the central nervous system. VIII. Role of lipids in the binding and release of neurohormones by synaptic vesicles. Ann. N.Y. Acad. of Sci., 1967, 144, 411-432.

20. Burton, R.M. and Gibbons, J.M. Lipid composition of a rat brain synaptic vesicle fraction. Biochem. Biophys. Acta, 1964, 84, 220-223.

21. Whittaker, V. The binding of acetylcholine by brain particles in vitro. In U. von Euler (Ed.) Mechanisms of release of Biogenic amines, Proceedings of the International Wenner Gren Centennial Symposium. Stockholm, Sweden, Pergamon Press, 1965. pp. 147-163.

22. Whittaker, V. The binding of acetylcholine by brain particles in vitro. In U. von Euler (Ed.) Mechanisms of release of biogenic amines, Proceedings of the International Wenner Gren Centennial Symposium. Stockholm, Sweden, Pergamon Press, 1965. pp. 147-163.

23. Koelle, G.B. Neurohumoral transmission and the autonomic nervous system. In Goodman, L. and Gillman, A. (Ed.) The pharmacological basis of therapeutics. 1965, p. 406.

24. Koelle, G.B. Neurohumoral transmission and the autonomic nervous system. In Goodman, L. and Gillman, A. (Ed.) The pharmacological basis of therapeutics. 1965, p. 406.

25. Koelle, G.B. Neurohumoral transmission and the autonomic nervous system. In Goodman, L. and Gillman, A. (Ed.) The pharmacological basis of therapeutics. 1965, p. 406.

26. Koelle, G.B. Neurohumoral transmission and the autonomic nervous system. In Goodman, L. and Gillman, A. (Ed.) The pharmacological basis of therapeutics. 1965, p. 406.

27. Hunt, R. and Tarveau, M. The relationship between the toxicity and chemical constitution of choline and analogous compounds. J. Pharm. and Exp. Therap., 1909, 3, 303-337.

28. Dale, H.H. The action of certain esters and ethers of choline, and their relation to muscarine. J. Pharm. and Exp. Therap., 1915, 6, 417-436.

29. Loewi, O. Uber humoral ubertragbarkeit der herznervenwirkung. I. Mitteilung, Pfluegers Arch. Ges. Physiol., 1921, 189, 239-242.

30. Feldberg, W. and Gaddum, J.H. The chemical transmitter at synapses in a sympathetic ganglion. J. Physiol. (London), 1934, 81, 305-319.

31. Dale, H. The William Henry Welch Lectures, 1937, Acetylcholine as a chemical transmitter of the effects of nerve impulses. I. History of the ideas and evidence. Peripheral autonomic actions. Functional nomenclature of nerve fibres. II. Chemical transmission at ganglionic synapses and voluntary motor nerve endings. Some general considerations, J. Mt. Sinai Sosp. N.Y., 1938, 4, 401-429.

32. Katz, B. and Miledi, R. The measurement of synaptic delay, and the time course of acetylcholine release at the neuromuscular junction, Proc. Roy. Soc. (London), 1965, Ser. B 161, 483-495.

33. Fatt, P. and Katz, B. Spontaneous subthreshold activity at motor nerve endings. J. Physiol. (London), 1952, 117, 109-128.

34. Whittaker, V.P., Michaelson, I.A. and Kirkland, R.J., The separation of synaptic vesicles from nerve-ending particles ("synaptosomes"). Biochem. J., 1964, 90, 293-305.

34. De Robertis, E.D.P., Pelligrino De Iraldi, A., Rodriguez De Lores Arnaiz, G., and Salganicoff, L. Cholinergic and non-cholinergic nerve endings in rat brain. I. Isolated and subcellular distribution of acetylcholine and acetylcholine esterase. J. Neurochem., 1962, 9, 23-35.

35. De Robertis, E.D.P., Pelligrino De Iraldi, A., Rodriguez De Lores Arnaiz, G., and Salganicoff, L. Cholinergic and non-cholinergic nerve endings in rat brain. I. Isolated and subcellular distribution of acetylcholine and acetylcholine esterase. J. Neurochem., 1962, 9, 23-35.

36. Bodian, D. Cytological aspects of synaptic function, Physiol. Rev. 1942, 146-169.

37. Brodkin, E. and Elliot, K. Binding of acetylcholine, Am. J. Physiol. 1953, 173, 437-442.

38. Fatt, P. and Katz, B. Spontaneous subthreshold activity at motor nerve endings. J. Physiol. (London), 1952, 117, 109-128.

39. Katz, B. and Miledi, R. The measurement of synaptic delay. Proc. Roy. Soc. (London), 1965, Ser. B 161, 483-495.

40. Krnjevic, K. and Mitchell, J.F. The release of acetylcholine in the isolated rat diaphragm. J. Physiol. (London), 1961, 155, 246-262.

41. Fatt, P. and Katz, B. Spontaneous subthreshold activity at motor nerve endings. J. Physiol. (London), 1952, 117, 109-128.

42. Martin, A.R. A further study of the statistical composition of the end-plate potential, J. Physiol. (London), 1955, 130, 114-122.

43. Nachmansohn, D. Chemical control of bioelectric currents in membranes of conducting cells. J. Mt. Sinai Hospital, 1964, 31, 549-583.

44. Ehrenpreis, S. Possible nature of the cholinergic receptor, Ann. N.Y. Acad. of Sci., 1967, 144, 720-734.

45. Thesleff, S. Supersensitivity of skeletal muscle produced by botulinum toxin. J. Physiol. (London), 1960, 151, 598-607.

46. Browning, E.T. and Schulman, M.P. [^{14}C] Acetylcholine synthesis by cortex slices of rat brain. J. of Neurochem. 1968, 15, 1391-1406.

47. Fellman, J.H. Personal Communication, 1969.

48. Scheuler, F.W. A new group of respiratory paralyzants. I. The hemicholiniums, J. Pharm., 1955, 115, 127.

49. MacIntosh, F.C. Synthesis and storage of acetylcholine in nervous tissue. Can. J. of Biochem. and Physiol. 1963, 41, 2555-2571.

50. Keynes, R.D. The nerve impulse and the squid. Am., 1958, 199, 83-90.

51. Birks, R.I. The role of sodium ions in the metabolism of acetylcholine, Can. J. of Biochem. and Physiol. 1963, 41, 2573-2597.

52. Whittaker, V.P., Michaelson, I.A. and Kirkland, R.J. The separation of synaptic vesicles from nerve-ending particles ("synaptosomes"). Biochem. J., 1964, 90, 293-305.

53. De Robertis, E.L. Salganicoff, L., Ziehr, L.M. and Rodriguez, De Lores Arnaiz, G. Acetylcholine and cholineacetylase of synaptic vesicles, Science, 1963, 140, 300-301.

54. Tucek, S. Subcellular localization and binding of choline acetylase in the cholinergic nerve endings of the brain. J. of Neurochem., 1966, 13, 1317-1327.

55. Fonnum, F. The compartmentation of choline acetyl transferase within the synaptosome, Biochem. J., 1967, 103, 262-270.

56. Fonnum, F. Choline acetyltransferase binding to and release from membranes, Biochem. J., 1968, 109, 389-398.

57. Whittaker, V.P., Michaelson, I.A. and Kirkland, R.J., the separation of synaptic vesicles from nerve-ending particles ("synaptosomes"). Biochem. J., 1964, 90, 293-305.

58. MacIntosh, F.C. Synthesis and storage of acetylcholine in nervous tissue. Can. J. of Biochem. and Physiol., 1963, 41, 2555-2571.

59. Birks, R.I. The fine structure of motor nerve endings at frog myoneural junctions. Ann. N.Y. Acad. of Sci., 1966, 135, 8-20.

60. Katz, B. The Croonian Lecture - The transmission of impulses - from nerve to muscle and the subcellular unit of synaptic action. Proc. Roy. Soc., B 155, 455-479.

61. Nachmansohn, D. Chemical control of the permeability cycle in excitable membranes during electrical activity. Ann. N.Y. Acad. of Sci., 1966, 137, 877-900.

62. Birks, R. I. The role of sodium ions in the metabolism of acetylcholine. Can. J. of Biochem. and Physiol., 1963, 41, 2555-2571.

63. Abadom, P.N. and P.G. Scholefield, Amino acid transport in brain-cortex slices. I. The relationship between energy production and glucose dependent transport of glycine. Can.J. of Biochem. and Physiol. 1962, 40, 1575-1590.

64. Del Castillo, J. and Engback, L. The nature of the neuromuscular block produced by magnesium. J. Physiol. (London), 1954, 124, 370-384.

65. Keynes, R.D. and Ritchie, J.M. The movement of labelled ions in mammalian non-myelinated nerve fibers. J. Physiol. (London), 1965, 179, 333-367.

66. Katz, B. and Miledi, R. The measurement of synaptic delay, and the time course of acetylcholine release at the neuromuscular junction. Proc. Roy. Soc., 1964, 161, 483-503.

67. Del Castillo, J. and Katz, B. Quantal components of the end-plate potential. J. Physiol. (London), 1954, 124, 560-573.

68. Hutter, O.F. and Kostial, K. Effect of magnesium and calcium ions on the release of acetylcholine. J. Physiol. (London), 1954, 124, 234-241.

69. Birks, R.I. The role of sodium ions in the metabolism of acetylcholine. Can. J. of Biochem. and Physiol. 1963, 41, 2573-2597.

70. Hodgkin, A.T. and Huxley, A.F. A quantitative description of membrane current and its application to conduction and excitation in nerve. J. Physiol. (London), 1952, 117, 500-544.

71. Chandler, W.K. and Meves, H. Voltage clamp experiments on internally perfused giant axons. J. Physiol. (London), 1965, 180, 788-820.

72. Katchalsky, A. Membrane thermodynamics. In Gardner, C. Quarton, Theodore Melnechuk, and Francis O. Schmitt (Eds.) The Neurosciences. N.Y. Rockefeller Press, 1967, 326-343.

73. Rojas E. and Ehrenstein, G. Voltage clamp experiments on axons with potassium as the only internal and external cation. J. Cellular Comp. Physiol., 1965, 66, Suppl. 2, 71-77.

74. Taylor, R.E. The role of inorganic ions in the nerve impulse, The Neurosciences, 1967, Rockefeller University Press, N.Y. p. 311.

75. Del Castillo, J., Rodriguez, A., Romero, C.A. and Sanchez, V. Lipid films as transducers for detection of antigen-antibody and enzyme substrate reactions. Science, 1966, 153, 185-188.

76. Bean, R.C. Characterization of lipid-protein excitable membrane and its application to problems in molecular biology, Publication No U3494, Philco Corporation.

77. Gray, E.G. and Whittaker, V.P. The isolation of synaptic vesicles from the central nervous system. J. Physiol., 1960, 153, 35-37P.

78. Whittaker, V.P., Michaelson, I.A. and Kirkland, R.J. The separation of synaptic vesicles from nerve-ending particles ("synaptosomes"). Biochem. J., 1964, 90, 293-305.

79. Marchbanks, R.M. Exchangeability of radioactive acetylcholine with the bound acetylcholine of synaptosomes and synaptic vesicles. Biochem. J., 1967, 106, 87-95.

80. Barker, L.A., Amaro, J., and Guth, P.S. Release of acetylcholine from isolated synaptic vesicles-I. Biochem. Pharmacol., 1967, 16, 2181-2187.

81. Whittaker, V.P. Some properties of synaptic membranes isolated from the central nervous system. Ann. of N.Y. Acad. of Sci, 1966, 137, 982-998.

82. Svennerholm, L. On the isolation and characterization of N-acetyl-sialic acid. Acta Soc. Med., 1956, 61, 75-85.

83. A modified assay for phosphorous. Worthington Manual, 1968, 3.1.4.3.

84. Whittaker, V.P. Some properties of synaptic membranes isolated from the central nervous system. Ann. of N.Y. Acad. of Sci., 1966, 137, 982-998.

85. Marchbanks, R.M. Exchangeability of radioactive acetylcholine with the bound acetylcholine of synaptosomes and synaptic vesicles. Biochem. J., 1967, 106, 87-95.

86. Barker, L.A., Amaro, J. and Guth, P.S. Release of acetylcholine from isolated synaptic vesicles-I. Biochem. Pharmacol., 1967, 16, 2181-2187.

87. Hosein, E.A. and Levy, R. The effects of temperature on the release of material with acetylcholine-like activity from brain subcellular particles. Can. J. of Physiol. and Pharmacol., 1966, 45, 401-405.

88. A modified assay after McFarlane and Knight (1941). Worthington Manual, 3.1.4.3.

89. A modified assay after McFarlane and Knight (1941). Worthington Manual. 3.1.4.3.

90. Katz, B. and Miledi, R. The effect of calcium on acetylcholine release from motor nerve terminals. Proc. Roy. Soc. (London), Ser. B, 161, 496-503.

91. Bloedel, J., P.W. Gage, R. Llinas, and D.M.J. Quastel. Transmitter release at the squid giant synapse in the presence of tetrodotoxin. Nature (1966), 212, 49-50.

92. Birks, R. The role of sodium ions in the metabolism of acetylcholine. Can. J. of Biochem. and Physiol., 1963, 41, 2555-2571.

93. Burton, R.M. Gangliosides and acetylcholine of the central nervous system. III. The binding of radioactive acetylcholine by subcellular particles of the brain. Int. J. Neuropharmacol., 3, 13-21.

94. Whittaker, V. The binding of acetylcholine by brain particles in vitro. In U. Von Euler, (Ed.). Mechanisms of release of biogenic amines. Proceedings of the International Wenner Gren Centennial Symposium. Stockholm, Sweden. Pergamon Press, 1965, pp. 147-163.

95. Katchalsky, A. Membrane thermodynamics. The Neurosciences, Rockefeller University Press, 1967, 326-343.

96. Burn, J.H. and Rand, M.J. Acetylcholine in adrenergic transmission. Ann. Rev. Pharmacol., 1965, 5, 163-182.

97. Katz, B. and Miledi, R. The measurement of synaptic delay, and the time course of acetylcholine release at the neuromuscular junction. Proc. Roy. Soc., 1964, 161, 483-495.

98. Birks, R. and MacIntosh, F.C. Acetylcholine metabolism of a sympathetic ganglion. Can. J. of Biochem. & Physiol., 1961, 39, 787-827.

99. Goodman, L. and Gillman, A. The pharmacological basis of therapeutics. New York, Macmillan, 1955, 619.

100. Fellman, J.H. A chemical method for determination of acetylcholine: its application to a study of presynaptic release and a choline transacetylase assay. J. of Neurochem., 1969, 16: 135.

101. Kusano, K., Livengood, D.R. and Werman, R. Tetraethylammonium ions: effect of presynaptic injection on synaptic transmission. Science, 1967, 155, 1257-1259.

102. Robinson, J.D., Anderson, Julius H. and Green, J.P. The uptake of 5-hydroxytryptamine and histamine by particulate fractions of brain. J. of Pharm. and Expt'l. Therap., 1965, 147, 236-243.

103. Green, J.P., Furano, A.V. Two pools for amines in neoplastic mast cells. Biochem. Pharm., 1962, 11, 1049-1053.

104. Robinson, J.D., Carlini, E.A. and Green, J.P. Effects of ganglioside preparations on smooth muscle. Biochem. Pharmacol., 1963, 12, 1219.

105. Green, J.P., Robinson, J.D. and Day, M. Interaction between cerebrosides sulfates and amines. J. Pharmacol. 1961, 131, 12-17.

106. Von Euler, U.S., Stjarne, L., and Lishajko, F., Uptake of radioactivity labelled DL catecholamines in isolated adrenergic nerve granules with and without reserpine. Life Sci., 1963, 11, 878-885.

107. Von Euler, U.S., Stjarne, L., and Lishajko, F., Uptake of radioactivity labelled DL catecholamines in isolated adrenergic nerve

108. Burton, R.M. and Howard, R.E. Gangliosides and acetylcholine of the central nervous system. VIII. Role of lipids in the binding and release of neurohormones by synaptic vesicles. Ann. N.Y. Acad. Sci., 1967, 144, 411-432.

109. Kennedy, W.R. and Jimenez-Pabon, E. The myasthenic syndrome associated with small cell carcinoma of the lung (Eaton-Lambert syndrome). Neruol., 1968, 18, 757-766.

110. Marchbanks, R.M. Exchangeability of radioactive acetylcholine with the bound acetylcholine of synaptosomes and synaptic vesicles. Biochem. J., 1968, 106, 87-95.

111. Vos, Johan, Kuriyama, Kinya and Roberts, Eugene. Electrophoretic mobilities of brain subcellular particles and binding of aminobutyric acid, acetylcholine, norepinephrine, and 5-hydroxytryptamine.

112. Kuriyama, Kinya, Roberts, Eugene and Vos, Johan. Some characteristics of binding of γ-aminobutyric acid and acetylcholine to a synaptic vesicle fraction from ouse brain. Brain Res., 1968, 9, 231-252.

113. Svensson, H. Zonal density gradient electrophoresis. In P. Alexander and R.J. Block (Eds.). A Laboratory Manual of Analytical Methods of Protein Chemistry. London, England. Pergamon Press, 1960, 193-244.

114. Svensson, H. Zonal density gradient electrophoresis. In P. Alexander and R.J. Block (Eds.). A Laboratory Manual of Analytical Methods of Protein Chemistry. London, England. Pergamon Press, 1960, 193-244.

115. Takeno, K., Nishio, A. and Yanagiya, I. Bound acetylcholine in the nerve ending particle. J. of Neurochem., 1969, 16, 47-52.

116. Sellinger, O.S., Borens, R.N. and Nordum. L.M. The action of trypsin and neuraminidase on the synaptic membrane of brain cortex. B. and B. Acta., 1969, 173, 185-191.

117. Sellinger, O.S., Borens, R.N. and Nordum. L.M. The action of trypsin and neuraminidase on the synatpic membrane of brain cortex. B. and B. Acta., 1969, 173, 185-191.

118. Whittaker, V.P. Some properties of synatpic membranes isolated from the central nervous system. Ann. of N.Y. Acad. Sci., 1966, 137, 982-998.

119. Lapetina, E.G., Soto, E.F. and De Robertis, E. Gangliosides and acetylcholine esterase in isolated membranes of the rat-brain cortex. B. and B. Acta., 1967, 135, 33-43.