STUDIES ON THE INCUBATION OF BRAIN SUB-CELLULAR PARTICLES UNDER VARIOUS CONDITIONS WHICH AFFECT THE RELEASE OF MATERIALS WITH ACETYLCHOLINE-LIKE ACTIVITY

by

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ABBREVIATIONS

AC	Acetylcarnitine
ACh	Acetylcholine
ACh-Cl	Acetylcholine-chloride
ATP	Adenosine-tri-phosphate
BuCh	Butyrylcholine
CoA	Co-enzyme A
DFP	Diiso-fluoro-phosphate
GBB	Gamma-butyrobetaine
PCh	Proprionylcholine

INTRODUCTION

A. Historical Background.

The synthesis of acetylcholine (ACh) was achieved by Baeyer (1) as early as 1867. However, there was complete lack of interest in this compound for almost forty years. Then, in 1906, Hunt and Taveau (2) displayed its marked depressor effects on the heart and blood pressure and they further showed that these pharmacological effects could be abolished by the administration of atropine.

Paralleling these findings was Elliott's (3) formulation of the concept of the chemical mediation of humoral transmission in sympathetic nerve. He discovered marked functional similarities between the responses following sympathetic nervous stimulation and administered adrenaline and suggested that this substance might be released from nerve endings upon stimulation. Several years later, Dixon (4) presented evidence that the heart contained a substance "proinhibitin" which, upon vagal stimulation, was converted to the active form "inhibitin". He (5) observed that the active form of this material was capable of producing cardiac arrest. He further proposed that physiological activity of muscles and glands could be due to "specific hormones" released locally upon nerve stimulation although at no time was reference made to ACh. His work, however, presented the first notions on free and bound or occluded active substances in nervous tissue. Weiland (6) in 1912, demonstrated that the application of material

released from a stimulated intestine, to a second strip of intestine produced effects similar to those observed during nerve stimulation.

The isolation of ACh was first performed by Ewins in 1914 who extracted it from ergot. Dale (7) employing Ewin's ergot extract showed that it was an active material which strongly inhibited the heart beat of animals. Dale (8) first reported the vasodilator and nicotine-like action of ACh. Sometime later, Loewi (9) demonstrated the role of chemical mediators in humoral transmission. In these experiments, Loewi stimulated the vagus nerve in the perfused frog's heart and inhibited its beat. Upon perfusing of this perfusate (obtained during stimulation) into a second heart, the beat of the latter was also inhibited. Loewi (10) termed this inhibitory substance released in the perfusate during stimulation "vagusstoff". He further showed that this material was readily destroyed by an esterase present in the heart and that the alkaloid eserine could inhibit the action of the enzyme. Acetylation of the hydrolyzed products of vagusstoff restored its activity on the frog heart. Thus, it appeared that ACh or some other choline ester was the active component in vagusstoff.

The first real evidence for the existence of ACh in the animal tissues came from the work of Dale and Dudley (11) who, in 1929, demonstrated its presence in an extract of horse spleen. Several years later Chang and Gaddum (12) applying the method of parallel bioassay confirmed the presence of ACh in tissue extracts.

Finally ACh was chemically identified in nerve by Bacq and Mazza (13) and in brain tissue by Stedman and Stedman (14).

B. Release of Acetylcholine from Nervous Tissue.

In 1934, (15) Dale and his group extended the hypothesis of chemical mediation in humoral transmission to include the transmission of nerve impulses across the neuromuscular junction and ganglionic synapses. Dale (16) showed that stimulation of motor nerve fibers to perfused voluntary muscles resulted in the appearance of ACh in the venous fluid. Curarine prevented the transmission from nerve to perfused muscle although the stimulation of the motor nerve fibers caused the usual release of ACh.

Feldberg and Vartainen (17) presented evidence that stimulation of the preganglionic fibers of the cervical sympathetic nerve in the cat resulted in the liberation from the ganglion of a substance which on pharmacological assays was indistinguishable from that of ACh. They concluded that the liberation of this material occurred at the synapses. Later studies (18) indicated that stimulation of pre-ganglionic fibers resulted in a high ACh level, but fell off rapidly during the first twenty to sixty minutes of a three hour stimulation experiment. In the presence of eserine the amount of ACh produced during the initial stages of stimulation was of the order to cause partial paralysis of the ganglion cells.

Kalson and MacIntosh (19, 20) investigated the

effect of varying the composition of the perfusion fluid on the release and synthesis of ACh from the cervical sympathetic ganglion. They observed only a limited synthesis of ACh when the media contained inorganic salts and fatigue produced by continuous stimulation could be removed upon the addition to the perfusion fluid of glucose, galactose, lactate or pyruvate. Further studies by Emmelin and MacIntosh (21) have indicated that altering the pH of the Locke perfusion medium (8, 5 or 7) and substituting it for heparinized plasma or defibrinated blood resulted in values that were within close limits as long as cholinesterase inhibitors were present in the medium in adequate amounts. Stimulation of the cat's tibialis muscle in the presence of these various media produced similar results as far as ACh liberation was concerned.

Birks and MacIntosh (22) have shown that there are several different states of ACh in existence in ganglia, of which these authors have characterized five. These are:

1. Depot ACh
2. Surplus ACh
3. Easily extractable ACh
4. Firmly bound ACh
5. Available ACh

The absence of calcium ions in the perfusion fluid resulted in the appearance of a long continued spontaneous activity of the cells of the sympathetic ganglion in the form of a repetitive discharge of impulses along the post-ganglionic axons (23). At the same

time there occurred a failure of transmission of excitation from the synaptic endings of the pre-ganglionic fibers. Finally these workers observed a corresponding failure of the output of ACh. Hutter and Kostial (24) observed that an increase in calcium ion concentration in Locke medium to 8 - 10 mM, caused the liberation of twice the usual amount of ACh by pre-ganglionic stimulation. These workers found that magnesium ions in concentrations which produce block of ganglionic transmission (15 -24 mM) reduced the output of ACh from the perfused ganglion. Calcium ion concentrations of 8 - 10 mM relieved the block produced by magnesium and restored ACh output.

Studies on ACh liberation were extended to include other tissues. Brooks (25) has shown that ACh is released from the isolated guinea-pig diaphragm in the absence of nerve stimulation. Straughan (26) observed a comparative resting release from the rat and guinea-pig diaphragm preparations. Optimal release was obtained at 37°C in Krebs' medium containing 5×10^{-6} neostigmine sulfate, when the diaphragm was stimulated through the phrenic nerve. Decrease in temperature from 37°C to 20°C reduced the amount of ACh liberated by a 20 minute tetanus. Continued stimulation at 25 pulses per second resulted in the depletion of ACh and this was unaffected by the addition of 1×10^{-6} choline. These results were confirmed by Mitchell and Silver (27), and these authors further demonstrated that increased potassium ion concentration in the medium resulted in the increased

liberation of ACh.

Burgen, Dickens and Zatman (28) investigated the action of Botulinum Toxin on the neuromuscular junction. This toxin produced irreversible paralysis on the isolated rat phrenic nerve diaphragm preparation. These authors concluded that this paralysis was due to neuromuscular block differing from that produced by d-Tubocurarine, in that motor nerve tetani were well maintained. Furthermore, the paralysis was unaffected by anticholinesterases. The output of ACh during motor nerve stimulation was greatly reduced and these authors concluded that Botulinum Toxin acts by decreasing the liberation of ACh resulting in a block in transmission. Brooks (29) believes that the toxin acts by rendering the motor nerve filament impermeable to ions by hyperpolarizing its cellular membrane.

C. Release of Acetylcholine from the Central Nervous System.

Angelucci (30) investigated the liberation of ACh from the perfused frog spinal cord. The amount released was observed to vary with the extent of the reflex activity of the cord and the addition of eserine had little or no effect upon the recovery of ACh from the perfusion fluid. However, the material released could be detected on the leech muscle only if this biological preparation was treated with eserine. Angelucci therefore concluded that the substance released during spinal cord perfusion was ACh.

Mitchell and Silver (31) observed that the application of ACh to the dorsal roots had little effect on the frog spinal

cord, unless applied in very high concentrations. They concluded that ACh is therefore not involved to any great extent in the synaptic activity of this preparation.

Several workers have reported that ACh can be detected in eserinized fluids which have been in contact with the central nervous system. Such reports have come from blood flow studies through dog and cat brain (32, 33), Ringer perfused through dogs cerebrospinal fluid (34) and from fluid held in small cups (35, 36) against the surface of the cortex in cats and sheep. A significant piece of work in this area was carried out by MacIntosh and Oborin (37). Results were obtained with plastic cylinders or cups containing fluid and placed in a position on the cortex so as to produce minimal amount of surgical damage. In the presence of eserine, the amount of ACh released into the fluid was of the order of 10^{-6}M. During narcosis the ACh accumulating in the small saline filled cup placed in contact with the exposed cerebral cortex of the cat decreased as the depth of anesthesia deepened. Mitchell (36) applied anticholinesterase drugs within the cylinders placed on the cerebral cortex of sheep, cats and rabbits under narcosis and obtained the release of ACh into these cups. Electrical stimulation of the cortex increased the rate of liberation of ACh from the primary somatosensory cortex; this rate was dependent upon the frequency of stimulation.

D. Pre-Synaptic Vesicle Theory of Humoral Transmission:

Early descriptions of nerve endings drew attention

to the presence of granules or vesicles within the synapses. In 1953, morphological investigations of synapses by Sjöstrand (38), Palay and Palade (39), de Robertis and Bennett (40), and about the same time, electrophysiological findings by del Castillo and Katz (41, 42, 43) confirmed the presence of these particles within synaptic nerve endings. These sub-cellular particles were thought to be the normal carriers of intracellular ACh. It was believed that such particles might either be discharged like secretion granules or alternatively, loose all of their ACh content when they reached the outer surface of the nerve membrane. Hence their breakage would provide the liberation of limited amounts of active substances and would favor transmission across the synapse. The minature end-plate potentials observed during stimulation of motor nerves was postulated by del Castillo and Katz (41) to be due to a localized bombardment of the post synaptic membrane by packets or "quanta" of ACh released from the vesicles within the nerve terminals. These workers found that the amount of ACh in a quantum is approximately fixed and changes in the overall rate of its release are determined by variations in the number of quanta released. The spontaneous discharge of ACh was quite sensitive to conditions such as temperature, osmotic pressure of the medium and the calcium; magnesium ratio in the medium.

In non-cholinergic neurons, Koelle (45, 46) has attempted to explain the presence of moderate quantities of cholinesterase. He proposed that the liberation of ACh combined with its subsequent

action on the presynaptic membrane may trigger the release of the actual transmitter substance. According to Koelle, the sequence of events is as follows:

The nerve action potential liberates ACh from the pre-synaptic terminals which then acts at the same terminals to effect the release of the synaptic transmitter. This latter substance produces the post-synaptic potential which initiates a nerve action potential. One serious objection to Koelle's Theory of Additional Quanta of ACh release, is that d-Tubocurarine and other blocking agents do not interfere with ACh release while they do abolish its excitatory action. By these findings, an interference with the liberation of the transmitter substance from its pre-synaptic site should then occur.

E. Studies on the Isolation of Sub-Cellular Fractions:

Early investigations by de Robertis and Bennett (40) suggested that ACh was associated with nerve granules or particles. Later work by Hebb and Whittaker (47) presented evidence that ACh was mainly associated with the mitochondrial fraction prepared from guinea-pig or rat brain homogenates. Whittaker (48), using brain suspensions in 0.32M sucrose, employed differential centrifugation techniques which resulted in the separation of the homogenate into several distinct fractions designated P_1, P_2, P_3, S_3. The heaviest fraction P_1, sedimented at 600-g for five minutes, consisted largely of myelin fragments, nuclei and cellular debris. The second fraction P_2 sedimented

at 22,000 g for 30 minutes was primarily a crude mitochondrial fraction containing myelin fragments, mitochondria and nerve ending particles. The lightest microsomal fraction, P_3, was sedimented at 105,000 g for 60 minutes, leaving the S_3 or particle-free cytoplasmic fraction as the supernatant fluid.

Whittaker (48) found that about 70% of the ACh activity was confined to the P_2 fraction. In order to obtain a more concise picture of the localization of ACh, density gradient differential centrifugation of the P_2 fraction in sucrose was carried out. The P_2 suspension in 0.32 M sucrose was layered over equal quantities of 0.8 and 1.2 M sucrose solutions. Centrifugation of this suspension at 100,000 g for 60 minutes in the ultracentrifuge resulted in the distribution of the material in P_2 into the three separate layers. Electron-microscopic examination (49) revealed that the most dense fraction C, consisted mainly of mitochondria. The less dense fraction B, comprised the nerve ending particles including the pre-synaptic vesicles. The least dense fraction A, resembled glial cells. Careful analysis of the B sub-fraction seemed to indicate that it was not however a pure pre-synaptic vesicle fraction, but rather pinched-off nerve endings. However, it appeared to contain about 80% of the total ACh and choline-acetylase normally present in the P_2 fraction.

De Robertis (50, 51) followed up Whittaker's preliminary work. He was able to obtain a purer pre-synaptic vesicle

fraction. Essentially the three primary fractions (nuclei N, mitochondria M and supernatant S) were separated by similar methods. However, the mitochondrial fraction M was centrifuged in solutions of sucrose with densities varying between 0.8 and 1.4 M. Five sub-fractions were obtained.

Sub-fractions (i) Myelin fragments.
(ii) Small fragments of nerve endings. Synaptic vesicles.
(iii) Pinched-off nerve endings containing numerous synaptic vesicles.
(iv) Most pure and abundant fraction of nerve endings.
(v) Practically a pure fraction of nerve endings.

The distribution of ACh in these fractions showed that it was almost entirely absent from (i), little in (ii), reached the highest concentration in (iii), tapering off in (iv) and (v).

F. The Nature of Acetylcholine in Tissue:

Early investigations of ACh in situ suggested that it existed in a bound or "precursor" form which was released in a free and diffusable form upon nerve stimulation. Beznak (52) was one of the first workers to demonstrate this binding in tissues. He postulated that ACh would not exist in significant quantities in a free and diffusable form, and that this binding protected it from immediate destruction by hydrolyzing enzymes. Furthermore, he argued that it is this binding that restrained the powerful action of ACh itself. Mann, Tennenbaum and Quastel (53) also provided evidence for the existence in

brain of a pharmacologically inactive substance which was broken down under a variety of conditions to form ACh. Conditions known to convert bound ACh to free ACh include denaturing agents (ether, chloroform or acetone) mineral acids and heat. For example, Cortegianni et al. (54, 55) noted that heating of brain extracts to 70°C for 3 minutes led to the release of free ACh. Stedman and Stedman (14) demonstrated that macerated brain suspended in saline-chloroform or saline-ether mixtures liberated free ACh, which they claimed for the most part was derived from the bound fraction. The action of alcohol or of acid-alcohol to liberate free ACh was first described by Loewi (56).

Braganca and Quastel (57) have pointed out that bound ACh could simply be ACh held within the mitochondria. Evidence for this was shown by Bellamy (58, 59) who isolated ACh and its synthesizing enzyme in the mitochondrial fraction prepared from locust head. He presented evidence that only a portion of the total ACh normally extracted in the presence of eserine was destroyed in its absence. He reasoned that had the active material been in free solution, total destruction of the ester would have occurred under these conditions. It appeared therefore that in vivo a large proportion of the total ACh was inaccessible to the destructive enzyme and was therefore present in a bound or occluded form.

Elliott and Henderson (60) concluded that all the ACh is initially in a bound form. These authors feel that where high values for free ACh have been reported, this may be due entirely to

artifacts in the experimental procedures.

The association of ACh with sub-cellular particles led Whittaker (48) to investigate factors liberating ACh from this vesicle fraction. Previous findings had indicated that about 50% of the particle bound ACh was in a labile form which was readily releasable by relatively mild treatment (osmotic dilution, freezing and thawing, and dialysis) while the rest was in a more stable form requiring treatment with trichloroacetic acid or organic solvents for total release.

He observed that conditions which liberated the labile form only, included:

(i) Ageing at 0°C.
(ii) Osmotic dilution.
(iii) Freezing and thawing.
(iv) Cobra venom treatment.
(v) Shaking with glass beads.

Total release of the contained ACh-like activity was obtained by incubating for one hour at 37°C, treatment with organic solvents and exposure to mild acids. However, electron microscopic examination produced evidence of extensive damage to these sub-cellular structures and hence disruption of the particles with the concomitant release of ACh. These studies produced evidence for the existence of two forms of ACh - a labile form which was released by mildly disruptive techniques which likely altered the permeability of the particles permitting the outward diffusion of free ACh and secondly, a more stable fraction which was held together by chemical forces.

6. A Criticism of Acetylcholine Identification.

The primary basis for the establishment of ACh as the parasympathetic transmitter was evidenced by:

(i) Maximal nicotinic and muscarinic potency of ACh.

(ii) Bioassay of rat brain extracts by the use of the Frog rectus abdominis preparation, Richter and Crossland (61).

(iii) Bioassay on various test objects in comparison to pure ACh, Chang and Gaddum (12).

(iv) Demonstration of ACh activity in horse spleen extracts, Dale and Dudley (11).

(v) Extraction of ACh synthesized by mammalian brain tissue in vitro. Stedman and Stedman (14).

(i) Maximal Nicotinic and Muscarinic Potency of ACh.

The potent nicotinic and muscarinic activities of ACh were known even before this substance was discovered in animal tissue. These pharmacologic properties were associated or related in order to fit the proposed theory of neurohumoral transmission. Evidence against such a proposal was that the biological activity of ACh was overshadowed by that of PCh (12).

(ii) Bioassay of Rat Brain Extracts by the Use of the Frog Rectus Abdominis Preparation, Richter and Crossland (61).

The investigation of Richter and Crossland (61) is a prime example where ACh has been identified in extracts or perfusates on the sole basis of bioassay upon a single test object. This leaves a definite uncertainity as to whether the substance is really ACh.

It was shown that the product of the choline acetylase system is predetermined by the nature of the substrates. Korey, de Braganza and Nachmansohn (62) demonstrated that purified choline acetylase can utilize propionic acid to synthesize an active choline ester which Gardiner and Whittaker (63) showed to be PCh.

The first possible occurrence of pharmacologically active non-choline esters in nervous tissue was presented by Nachmansohn, Hestrin and Voripaieff (64), in which their enzymatic factor was synthesized in the absence of choline as a substrate. Middleton and Middleton (65) illustrated that this active material had less activity (relative to ACh) on the frog rectus than on the cat's blood pressure and the frog heart preparation.

Hosein and Smoly (unpublished) have synthesized acetyl-l-carnitine using the Feldberg's ACh synthesizing system but employing the substrates carnitine and acetate. Their results indicated that the nature of the substrate determines the path of the acetylating system and that bioassay based on a single test object cannot determine the nature of the product in tissue extracts.

(iii) Bioassay on Various Test Objects in Comparison to Pure ACh. Chang and Gaddum (12).

Chang and Gaddum compared the relative biological potencies of several pure sympathetomimetic choline esters in different biological test objects. From their experiments they found that these choline esters have different potencies (relative to ACh) on

the normal and eserinized frog rectus abdominis, the eserinized dorsal muscle of leech, the rabbit intestine and the rabbit's blood pressure preparation. These authors concluded that the biologically active materials in tissue extracts could be considered as ACh when, on several different test preparations, the same quantitative results in terms of ACh were obtained in each case. However, they never illustrated any parallel quantitative assay which could differentiate between several bioactive substances in admixtures with ACh and pure ACh alone.

In experiments where differential assay results of biological activities of tissue extracts may agree, no reliable conclusion can be drawn. Alternatively, in experiments in which differential assay results differ, several active substances in the extract are indicated. Traditionally, the ACh activity of brain extracts is considered to be identical to ACh when a 1:1 ratio is obtained on parallel bioassay. However, reports in the literature in which the use of distinguishing test preparations such as the guinea-pig ileum and the frog heart have shown this identical comparison does not hold (66). Similarly, the relationship between extracts from normal, anoxic and anesthetized rats assayed on the Venus Mercenaria and frog heart preparations does not show a 1:1 relationship (67). Ryall (68) found that the TCA extract from P_2 fractions of brain homogenates assayed identically on four test objects, but not on the toad atrium. Finally, Crossland, Pappius and

Elliott (69) revealed that TCA extracts of frozen brain tissue produced identical results on the eserinized frog rectus and the eserinized leech dorsal muscle. However, acid-saline extracts gave a value of 25% higher on the leech than on the frog rectus preparation.

Chang and Gaddum also provided two specific tests for the identification of ACh.

(a) Production of a vasopressor action which is abolished by atropine.

(b) Production of a contraction of the isolated frog rectus abdominis which is enhanced by eserine.

However, several years later, Weger (70) found that these tests applied to acetyl-l-carnitine as well as acetylcholine. In addition, Nachmansohn's enzymatically formed factor, although not a choline ester, depressed the amplitude of the frog heart beat and the cat's blood pressure, effects which were abolished by atropine (62).

(iv) Demonstration of ACh Activity in Horse Spleen Extracts, Dale and Dudley (11).

Although Dale and Dudley demonstrated the likely presence of ACh in spleen extracts, contrary to common belief they did not chemically identify ACh in this tissue. On pharmacological identification the spleen extract assayed identically on 3 test preparations (rabbit jejunum, cat's blood pressure and cat's denervated gastrocnemius). The active material was considered to be entirely ACh. However, about 25 years later Gardiner and Whittaker (63) chemically

identified PCh in spleen extracts. These authors successfully used the guinea-pig ileum and the frog rectus preparation to differentiate pharmacologically between PCh and ACh.

(v) Extraction of ACh Synthesized *in vitro* by Mammalian Brain Tissue. Stedman and Stedman (14).

Perhaps the greatest drawback in the ACh theory is the decided lack of chemical identification. Stedman and Stedman (14) identified ACh in ox brain. However, on conversion of 1400 mg. of chloroplatinate to its tetrachloroaurate derivative and subsequent identification of ACh, 99% by weight of the original chloroplatinate was lost. Since almost total loss occurred, it is likely that other active materials were discarded.

Bischoff, Grab and Kapfhammer (71) believed that they had isolated choline and ACh from skeletal muscle while Strack *et al.* (72) are of the opinion that the substances claimed by the former group of workers might be the reineckate of carnitine whose gold content is similar to that of choline and ACh.

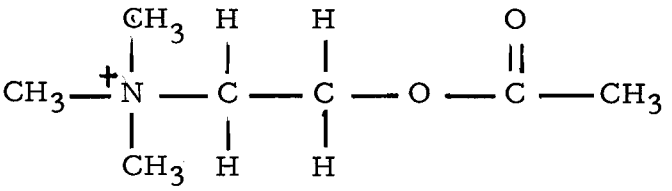
The chemical structures of the choline esters described in the above discussion are presented along with the chemical structures of the synthetic and naturally occurring betaine esters to be discussed in Sections H and I.

Choline Esters

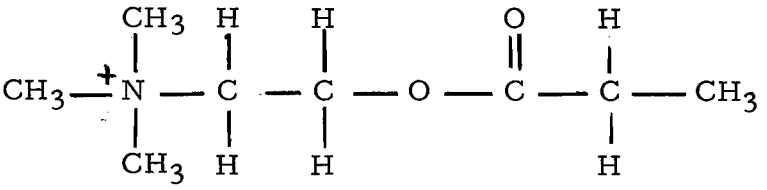
Positions of atoms

1 2 3 4 5 6

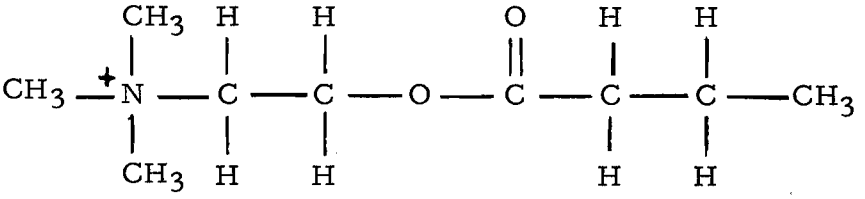
ACh



PCh

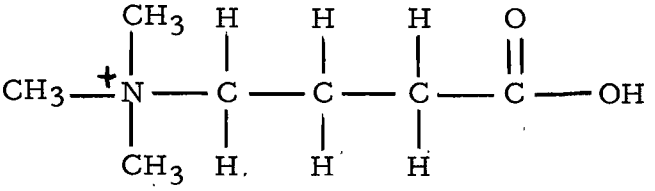


BuCh

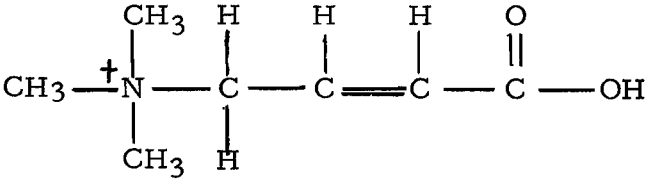


Unconjugated Betaines

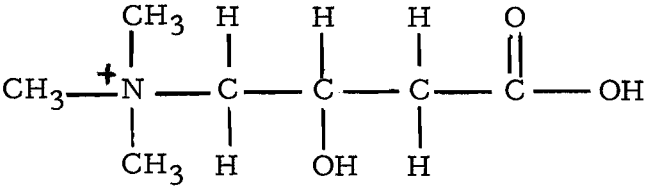
GBB (gamma-butyrobetaine)



Crotonbetaine

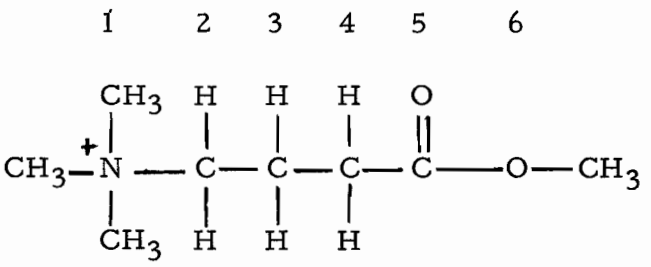


Carnitine



Synthetic Aliphatic Betaine Esters

GBBM
(Methyl ester of
gamma-butyrobetaine)



GBBE
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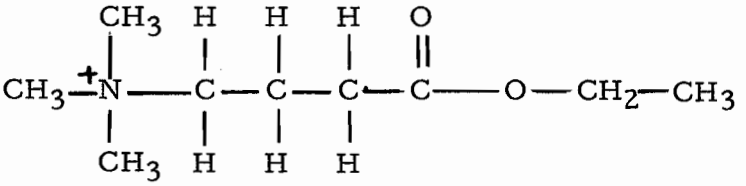
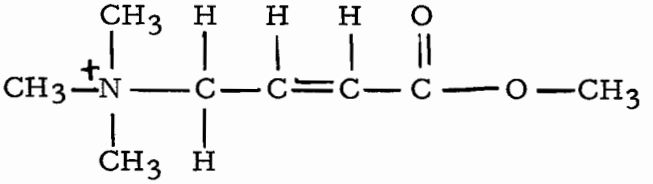
gamma-butyrobetaine)



Crotonbetaine M
(Methyl ester of
crotonbetaine)



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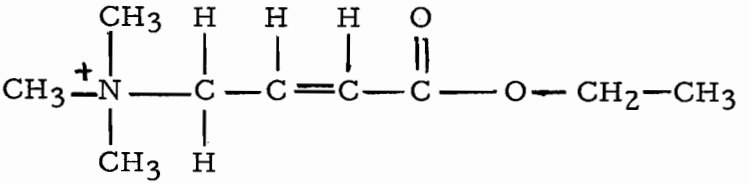
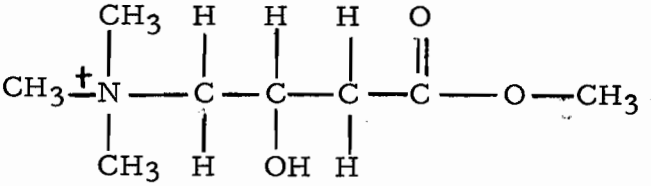
crotonbetaine)



Carnitine M
(Methyl ester of
carnitine)



Carnitine E
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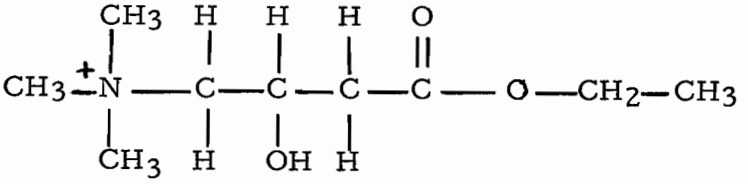
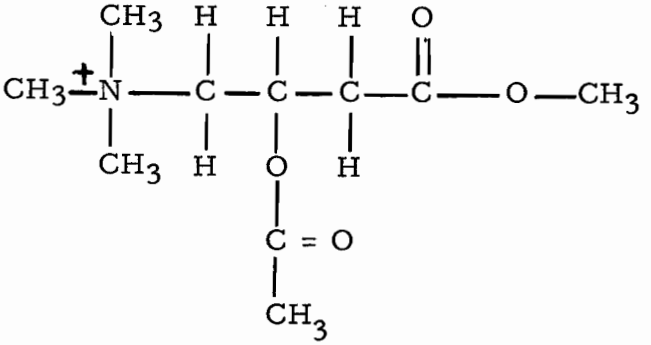
carnitine)

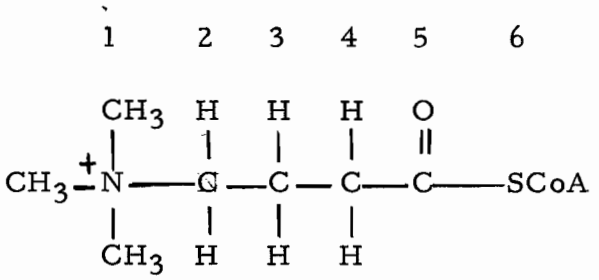


Acetylcarnitine M
(Methyl ester of
acetylcarnitine)

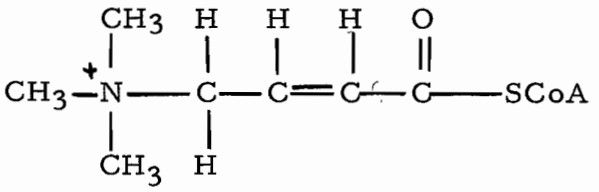


Naturally Occurring Betaine CoA Esters and acetyl-l-carnitine

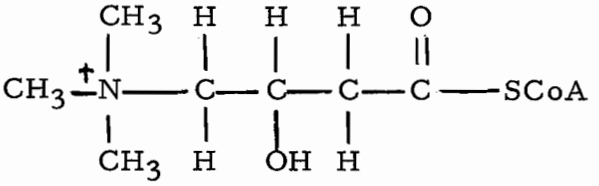
GBB CoA ester



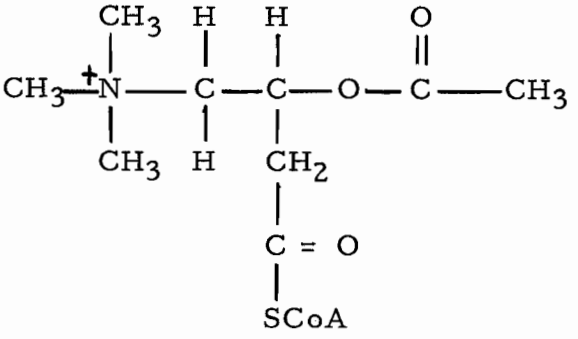
Crotonbetainyl CoA ester



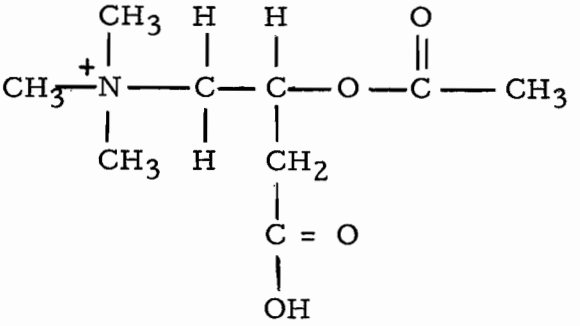
Carnityl CoA ester



Acetyl-l-carnityl CoA ester



AC (acetyl-l-carnitine)



H. Discovery and Pharmacological Properties of Betaine Esters.

About the time that Hunt and Taveau (2) had discovered the marked pharmacological properties of ACh, Krimberg (73) demonstrated the presence of various betaines in tissue when he discovered gammabutyrobetaine (GBB) and carnitine in extracts of mammalian muscle. Studies by Breiger (74) illustrated that GBB was the toxic factor in rotting horse meat and was responsible for the following symptoms in dogs that ingested this meat:-contraction of the blood vessels, salivation, excessive tearing, dilation of the pupils and nerve paralysis. Linneweh (75) confirmed these findings by injecting GBB into dogs and producing the same symptoms. Furthermore, he was able to isolate from the urine gammabutyrobetaine, carnitine, ethyl carnitine and crotonbetaine, and suggested that GBB was oxidized to carnitine and crotonbetaine.

Weger showed in 1936 (76) for the first time that carnitine in large doses itself caused an irreversible damage to the heart, resulting in negative chronotrophy which was only partially inhibited by atropine, Acetyl-l-carnitine, however, in small doses has a negative inotropic and chronotropic effect on the heart, which was enhanced by eserine and abolished completely by atropine. The activity of acetylcarnitine was destroyed after incubation with a heated tissue extract. This substance had, like ACh, only a slight alkali resistance.

Strack and Forsterling (77) could not repeat the work of Weger and came to the conclusion that the ACh-like effects of acetylcarnitine were caused by contamination of preparations of natural carnitine with choline and, after acetylation, with ACh.

Dallemagne et al. (78) undertook a study of the effects of l-carnitine, its methyl and ethyl esters, acetylcarnitine and the dihydrochloride of the disulphide of β-mercapto-α-butyrobetaine on different elements of the cholinergic system. They demonstrated that acetylcarnitine had between 1/4 to 1/10 the activity of ACh on the frog rectus; while the ethyl ester was less active than the methyl ester and ACh. Acetyl-l-carnitine and the methyl ester of l-carnitine also stimulate synaptic transmission (nictitating membrane). The methyl and ethyl ester of l-carnitine also exhibited muscarinic action (fall of blood pressure).

Hosein and McLennan (79) were able to confirm these earlier findings of Linneweh (75) by studying the effects of synthetic GBB esters injected into mice. The former workers employed much smaller doses than those employed by Linneweh in order to produce the same effects. Furthermore, Hosein and McLennan revealed that the synthetic aliphatic methyl and ethyl esters of GBB possessed most of the pharmacological properties of ACh on various biological preparations. For example, when assayed on the cat's blood pressure, preparation GBBE, like ACh, produced a fall in blood pressure,

although it was only 1/45 as potent as ACh on this particular test object. Similar ACh-like properties however, were obtained with other preparations such as the heart and rectus abdominis muscle of the frog. Subsequent tests by Hosein (unpublished) demonstrated that injection of the ethyl ester derivatives into rats not only produced the typical ACh response, but also the effect could be abolished by further injection of atropine. His experiments demonstrated that intracranial injections of GBB esters produced convulsions in unanesthetized rats. Other pharmacological studies by Hosein, Ottolenghi and Dorfman (80) revealed that the addition of 4×10^{-5} M ethyl ester of GBB to the Locke perfusion fluid produced a stimulation of the ganglion cells as observed by contraction of the nictitating membrane.

I. Naturally Occurring Betaine Esters.

In 1953, Bannister et al. (81) reported that 26% of the ACh activity found in alcoholic extracts of ox spleen was due to some unidentified material which they termed component F having an R_F of 0.5 in the butanol-water chromatographic system. Whittaker (82) concluded that this F component was a mixture of varying proportions of PCh and ACh in combination with an unknown zwitterion X. Later studies by Hosein (83) demonstrated the presence of this component in the tissue debris fractions prepared from extracts of normal rat brain and he thought this material in the F component to be betaine esters. Subsequent chromatographic analysis of the acid hydrolysates of

these materials revealed the presence of GBB and carnitine. Hosein and Proulx (84) chemically identified these materials by preparing chloroaurate derivatives from acid extracts of normal and convulsing rats. They observed the melting points of these derivatives to be the same as those of the synthetic substances.

Analysis of the hydrolysates revealed the presence of ribose, phosphate and b-alanine suggesting the presence of coenzyme A and the possibility of betaine-CoA esters. In addition GBB-CoA was synthesized (85) and shown to possess an R_F value of 0.6 in the butanol-water chromatographic system as well as pharmacological activity on the frog rectus preparation. The fact that this activity could be blocked by atropine further suggested an ACh-like action.

Further work by Hosein, Proulx and Ara (86) and Hosein and Orzeck (87) on extracts of normal brain has shown that choline, proprionylcholine, butrylcholine and a mixture of the CoA esters of gammabutryobetaine, crotonbetaine, carnitine and acetyl-l-carnitine were present. These workers observed that the betaine-CoA ester fraction accounted for about 75% of the total ACh-like activity while the choline ester fraction contributed less than 20% of the assayed activity.

Finally Hosein and Proulx (88) investigated the distribution of ACh-like substances in sub-cellular particles of normal

rat brain. Most of this ACh-like activity was localized in nerve ending fractions to the extent of about 80%. Chemically identifiable ACh accounted for no more than 10% of the total activity of this fraction. Exposure of this crude nerve ending fraction to water, resulted in the liberation of 50% or more of the total ACh activity. These workers observed that of the material released, only half was pharmacologically active, the other portion was bound to some substance and could be made pharmacologically active when released from this complex by TCA. Three forms of bound active substances were recognized, one which remained in the P_2 fraction after water treatment (designated firmly bound ACh-like substances), a second form which was released by water and was pharmacologically active, and the third form which was also released by water, but inactive. The betaine-CoA esters accounted for most of the activity associated with any one form of the bound substances.

J. Narcosis and the Acetylcholine System.

Earliest recordings on the effect of narcotics in relation to variations of ACh content in brain were reported by Welsh (89) who found an increase of free ACh in pentobarbital narcotized rats. Later, Tobias, Lipton and Lepinat (90) illustrated similar findings, an increase of brain ACh during nembutal narcosis. Experiments performed by Richter and Crossland (61) and Crossland and Merrick (91) showed that rats, killed by immersion in liquid air

during decreased cerebral activity induced by narcotic drugs, exhibited an increase in extractable ACh from brain tissue. A year later, Elliott et al. (35) also discovered that pentobarbital, ether or chloralose anesthesia increased the ACh content of rat and cat brain. In addition, these workers found that the ACh content rises fairly rapidly to a maximum during deep anesthesia. Further, decreasing the amount of anesthetic will decrease the elevated level of ACh which can again be elevated by administration of the drug.

Ether, in concentrations which can depress the respiration of rat brain cortex slices, reduces the rate of ACh synthesis (92). Further studies by McLennan and Elliott (93), and Johnson and Quastel (94) have presented evidence for a depression of ACh synthesis in rat brain cortex slices incubated in the presence of various narcotic agents.

The results presented by these various groups of researchers, obtained during administration of light and deep anesthesia required reexamination in the light of the discovery by Hosein, Proulx and Ara (85) regarding the nature of the ACh-like activity in brain tissue. Hosein and Ara (139) showed by biological assay of material in brain extracts prepared from narcotized rats and separated by chromatography, that the increased ACh activity in brain during deep anesthesia was due either to one or several of the Coenzyme A ester derivatives of betaines in the band of the chromatogram (butanol-water

system) with an R_F 0.5-0.7. Hosein and Proulx (88) showed that during nembutal or ether anesthesia, the betaine-CoA esters in the nerve ending fraction from brain homogenates increased appreciably whereas the chemically identifiable ACh did not noticeably change.

Recent work by Koh (95) on the chemical identification of materials with ACh-like activity, has revealed the presence of only one compound, acetyl-l-carnityl-CoA in the brains of narcotized rats. Other tests such as differential assays and colorimetric analysis showed this material, extracted during narcosis, was different from ACh and would seem to provide additional evidence for the identification of acetyl-l-carnityl-CoA.

K. Theories of Narcosis.

Narcosis has been defined as "the reversible decrease or abolition of the normal cell activities by agencies that are not highly selective" (96).

Many theories of narcosis have been put forward in an attempt to evaluate the mechanism of action of narcotic drugs. A summary of these theories of narcosis follows:

(i) Overton-Meyer Lipoid Solubility Theory.

Among the first theories to explain the laws governing the action of drugs on cells were those proposed by Overton (97) and by Meyer (98). These workers measured the comparative distribution of several agents known to have remarkable anesthetic properties, between oil and water phases. They observed a remarkable

correlation between the anesthetic activity and the oil/water distribution for a limited number of compounds. They believed that drugs possessing anesthetic action must be lipoid soluble and thus depend largely on the relative solubility in the fatty material as compared to that of water.

The chief drawback to this theory is that many substances which are more soluble in oil than in water possess no narcotic action such as camphor or dieldrin which are in fact convulsant agents. The Overton-Meyer correlation can hardly be considered a universal theory for the mechanism of narcosis. It is, in fact, an expression of an experimentally observed relationship which applies to a limited number of anesthetic agents.

(ii) Surface Tension and Adsorption Theory.

In 1904 Traube (99) demonstrated that a large group of surface-active substances, which can lower the interfacial tension between water and another liquid phase, included many narcotics. He was able to demonstrate clearly that within a series of alcohols, there was a striking correlation between their pharmacodynamic depressant activity and their ability to lower surface tension. This property would account for their accumulation at cell surfaces thus altering the permeability of the cellular membrane. There have been two serious objections to this theory. First, substances can produce narcosis that are not adsorbed onto the cell surface and secondly,

detergents have the ability to lower surface tension, but do not possess narcotic properties.

(iii) Lillie's Cell Permeability Theory.

This theory, developed over the years 1909-23 (100, 101, 102) has more than any of the others survived much criticism. It has often been referred to as the theory of membrane stabilization and has its basis primarily in excitable tissues.

It suggests that anesthetic agents raise the threshold of excitable membranes by penetrating into the membrane. They act by exerting pressure so that the pore size increases with less ease and hence rapid ion exchange is blocked. This is compatible with microelectrode measurements, which has revealed anesthetic agents elevating the threshold and increasing the resistance while the capacitance remains unchanged.

The theory supposes that in the membrane the charged groups on proteins and lipids overlap and thus are responsible for the electrostatic field. An oncoming depolarization will change the electrostatic field and will overlap in a manner such that the permeability to sodium is increased. The penetration into this system of a lipoid soluble anesthetic agent will result in a change in the overlapping of the side chains in a manner such that depolarization cannot result in an increase in the negative electrostatic field. This would further resolve itself into decrease in sodium permeability

resulting in nerve block. The main objection here is that there is no proof that changes in the permeability of the cell membrane are related in any way with the production of the state of narcosis.

(iv) Pauling's Hydrated Micro-Crystal Theory.

This recent theory put forward by Pauling (103, 104) differs from the earlier ones in that it involves primarily the interaction of the anesthetic agent with water rather than lipoid molecules. He postulated that these agents through hydrogen bonding form hydrated microcrystals with protein side chains within the cell. These crystals are similar in structure to known hydrate crystals of ether, chloroform, nitrous oxide, xenon and other anesthetic agents, as well as substances related to protein side chains. They are believed capable of entrapping ions and electrically charged side chains in such a manner as to decrease the energy of electric oscillations in the brain thus producing the loss of consciousness.

The chief criticism of this theory is that it is not a proposed mechanism of action for all anesthetic agents, for example, barbiturates do not form hydrated microcrystals and hence it cannot be considered as a universal theory of narcosis governing the action of all narcotic agents.

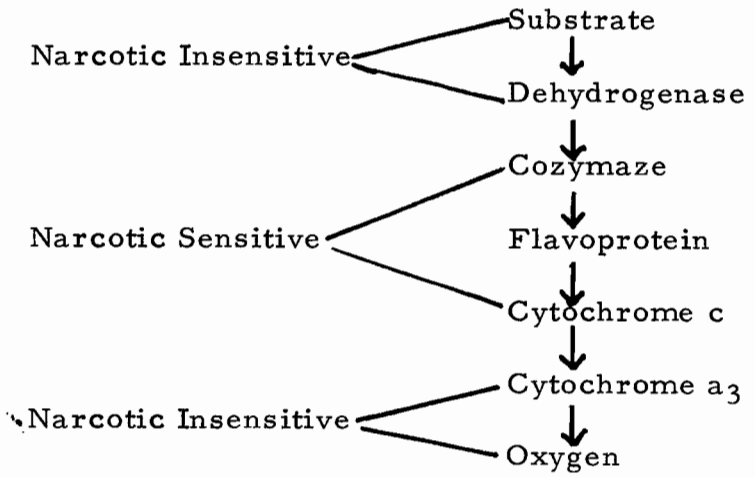
(v) Chemical Findings During Narcosis.

Quastel and Wheatley (105) formulated the concept of the mechanism of narcosis based on the inhibition of the

enzyme system necessary for the oxidation of carbohydrates in brain. These authors showed that with brain tissue, narcotics inhibited the oxidation of glucose, lactate and pyruvate, but not that of succinate. These findings were confirmed by Jowett and Quastel in 1937 (106). However, more recently Quastel (107) has taken the view that the major effect of a narcotic is not the suppression of the respiration as a whole, but is mainly concerned with the suppression of that aspect of the respiratory process responsible for the formation of the high energy phosphate bonds. His scheme for the probable site of action of narcotics is shown in Figure 1.

Figure 1

Possible Site of Action of Narcotic Agents



Low concentrations of narcotics possess the property of inhibiting the respiration of brain tissue either in the form of a mince or slices (106). Studies on the effect of several barbiturates on oxygen uptake of minced guinea-pig brain demonstrated a rough

parallelism between hypnotic power and inhibitory effect on brain metabolism (105).

The addition of pentobarbital (0.002M) brings about a large inhibition of oxygen uptake by human cerebral cortex slices in the presence of glucose (108). When more dilute concentrations of this narcotic agent were investigated, the effect sometimes brought about a small increase in respiration of the brain cortex slices (109).

L. Theories on Convulsive Phenomena.

(i) General Physiological Changes During Convulsions.

During convulsive activity, a large increase in both cerebral oxygen consumption and blood flow have been observed and are believed necessary for the maintenance of this activity (110). The increased cerebral circulation is thought to be due to powerful vasodilator effects of surplus carbon dioxide (111). However, other vasodilator products such as lactic acid and liberated ACh have also been implicated in this action.

Perfusion studies on cat brain have indicated that this increased rate of CO_2 - production during convulsions is mainly due to the oxidation of non-carbohydrate materials (112). Later studies have, in fact, demonstrated that incorporation of glucose into amino acids and protein occurs during convulsive activity (113, 114). However, the mechanism whereby an increased rate of combustion of metabolites occurs is totally unknown.

A satisfactory technique for the study of labile constituents of brain involved rapid freezing of brain in situ in liquid air. Such investigations have demonstrated, during metrazol-induced seizures, a decreased amount of high energy phosphate compounds and a corresponding increase in inorganic phosphate and lactic acid content (115). Similarly a fall in ATP, glycogen and glucose was observed following the administration of convulsive agents and electroshock in cats (116).

Convulsive agents such as metrazol, picrotoxin and sodium fluoroacetate when added to respiring cerebral tissue slices did not alter their respiration, glycolysis or phosphate content except for some inhibition at very high concentrations (117, 118).

Conditions such as hypoxia, hypoglycemia and cyanide poisoning have produced convulsive activity. This activity has been attributed to an interference in energy metabolism and this view is held by many workers to be a primary event in the initiation of seizures.

(ii) The Acetylcholine System and its Relationship to Convulsive Activity.

Early investigations (119, 120) have demonstrated the presence of spike discharges from cerebral cortex which closely resemble those seen during epilepsy, which were produced by the local application of unphysiological concentrations of ACh to the cerebral cortex of cats. Furthermore, intraventricular or intracisternal injections of ACh

and anticholinesterases into cats and man have produced generalized convulsions (121, 122). More recent findings by Feldberg and Sherwood (123) revealed a state of catatonia or stupor in cats following the intraventricular injections of ACh, physostigmine and DFP. These results were interpreted as involving the stimulation of cholinoceptive neurones in subcortical structures close to the ventricles and the rostral extensions of the reticular formation.

Although ACh is not normally present in the cerebrospinal fluid in detectable amounts, a number of workers have provided evidence for its presence in uneserinized animals (124). Intensified hyperactivity of the brain appears to be associated with the release of ACh in sufficient amounts for some of it to escape destruction by cholinesterases and hence diffuse into the subarachnoid space, or into fluids outside the pia surface of the cortex (35).

The ACh content of brains of anesthetized animals was found to be considerably increased and after administration of metrazol or picrotoxin, it decreased to normal values (35, 90). Richter and Crossland (61) found that the ACh level in the brain of young rats which were killed by being dropped into liquid air, varied with the physiological state of the animal. Convulsions initiated by electroshock for a one to three second period resulted in a rapid fall in ACh content of the order of 50%, followed in 15 seconds by a return to the normal level. Convulsions did not begin again until the ACh level had returned to

normal after the initial fall and the convulsions terminated with this second fall in ACh. These findings indicated that the two effects might be related, and that an adequate ACh level would be one of the requirements for convulsive activity of this type. This convulsive activity appeared to disappear when the ACh level fell to too low a value as the rate of synthesis could not keep pace with the rate of ACh liberation and breakdown.

Naruse et al. (125) demonstrated in a convulsive (ep) strain of mice, that see-saw stimulation for a period of 8 seconds lowered the total brain ACh activity by 25%. Prior to the onset of convulsions, the fall in ACh level was about 40% both in the so-called "free" and "bound" ACh containing fractions. The total brain ACh level returned to normal about 60 seconds after convulsive activity ceased. Injection of pentamethyltetrazol resulted in a 40% fall in the ACh activity in both fractions. Further studies by Kurokawa et al. (126) on the sub-fractionation of brain tissue after metrazol induced convulsions, revealed that changes in the osmotically "labile" fraction of bound ACh was responsible for the increased liberation in ACh. The decrease in bound ACh activity occurred mainly in the M fraction or crude mitochondrial fraction containing the synaptic vesicles and nerve ending particles.

The discovery by Hosein (83) of the chemical nature of the substances with ACh-like activity released during convulsive activity resulted in a re-examination of previous findings. Hosein

and Proulx (84) observed free GBB in supernatant fractions prepared from the brains of rats killed during convulsions induced by either picrotoxin or dieldrin. Investigations on the nature of material released during convulsions revealed an increase in ACh activity entirely accountable for by the betaine-CoA esters (84).

Hosein and Ara (127) observed an increase in the ACh-like activity in the betaine-CoA ester band on the chromatograms of material obtained from dieldrin convulsed rat brains. In the supernatant fractions prepared from camphor, metrazol or electroshock convulsed rat brain extracts, there was an increase in ACh-like activity in the betaine-CoA ester band of the chromatogram. An interesting finding was that there was almost little or no activity in the choline ester bands of the chromatogram. No increase in total ACh activity was observed in picrotoxin or strychnine convulsed brain extracts. Further experiments, in which rats were made to convulse either chemically or electrically and were then immobilized in liquid air, revealed a decrease in total brain ACh activity, again entirely accountable as a fall in the activity of the betaine-CoA ester fraction.

M. Pharmacological Properties of Narcotic Agents:

Many compounds differing widely in their chemical nature are capable, nevertheless, of producing a state of narcosis when administered to an animal. However, it appears that there is no pharmacological property common to all narcotic agents which can provide

evidence for a mechanism of action of narcotics.

Accordingly, a summary of the important pharmacological properties associated with the chemical substances investigated by this author will be discussed.

(i) Magnesium

It has been known for many years that nerve function, both central and peripheral, is depressed by high concentrations of magnesium ions. In 1905, Meltzer and Auer (128) found that general anesthesia involving the abolition of reflexes can be produced in animals by subcutaneous injection of magnesium salts. These workers revealed that, like d-Tubocurarine, magnesium can also antagonize the physostigmine-induced twitchings in skeletal muscle. Similarly in the spinal dog, the central narcotic action of magnesium resulted in complete loss of the flexion reflex (129).

Experiments have revealed that anesthesia can be reached in dogs and cats when the serum levels of magnesium range between 15 to 33 mg %. Perfusion of the cervical sympathetic ganglion of the cat with Locke solutions containing high concentrations of magnesium ions (15-25 mM) resulted in a decrease in transmission of impulses when the ganglion was stimulated through its nerve fiber and, at the same time, in a fall in the rate of liberation of ACh (24).

del Castillo and Engbaek (130) have further concluded that magnesium ions have 3 distinct effects on the neuromuscular junction: (i) they decrease the amount of transmitter liberated

at the motor nerve terminal (ii) they diminish the depolarizing action of ACh at the end plate and (iii) they depress the excitability of the muscle fiber membrane.

Numerous investigations by del Castillo and Katz (41, 42, 43, 44) and Fatt and Katz (131, 132) have provided evidence that magnesium reduces the quantal release of ACh from the neuromuscular junction during motor nerve stimulation.

(ii) Bromides

The manner in which bromides act in producing depression of the central nervous system has been rather obscure. Experiments with radioactive bromide have shown that bromide ions can gain access to nerve cells in the cerebellar cortex (133) and it would appear that the mechanism of bromide narcosis tends to be a direct effect of the bromide ion exerted at the cell surface.

In man, doses of sodium bromide larger than those used merely to produce sedation have resulted in the impairment of mental processes, difficulty in speech, a reduction of the capacity to perform processes requiring mental thought and finally impairment of memory. Very large doses result in the inco-ordination of motor activity, lethargy, confusion, disorientation, delerium or stupor (134). Intravenous injection of sodium bromide suppresses epileptic convulsions produced by camphor, but not the spinal convulsions from strychnine or picrotoxin (135).

(iii) Pentobarbital

The first introduction of barbiturates as narcotic agents was carried out in 1903 by Fisher and Von Mering (136) when they demonstrated the properties of di-ethyl barbituric acid as a narcotic agent. Since then over 100 of these ureide derivatives have been synthesized, the most widely used being 5,5' ethyl, 2-secondary amyl barbituric acid (pentobarbital or nembutal). This agent is readily absorbed and reversibly bound by serum albumin, passing into all tissues of the body (137).

Pentobarbital has a direct effect on certain tissues. It was shown to block the action of ACh on the heart of the Venus Mercenaria. Further studies have demonstrated an interference with transmission in the sciatic nerve of the frog, and concentrations of pentobarbital lower than those required to depress conduction in the sciatic nerve produced a decrease in temporal summation and facilitation in the superior cervical ganglion of the turtle (138).

Anesthesia induced by pentobarbital in rats caused an increase in concentration of materials with ACh-like activity in brain (139). Investigations performed in vitro revealed that low concentrations of this narcotic stimulated and high concentrations depressed both glycolysis and the synthesis of ACh in rat brain cortex slices, respiring in a Locke-bicarbonate or phosphate buffered medium (118, 93).

(iv) Ether

Diethyl ether, commonly referred to as ether,

was first prepared by Valerius Cordus in 1543, yet three centuries passed before its anesthetic properties were discovered. Like all other volatile hydrocarbons, it produces anesthesia by a reversible paralysis of the central nervous system, more specifically by the loss of consciousness, pain and voluntary movement without serious impairment to respiration and circulation (140).

Ether is not oxidized in the body, 79-92% is removed with the expired air, the rest being removed with other excretions.

Investigations carried out in vitro by Quastel and Jowett (106) have shown that ether depresses brain oxidation in the concentration range of 0.05 to 0.1 M and that the oxidation of glucose, pyruvate and lactate in rat brain cortex slices is depressed in the presence of ether. Both Mann et. al. (92) and Stedman and Stedman (14) have shown that ether depresses ACh synthesis in both respiring brain minces and slices.

Hosein and Ara (139) have revealed that, like nembutal, ether narcosis resulted in an increase of about 65% of the ACh-like activity in rat brain, this increase being exclusively confined to the betaine-CoA esters.

(v) Ethyl Alcohol

The effect of ethyl alcohol is one of depression, beginning with the higher functions, and later extending to the more vegitative mechanisms as the percentage of alcohol in the central nervous

system increases. The concentration of ethanol in the blood of living animals has been found to be 0.12% in slightly confusing and 0.7% in deeply narcotized states (141).

The addition of small concentrations of ethanol in vitro to rat brain cortex slices incubating in a glucose-phosphate medium whose respiration rate had previously been stimulated by the presence of 100 meq. Potassium/L caused a diminution of the oxygen consumption (142). Potassium-stimulated respiration of rat brain slices is much more affected by low ethanol concentrations than normal respiration, the alcohol concentration being of the same order as that necessary to bring about the narcotic state in the rat (143, 144). Ethanol produced a decrease in the respiration of electrically stimulated brain tissue in the presence of glucose (145).

(vi) Nitrous Oxide

Nitrous oxide was first prepared by Joseph Priestley in 1776 and three years later Sir Humphrey Davy discovered that it would obtund pain.

Significant differences from typical narcotics are shown in the failure of nitrous oxide to narcotize excised tissue (muscle or heart) and invertebrates. On these it merely acts as an indifferent gas.

In the in vitro respiration of guinea-pig brain preparations, oxidation of glucose and pyruvate, and oxidative phosphorylation are not affected by a mixture of 20% nitrous oxide, 78% of

nitrogen with 2% of oxygen (146). However, a mixture of 80% nitrous oxide and 20% oxygen inhibited oxygen consumption of rat brain cortex slices (147).

N. Pharmacological Properties of Convulsant Agents

Like the narcotics, many substances of diverse chemical nature can, when administered to animals, produce convulsive activity. In general, the mechanism of action of convulsant agents is rather obscure. Hence, as discussed above for narcotics, a summary of the important pharmacological properties of these agents related to this study follows.

(i) Picrotoxin

Picrotoxin is a non-nitrogenous bitter substance from the berries of Ananmirta Cocculus, a shrub found in the East Indies (148).

The location of action of picrotoxin convulsions is chiefly in the medulla oblongata in mammals as well as in frogs. These convulsions persist even after removal of the cerebellar hemispheres and on transection below the optic thalami. Local application of picrotoxin to the surface of the brain causes discharge of the underlying neurons.

McLennan and Elliott (93) have demonstrated increased ACh synthesis in rat brain cortex slices incubated in a high potassium medium containing 0.02 M picrotoxin. Elliott, et al. (35)

revealed that picrotoxin lowered the ACh content in brains of nembutalized cats, but not in unanaesthetized animals. For this reason it has found sole value in the treatment of severe depression following barbiturate poisoning.

(ii) Strychnine

Strychnine, an alkaloid extract from the hard button-shaped seeds of Strychnine Nux Vomica, has the outstanding pharmacological effect of producing strong tonic convulsions. Local application of large amounts of this alkaloid to the spinal cord of a frog results in convulsions (149). In an animal treated with strychnine, any tactile impulse such as the jarring of a table or even a gentle gust of wind can precipitate a convulsion (150).

Eccles (151) has postulated that strychnine acts by reducing the inhibitory action on motoneurons of several local circuits in the spinal cord resulting in the overbalance of excitatory actions.

In a concentration of 1.6×10^{-4} M (1:20000) strychnine acts as a competitive inhibitor of the hydrolysis of ACh by acetylcholinesterase. Nachmansohn has suggested that it is consistent with the view that the action of strychnine can be related to an accumulation of ACh at some crucial site at the surface of the neuron and hence to depolarization of the neuron (152).

(iii) Camphor

d-Camphor is the chief constituent of oil of camphor and has found employment as a mild irritant and antiseptic. It stimulates the central nervous system at all levels and produces clonic convulsions similar to those of metrazol. In mammals the convulsive action of camphor is localized above the optic thalami and motor cortex. Fatal camphor poisoning in both man and mice results in extensive neuronal necrosis which can be prevented by previous administration of barbiturates (153).

(iv) Metrazol

Metrazol produces convulsions which closely resemble those caused by picrotoxin. It stimulates the mid-brain, the medullary centers and most probably the spinal cord. Convulsions produced by metrazol are brief, irregular and resemble those of epilepsy and electroconvulsive seizures. Sub-convulsive doses of metrazol increase the susceptibility of rats to sound-induced convulsions (154).

Webb and Elliott (118) found that pharmacological concentrations of metrazol had no effect on either glycolysis or respiration of rat brain cortex slices incubated in Krebs' bicarbonate buffered medium. High concentrations resulted in a depression of glycolysis and respiration. McLennan and Elliott (93) noted an increase in ACh synthesis of rat brain cortex slices incubated in Krebs' medium containing 2.5 mM metrazol.

(v) d-Tubocurarine

Curare, the South American Indian arrow poison, acts primarily in paralyzing skeletal muscle by blocking synaptic transmission at the neuromuscular end plate (155). Topical application of the derivative, d-Tubocurarine to the cerebral cortex or, alternatively, intravenous or intracranial injections have resulted in spontaneous discharges similar to those produced by strychnine (156). Studies on the spinal cord of frogs, have shown that direct application of d-Tubocurarine results in typical strychnine-like convulsions (157). Similarly, Feldberg and Sherwood (123) noted that injection of d-Tubocurarine into the lateral ventricle of the cat results in a state of strong clonic convulsions. Similar findings on the cerebral convulsive effects of the picrotoxin type occur in rats and mice with 0.5-0.7 units/kg. These convulsions can be suppressed by treatment with barbiturates (158).

Coppée has shown that d-Tubocurarine does not suppress the formation of ACh in nerve stimulation, nor has it any inhibitory effect on cholinesterase (159).

(vi) Decamethonium

Decamethonium is a synthetic bis-quaternary ammonium compound. Like other members of this series, it produces neuromuscular blocks. Its pharmacological actions resemble ACh more closely than those of d-Tubocurarine (160). Unlike d-Tubocurarine,

its action is not antagonized by neostigmine, nor is it synergistic with ether.

Investigations (161) have shown that decamethonium blocks transmission through the autonomic (sympathetic and parasympathetic) ganglia and the muscle end-plate.

Jewell and Zaimis (162) have demonstrated that decamethonium blocks transmission in the red soleus and tibialis muscle motor end-plate of cats by a dual action in which depolarization is followed by a competitive phase for ACh. This results in a rapidly decreasing sensitivity of the muscle to repeated doses of decamethonium. Sensitivity is not, however, effectively antagonized by cholinesterase inhibitors. Like d-Tubocurarine, the central action of decamethonium when injected into the lateral ventricle of the cat was the production of convulsive activity (123).

(vii) Calcium

Numerous investigations have provided sufficient evidence that the central nervous system is dramatically affected by variations in the ionized calcium concentration. A decrease in ionized calcium results in a characteristic condition known as tetany (163).

On the other hand, perfusion of the cervical sympathetic ganglion of the cat in situ with Locke solution high in calcium (10-15 mM) resulted in an increased ACh liberation of the order

of 100% over values obtained with perfusion of normal Locke medium (24).

del Castillo and Katz (44) have provided evidence that calcium is essential for the quantal liberation of ACh from motor nerve terminals. Potassium-induced contractures of frog rectus abdominis muscles are dependent upon the presence of calcium and further indications are that these maintained contractures require extracellular calcium and that the brief response which appears under certain conditions requires a store of loosely-bound calcium (164).

Elliott and McLennan (165) have illustrated that the synthesis of ACh in rat brain cortex slices is largely dependent upon the calcium ion concentration of the incubating medium and that either increasing or decreasing the concentrations from physiological levels resulted in a fall in ACh synthesis.

Evidence was presented by Douglas and Rubin (166) that ionized calcium was necessary for the liberation of catecholamines from the adrenal medulla.

(viii) Semi-carbazide

The administration of this chemical agent produces clonic convulsions in both man and animal (167). These seizures are slow in onset, and usually develop over a period of one to two hours. Investigations on semi-carbazide treated cats reveal sub-cortical seizure activity preceding the appearance of changes in the cortex. These

subcortical structures (caudate nucleus and periaquedactal gray matter) contain large amounts of an enzyme system for which pyridoxal-phosphate is a co-enzyme and its administration is an effective antagonist of semi-carbazide convulsions. It is believed that semi-carbazide acts by blocking the formation of gamma-aminobutyric acid (GABA) (168). This results in the lack of inhibitory impulses providing the basis for the convulsive activity observed during semi-carbazide administration.

O. Pharmacological Properties of Drugs Affecting the Central Nervous System.

There are substances which although neither convulsants or narcotics, are nevertheless capable of affecting the metabolism of the central nervous system. A discussion of the important pharmacological properties associated with some of these chemical substances investigated by this author follows.

(i) Neostigmine.

The chemical structure of neostigmine contains a trimethylated quaternary ammonium ion attached to a substituted carbamic acid ester. This compound was first synthesized by Stedman in 1926 (169) and investigated pharmacologically by Aeschlimann and Reinert (170) in 1931.

Experiments on the kinetics of cholinesterase inhibition by neostigmine have confirmed the competitive nature of the

reaction (171). The inhibition of cholinesterase by neostigmine, like that by physostigmine, is reversible. Neostigmine can act also as an ACh analogue in that it can excite the effector cell directly. It antagonizes the myoneural block of d-Tubocurarine and other substances possessing similar action, and is widely used clinically for that purpose.

(ii) Cobra Venom

The Colubrid class of venoms, of which the Indian Cobra Naja Naja is an example, is known to produce pronounced effects on the nervous system. These venoms are characteristic members of the neurotoxic group and victims of these snake bites generally die through paralysis of the respiratory muscles. It was first suggested by Brueton and Fayrer (172, 173) that these venoms possessed an action similar to that of curare at the neuromuscular junction. Further studies showed that this curare-like action was more potent in the Colubrids than in the vipers (174, 175). The Indian cobra was found to be the most potent, being active in concentrations of 1:200,000.

Braganca and Quastel (176) reported that heated cobra venom caused an increase in total ACh synthesis by brain homogenates incubated in Locke medium. The activating effect was markedly increased in the presence of extra potassium and a large increase in the rate of liberation of "bound" to "free" ACh was observed.

When animals were subjected to certain physical conditions, a precipitation of convulsive activity followed due to a stimulating effect of these conditions in producing this type of activity in animals.

A summary of the pertinent literature regarding these physical conditions related to the investigations carried out by the author follows.

P. Conditions Related to the Production of Convulsive Activity in Animals.

(i) Electrical Stimulation of Nervous Tissue.

Early investigations revealed that strong faradic stimulation of the isolated spinal cord of rabbits and of brain-cord preparation of toads caused the release of ACh into the surrounding fluid (177).

Richter and Crossland (61) stimulated rats electrically by passage of an electric current through scalp electrodes for a period of 1 to 3 seconds. Immediate liquid air freezing of the animals after stimulation resulted in a loss of over 50% of the whole brain ACh.

A novel technique for the electrical stimulation of brain tissue was devised by McIlwain (178). He applied alternating electrical pulses to cerebral cortex slices, respiring in phosphate or bicarbonate medium in a specially constructed Warburg vessel fitted

with silver electrodes. He observed an increase up to 100% in the oxygen uptake by the electrically stimulated brain slices, and this stimulation further induced a variety of metabolic changes very similar to the effects of electrical stimulation of brain tissue in situ. Further studies by McIlwain and Narayanasawi (179) revealed that tissue suspensions, minces or sub-cellular particles did not respond to electrical pulses.

Roswell (180) applied McIlwain's technique for the electrical stimulation of brain slices and measured the ACh liberated from the tissue. Alternating electrical pulses for periods ranging from five to sixty minutes caused the liberation of ACh from the tissue into the surrounding medium. With eserine present in the medium, the free ACh was recovered in the medium, in non-eserinized media, little or no ACh could be recovered. In both instances, eserinized or non-eserinized medium, a fall in the bound ACh fraction was revealed after electrical stimulation of the slices.

(ii) Temperature.

The earliest demonstration of the effect of temperature on the release of ACh from nervous tissue was carried out by Cortegianni (54, 55). Heating brain tissue for three minutes at a temperature of 70°C resulted in the liberation of nearly all of the bound ACh fraction from the tissue.

Stimulation of frogs kept at elevated temperatures

through the vagus nerve resulted in the liberation of ACh which was responsible for the vagal inhibition observed during these elevated temperatures (181). The resting release of ACh from the isolated hemi-diaphragm showed a ten fold increase when the values obtained at 16°C are compared with those at 37°C (182).

Brown (183) showed that a decrease in temperature from 39 to 20°C produced a ten-fold reduction in the output of ACh from the cat's superior cervical ganglion. However, Kostial and Vouk (184) observed no effect in this temperature range, and they suggested that differences between their results and those of Brown were probably due to different frequencies of stimulation employed.

Finally, in infants, during high fever, convulsive activity can occur in which the increase in temperature might be responsible for the liberation of the bound ACh fraction.

(iii) Audiogenic Seizures.

It has been known that certain epileptics are susceptible to sound stress which has been amply exhibited by the so called "Church Bell Epilepsy" phenomenon (185). Here the vibrational frequency of church bells can produce convulsions in a number of epileptics. In addition, certain strains of mice, during infancy are susceptible to sound stress (186). Studies (187) have shown that at a range of 13,000 - 14,000 cycles/sec., a marked electrocortical discharge occurs as evidenced by the convulsive-like patterns at the onset

of the attack and a large discharge of both sympathetic and parasympathetic impulses. These seizures have been thought to be genetically controlled, while Ginsberg et. al. (188) have identified some of the neurochemical mechanisms involved. A correlation was reported between the susceptibility to seizures and the ACh level in the brain along with the capacity to bind this ACh.

Ultrasonic methods have found widespread use for the disruption of microorganisms or animal cells, which can yield extracts containing finer fragments than are obtained by other methods (189). Finally, ultrasound has been of use in the production of brain lesions and in the destruction of the labyrinth which results in the death of the cells after some delay following its application (190).

FORMULATION OF THE PROBLEM

Richter and Crossland (61) first demonstrated that the physiological state of an animal could be a reflection of the inverse proportion of its brain ACh content. Later, studies by other workers (38, 39, 48, 49) revealed that brain ACh was associated with physically discrete sub-cellular structures and several years later, Whittaker (191) put forward the suggestion that these sub-cellular particles could be used as a pharmacological tool for the investigation of the affects of drugs and toxins on the release mechanism of bioactive substances from nervous tissue.

The two findings coupled with the suggestion presented by Whittaker synthesized the problem to be investigated.

(1) Since narcotic agents were known to affect the metabolism of ACh in brain, a systematic study was carried out on the influence of representatives of various classes of narcotic agents on ACh metabolism during their incubation with brain sub-cellular particles. The bioactive substances were estimated initially by the colorimetric procedure provided by Hestrin (192) and later these results were supplanted by biological assay using the sensitized frog rectus preparation. Since no acceptable theory of narcosis exists, it was hoped that this investigation might provide some property common to all narcotic agents thus leading to an explanation of their mechanism of action.

(2) Many substances differing widely in chemical nature can produce convulsive activity when administered to an animal. Accordingly, as with the narcotic agents, experiments were carried out in which the influence of convulsant agents on ACh metabolism during the incubation with sub-cellular particles was investigated. As with the narcotics, it was hoped to obtain a single property or effect common to convulsants which might provide a clue to their in vivo mechanism of action.

(3) Several environmental conditions can produce convulsive activity in animals, and the two most prominent ones selected to be investigated were the influence (i) of increased temperature and (ii) of particular sound wave frequencies on brain sub-cellular particles, in determining what effect these treatments would have on their endogeneous ACh activity.

(4) Electroshock in animals can produce convulsions (61) although studies have shown that applied electrical pulses do not affect the metabolism of brain tissue suspensions (179). Thus rat brain cortex slices were electrically stimulated following the procedure of Roswell (180). These experiments were designed to obtain information on ACh metabolism in brain slices similar to those findings when convulsive agents or physical stimuli were applied to brain sub-cellular particles.

(5) Several other agents known to affect the metabolism of

ACh were investigated to provide further information on the influence of these agents on the endogeneous ACh metabolism of brain sub-cellular particles.

(6) The findings by Hosein et. al. (84, 88, 138) on the nature of the materials with ACh-like activity found in the brains of animals during narcosis or convulsions, prompted a study of the influence of these agents and conditions on the nature of the bioactive substances liberated or retained during their incubation with the sub-cellular particles. The nature of this material was investigated by the technique of paper chromatography as described by Banister et. al. (81) and Hosein et. al. (85). In addition some experiments on the chemical identification of the quaternary ammonium compounds present in the incubated supernatant were carried out by the technique of conversion to tetrachloroaurate derivatives and determination of their characteristic melting points.

METHODS

A. Isolation of Sub-Cellular Particles for In Vitro Studies.

In experiments to be described, black-hooded rats of either sex weighing approximately 200 to 300 grams wereob-tained either from the colonies at the Royal Victoria Hospital or the Quebec Breeding Farms, Inc. In several preliminary experiments, large white rats were employed, however, no difference in results were obtained using either strain.

Preparation of sub-cellular particles usually involved killing four rats by decapitation with a specially constructed guillotine and removing their brains within 45 seconds. Each brain was thoroughly homogenized to make a 10% suspension in cold 0.32 M sucrose containing 50 μM eserine sulphate to prevent hydrolysis of the bioactive materials. An all glass hand homogenizer was used (Ace Glass Co., Vineland, New Jersey) with a clearance of 0.025 cm.

The whole brain homogenate was centrifuged at 2,500 g for 3 minutes in the Precision Centrifuge kept cold in a refrigerator. The heavy debris containing the large blood vessels, nuclei and myelin and designated P_1, was discarded. The supernatant, S_1, was then centrifuged at 22,500 g for 20 minutes in the International Centrifuge, Model HR-I using the #1608 cups. This yielded the crude mitochondrial or P_2 fraction. The supernatant, S_2, was decanted off and the P_2 fraction was made up to 30 ml with glass distilled water (pH 7.4).

B. Incubation of Sub-Cellular Particles.

The P_2 suspension was divided into 10 aliquots of 2.5 ml and to the eight experimental samples the particular agent to be investigated was added in varying concentrations, the entire sample being made up to a total volume of 5 ml distilled water. The two remaining samples, the tissue controls, were prepared to 5 ml with distilled water only.

The samples were then incubated in a water bath at 37°C for a period of 15 minutes or in some experiments, for 30 minutes. The tubes were shaken gently once every five minutes to prevent sedimenting or clumping of the suspension. The samples were removed and the contents decanted into Spinco cellulose tubes (5/8" x 2-1/2"), placed in #40 head and centrifuged at 105,000 g force for a 30 minute period in the Spinco L Ultracentrifuge. This separated the suspension into two fractions, (i) the sedimented debris containing the vesicles and the nerve ending particles, designated the tissue residue; and (ii) the clear aqueous supernatant, designated incubation supernatant fluid.

C. Extraction of Acetylcholine from the Sub-Cellular Particles.

The method of TCA extraction of Chang and Gaddum (12), Banister et. al. (81) and of Hosein et. al. (85) was used.

The tissue residue was resuspended in 5 ml cold distilled water and, in the case of the incubation supernatant fluid, TCA was added to make a 5% TCA solution. The sample was thoroughly homogenized and the TCA treated homogenate was allowed to stand for 30 minutes at 0°C for protein precipitation. Periodic resuspension of the precipitate was made during this interval.

D. Removal of Trichloroacetic Acid from Extracts.

The precipitate formed from each tissue fraction after TCA treatment was collected by centrifuging for 5 minutes

at 2000 g, and then discarded. The clear supernatant produced was extracted (4-5 times) with 5 to 10 fold volumes of anhydrous ether to remove TCA and any lipid material. The pH of the extract usually was between 3.8 - 4.5 and was then brought to pH 6.5 - 7.0 with 0.1 N NaOH.

E. Preparation of Extracts for Chromatography.

The clear aqueous supernatants were evaporated to dryness in vacuo at 37°C. The dried extract was re-eluted with methanol and the contents mechanically shaken for five minutes. Impurities such as sucrose, nucleotides and protein remained undissolved although not all of the sucrose could be precipitated out. More prolonged shaking with either methanol or ethanol removed most of the sucrose. The methanolic or ethanolic extract was evaporated to 0.2 - 0.3 ml under a jet of air.

F. Chromatography

Paper chromatography was carried out as described by Banister et. al. (81) and Hosein et. al. (85). The concentrated extracts were carefully applied as a narrow band on a 6" x 22" sheet of Whatman #1 paper. The paper, secured between two glass plates, was placed in a trough and the entire assembly was transferred into a previously prepared chromatography tank and allowed to equilibrate for half an hour in the water-saturated butanol atmosphere. At the termination of the equilibrium period solvent consisting of

water-saturated butanol was poured into the trough as quickly as possible. The whole system was allowed to develop for 17-18 hours at room temperature (22°C). Development was considered complete when the solvent moved about 17-18 inches away from the starting line. The paper was then removed from the tank, the solvent front noted and it then was allowed to dry in air.

Individual bands with R_F values corresponding to those of the quaternary compounds were made visible by spraying with 2% iodine solution in ethanol. This resulted in the appearance of dark colored bands within a few minutes. In most cases, where the components were to be assayed on the frog rectus preparation, the material was eluted with methanol and assayed directly without the use of iodine. However, on occasion, narrow one inch strips were cut from the chromatograms and sprayed with iodine while the remainder of the paper could then be eluted and assayed as usual.

G. Elution of the Various Bands on the Chromatogram.

From previous investigations (85) and from preliminary studies carried out by the author in this laboratory, it was found that most of the ACh-like activity on developed chromatograms was recovered from two specific bands. These were the R_F 0.05 - 0.15 (ACh) and the R_F 0.45 - 0.65 (betaine-CoA esters); the latter contributing about 70 - 80% of the total ACh-like activity obtained when assayed on the sensitized frog rectus preparation, while the former band

contributed about 10 - 20% of the activity after recovery from the chromatogram. The other two regions, the starting line and R_F 0.15 - 0.45 contributed the remainder of the activity which usually amounted to less than 10% and for that purpose these bands were discarded. Therefore, from each chromatogram, two samples were obtained for assay purposes. Each zone of the paper was cut into tiny pieces and eluted by shaking in methanol for 2 hours. The methanol eluate was collected and the paper squares were shaken again in fresh methanol. The eluates were pooled and evaporated to dryness under air jets at 37°C. The dried material was then ready to be assayed.

H. Bioassay on the Frog Rectus Abdominis Preparation.

The method employed for the biological estimation of materials with ACh-like activity in the extracts was the sensitized Frog Rectus Abdominis muscle preparation. The general details have been described by MacIntosh and Perry (193), the method being that used by Richter and Crossland (61) and Whittaker et al. (48) and in general it has been found to be a very reliable method for the biological estimation of ACh.

The frog rectus abdominis muscle is divided longitudinally into two halves and the lower extremity of each half is tied to a metal hook at the base of a Feldberg leech bath, while the proximal end is tied to a Gimbal lever. The bath is then filled with

Amphibian Ringer solution, oxygenated with 95% O_2 and 5% CO_2 and the muscle suspended in this solution for a period of one hour. Generally tension of about 0.5 - 1.0 gram is applied to stretch the muscle. After one hour, the bath solution is replaced by neostigminized Ringer solution (4×10^{-6} M) and the muscle is allowed to sensitize in this solution for a further one hour period. Neostigminized Ringer is then used throughout the assay.

The following concentrations of salts were used in the preparation of the Amphibian Ringer Locke solution.

Sodium Chloride	9.0 gm
Calcium Chloride	0.24 gm
Potassium Chloride	0.42 gm
Sodium Bicarbonate	0.56 gm

These salts were made up to 1400 ml with distilled water and neutralized to pH 7.4 with 0.1 N HCl. It was found necessary to add the solids to the water; otherwise carbonates precipitated out. The pH of the solution increased above 8 on standing 24 hours or longer, hence it was necessary to prepare fresh solutions for each new assay attempted.

Acetylcholinechloride in concentrations of 10^{-6} gm/ml is prepared from standard ACh-Cl in ampoules containing 100 mg of ACh-Cl (Merck and Company). All the biological activity of the unknown materials is expressed in mμg equivalents of ACh-Cl.

Contractions were usually produced by adding

50 mμg of standard ACh-Cl solution by means of a syringe (1 ml capacity) to the bath solution, allowing 90 to 120 seconds for the resulting contraction to fully manifest itself. These contractions were elicited at 10 minute intervals until at least four consecutive contractions, more or less of equal height for the same concentration, were obtained. The preparation was then considered sensitized and ready for use.

The unknown sample was dissolved usually in 1 ml of Amphibian Ringer Locke solution and an aliquot of this solution was introduced into the bath for assay . If the contraction height exceeded the standard, the sample was further diluted or vice-versa. Each unknown aliquot was always bracketed between two ACh-Cl standards. These contractions were recorded on a kymograph moving at 2 mm/min. Upon termination of the assay, the paper was varnished with a shellac-methanol mixture in order to preserve the record. ACh-Cl equivalent of activity of the unknown samples was calculated from the recordings by the method of "bracketing".

Calculations were made from an average of 4 - 6 contractions for each unknown sample and expressed a mμg ACh-Cl/gm of fresh tissue.

I. The Modified Hestrin Procedure for the Measurement of Acyl Ester Derivatives.

This colorimetric procedure for the estimation of acyl ester compounds was developed by Hestrin (192) and later

modified by Friedman and Fraenkel (194).

The basic principle behind this method involved the formation of a ferric-hydroxyamic acid complex upon the addition of hydroxylamine to an extract in alkaline medium at either pH 8.5 or 13, which could then be estimated colorimetrically. Acetylcholine, possessing an acyl ester group, readily forms a hydroxamic acid derivative with hydroxylamine at an alkaline pH. Using ACh-Cl a standard curve was prepared and it was found that 1 μmole of ACh-Cl gave a reading of about 80 units when measured by the Klett-Summerson Colorimeter. The colorimetric method was valid between 0.1 and 2 μmoles.

In experiments to be reported, 2.5 ml of the incubation supernatant fluid were pipetted into a series of 15 ml conical centrifuge tubes. TCA was added to make a 5% solution which was allowed to stand for a 10 minute period to precipitate the protein. For the determination of the material with the acyl ester group present in the aliquots, formation of ferric-hydroxyamic acid complexes was carried out in which the required solutions were added to each aliquot in the following order:

1. 0.1 ml 28% hydroxylamine hydrochloride.
2. 0.5 ml 3N NaOH.
   Tubes shaken gently till precipitate disappears.
3. 1.0 ml 3N HCl.
4. 1.0 ml 5% $FeCl_3 \cdot 7\ H_2O$ (prepared in 0.1 N HCl).

The samples were centrifuged for 15 minutes

at 3000 g and the clear supernatant were estimated in the Klett-Summerson colorimeter using the green filter. The values were then calculated from the standard curve prepared with ACh-Cl and generally 0.3 - 0.6 μmoles of material with acyl ester linkages was found in each sample in the experiments to follow.

It was found that centrifugation of the solution after protein precipitation with TCA and then using the clear aqueous supernatant instead of the one already containing the precipitated protein produced the same results.

J. Chemical Identification of Materials with Acetycholine-like Activity.

The procedure for the preparation of the tetrachloroaurate derivatives of quaternary ammonium compounds was essentially the same as the one prescribed by Hosein et. al. (85). Initially it involved the preparation of TCA extracts from the incubation supernatant fluid, repeated extraction with ether, adjustment of the pH of the solution to between 6.5 - 7.0 and finally evaporation to dryness. From the dried eluates the tetrachloroaurate derivatives of the quaternary ammonium were prepared and their characteristic melting points were determined thus providing chemical identification of quaternary ammonium compounds liberated from the sub-cellular particles into the incubated supernatant fluid.

K. Procedure for the Electrical Stimulation of Brain Slices.

This method for the electrical stimulation of

rat brain cortex slices is similar to the one used by Roswell (180) in which he applied alternating electrical pulses to rat brain cortex slices respiring in a Locke-glucose phosphate medium (pH 7.4).

In the experiments to follow the apparatus used for the electrical stimulation of brain slices consisted of two platinum mesh screens mounted parallel to each other (1/8th inch apart) on a flat lucite plate. Wires were soldered to each screen which served as either anode or cathode when attached to a physiological stimulator. The tissue slices were layered carefully across both electrodes so that electrical pulses passed through the tissue slices when the current was applied.

Two rats were decapitated and brain cortex slices were prepared with a Stadie-Riggs microtome in which one outer top slice was cut from each cortex half. Each slice was rapidly weighed on a torsion balance and then one slice from each cortex was homogenized in cold TCA and served as a tissue control. The other two top slices were layered on the platinum grid and the entire apparatus was immersed in a 30 ml beaker containing 5 ml (an amount of solution to cover slices without causing them to float off the grid) of Locke-phosphate glucose medium (pH 7.4) oxygenated with 95% O_2 and 5% CO_2 at 37°C. A 10 minute equilibration period was allowed for temperature and gas equilibration of the vessel before the current was turned on. The slices were stimulated (15 volts, 100 pulses/sec.)

for periods of time varying from 5 to 60 minutes. After stimulation was completed, the slices were removed from the grid and immediately dropped into cold 5% TCA and homogenized. The grid was carefully washed and the washings and incubation medium were pooled together and similarly prepared as a 5% TCA solution. Extraction of materials with ACh-like activity present in both fractions was carried out by the methods described above.

CHAPTER I

PRELIMINARY EXPERIMENTS.

A. Homogenization

In the isolation of sub-cellular particles from brain homogenates, the initial stage of homogenization of the excised brain was particularly important for the extraction of ACh-like materials. If homogenization was too severe, most of the material with ACh-like activity was recovered in the S_2 (supernatant) fraction. Alternatively, poorly homogenized tissue resulted in a large amount of the ACh-like activity sedimenting at 600 g in the P_1 fraction. In experiments which follow, it was found that 30 excursions of the hand homogenizer resulted in the release of a consistent amount of substances with ACh-like activity in the P_2 fractions; of the order of that obtained by Hosein and Proulx (84). Since the clearance of the glass homogenizer became wider by constant erosion with use, it was necessary to replace the homogenizers at regular intervals about every two or three months of use.

B. Chromatography of P_2 Fractions.

With extracts treated with TCA and ether to remove protein and lipid material respectively, the application of this clear supernatant to the chromatograms was not difficult. However, non-TCA treated supernatant extracts contained much sucrose which, on application caked as a hard crust, preventing the development of the

chromatogram. Precautions were therefore taken to remove the sucrose in the sample before the extract was spotted on the chromatogram. The sucrose was precipitated out by repeated extraction with 100% ethanol and the clear ethanolic extract, after evaporation to a volume of 0.2-0.3 ml, was applied as a line (3 - 4 mm wide) on a 6" wide chromatogram strip.

C. Recovery of ACh-Cl After Chromatography in the Butanol-Water System.

Experiments carried out in our laboratory have shown that the R_F of crystalline ACh-Cl is 0.13 in the butanol-water system. To determine the amount of ACh recovered after chromatography, ACh-Cl in varying amounts was applied to chromatogram strips, run in butanol-water and a band with R_F 0.05-0.15 was cut, eluted and assayed on the frog rectus preparation for ACh activity.

Table I

Recovery of ACh from a chromatogram after chromatography in the butanol-water system, and bioassay on the frog rectus prepration.

μg ACh-Cl applied on chromatogram.	Amount recovered	% recovery
1.0	0.94	94
5.0	4.67	93
10.0	8.96	90

From these results, one can observe a recovery of about 90-95% of the ACh-Cl applied, in the band with R_F 0.05-0.15 on the chromatogram.

D. The Influence of Trichloroacetic Acid on the Mobility of ACh in the Butanol-Water Chromatography System.

The possibility that TCA accelerated the mobility of ACh-Cl was investigated since the results of the quantitative determination of ACh-like activity on the various bands of the chromatograms largely depended on adequate separation of the compounds. In particular, it was important to make certain that tissue ACh did not contaminate the band with R_F 0.5-0.7 normally occupied by the betaine-CoA esters. Experiments were performed in which 5 μgm ACh-Cl samples were prepared in 5% TCA, then extracted several times with ether, neutralized to pH 6.5, evaporated to a small volume and chromatographed. Table II illustrates the distribution of ACh-Cl, recovered from the various bands of the chromatogram.

Table II

Effect of Trichloroacetic Acid on the Mobility of ACh in a Butanol-Water Chromatographic System.

	μgm ACh-Cl Activity (Frog Rectus Assay)	
R_F 0.00 - 0.05 (Starting line)	0.05 - 0.15 (ACh band)	0.15 - 0.40
0.4	3.45	0.42
0.45	3.65	0.47
0.38	3.37	0.46

About 70% of the activity was recovered in the ACh band, while the rest of this activity (20%) was equally distributed over the two other bands of the chromatogram. No ACh activity was recovered beyond R_F 0.40.

Experiments in our laboratory in which reineckate salts prepared from ACh and then added to brain extracts, was found to be recovered in the band with R_F 0.05 - 0.15.

E. Reliability of the Bio-Assay Technique.

The frog rectus abdominis preparation is an accepted standard test object for the bio-assay of ACh and ACh-like substances (193). Using the technique of "bracketing", errors of not greater than 5% were obtained. The estimation of bioactive materials in part depended on the sensitivity to ACh of each particular preparation and therefore only muscles which responded to 30-80 mμgm ACh-Cl were used. In the instance that extracts contained interfering materials, these substances were added in equal quantities to the standard solution of ACh which was then assayed. They thus served as standards for assay of the extracts.

F. Ultra-Violet Spectrophotometric Analysis of the Material in the Band With R_F 0.5 - 0.70.

Since adenine-containing compounds have absorption peaks at 260 mμ, it would appear that betaine-CoA esters should also possess this property. The presence of the betaine-CoA esters in the eluate of the band with R_F 0.5-0.7 was therefore checked by ultra-violet spectrophotometric analysis. Their presence was indicated by

absorption peaks at 260 mμ and at 232 mμ which is characteristic of thiol ester linkages.

G. Chemical Identification of Material with ACh-like Activity Released from Incubated P_2 Fractions.

Attempts were made to determine the melting points of the tetrachloroaurate derivatives of the quaternary ammonium compounds extracted from P_2 fractions. The melting points of pure compounds are sharp and are characteristic for individual compounds and hence suitable for identification purposes.

Table III

Melting Point of Various Tetrachloroaurate Derivatives of Quaternary Ammonium Compounds.

Tetrachloroaurate Derivatives	Melting Point °C
ACh	169
PCh	250-255
BuCh	85-90
Choline	230
Carnitine	148-152
Acetyl-dl-carnitine	120-124
GBB	180-182
Crotonbetaine	208-211

To identify the nature of the materials released during incubation of the sub-cellular particles, a P_2 fraction was prepared from 20 rat brains. The tissue was made up in a fine suspension in glass distilled water, incubated at 37°C for a thirty minute period and the clear supernatant fluid after centrifugation was evaporated to

dryness. The particle debris was treated with 5% TCA, centrifuged and then extracted with ether to remove lipid and TCA. Both fractions were extracted with 100% ethanol to remove excess sucrose. Tetrachloroaurate derivatives of the quaternary ammonium compounds were prepared from the dried eluates. However, residual sucrose interfered with the crystal formation and difficulty was encountered in the determination of the melting points. On heating, the crystals blackened considerably. With the supernatant fraction, the melting point of only one compound was observed, namely, acetyl-l-carnitine. The tetrachloroaurate derivatives prepared from the tissue debris appeared as a slurry and displayed no distinct melting points.

H. The Use of the Hestrin Colorimetric Method for the Determination of ACh-Like Materials Released from the Sub-Cellular Particles.

The Hestrin method was used primarily to provide supplementary information about the release of ACh-like materials from the sub-cellular particles. In the instances when bio-assay could not be applied because of interfering materials in the supernatant fluid, this method served as an alternative for the detection of ACh-like substances released from the particles into the incubation medium. The rapidity with which results were obtained with this method made it advantageous in that results were obtained in several hours, while a comparable experiment with bioactive material assayed on the frog rectus preparation could take several days for completion.

The information obtained from this method

paralleled that observed with the bio-assay when the identical experiments were carried out and aliquots were subjected to these two methods of estimation.

An example of these parallel results can be seen in both figures 2 and 3 in which magnesium and bromide ions each gave similar patterns when the ACh-like activity was estimated by either method.

Samples containing the agents to be investigated were treated with Hestrin reagents to control any interference with the colorimetric estimation. A positive reaction resulted in the preparation of standards containing amounts of this material and these were employed as appropriate blanks for the particular concentrations used in the experiment. These samples were prepared for each experiment since it was found that the reagent blanks varied from one experiment to another.

The differences in colorimetric readings on the tissue blanks in each experimental run, varied from one experiment to another. Several factors were responsible for these results. First, it depended on the amount of tissue used in each experiment. Since it was not always possible to obtain rats of uniform weight, the amount of sub-cellular particles isolated from brain tissue was variable. Secondly, the actual rate of centrifugation varied slightly from one experiment to another because it was not always possible to use the particular centrifuge

because of constant use by others in our laboratory. Nevertheless, from the number of experiments performed, the same pattern of activity for any given experiment was consistently reproducible.

I. Release and Synthesis of Materials with ACh-like Activity from Sub-cellular Particles.

A number of experiments were designed to determine whether the amount of material with ACh-like activity recovered in the supernatant fluid after centrifugation of the particles was due to its release or alternatively to synthesis and release. A P_2 fraction prepared from 2 brains for each experiment was suspended in 20 ml of distilled water. The suspension was divided into two parts, one of which was extracted with 5% TCA and ether for release and estimation of total ACh-like activity, while the other half was incubated at 37°C for 15 minutes, centrifuged at 105,000 g, extracted with TCA and ether as above and the ACh-like activity of both the incubation supernatant fluid and tissue residue was estimated on the frog rectus preparation. Results are shown on Table IV.

Table IV

The Effect of Incubation of Sub-cellular Particles in Distilled Water on The Release of Material with ACh-like Activity.

mμgm ACh-Cl activity / P_2 fraction of brain tissue.				
Total (Control)	Tissue Residue	Incubation Supernatant Fluid	Total (Incubation)	% Change
1995	1108	1060	2168	6
1756	878	953	1831	6
1850	984	1116	2000	7.5
1944	955	1142	2097	6
1658	864	972	1836	9
1766	818	1064	1882	6

These results illustrate that most of the ACh-like activity in the supernatant was contributed by the liberation of materials with ACh-like activity although it appears that a low but negligible rate of synthesis may occur.

This synthesis occurred despite the anaerobic and hypotonic conditions in which the particles were subjected. It would appear, however, that the material with ACh-like activity recovered in the supernatant fluid was mainly liberated from the particles.

Similarly, the effect of incubation on the release or synthesis of ACh-like materials was measured by the Hestrin colorimetric method. As recorded in Table III, the results similar to those obtained with the bio-assay method were found.

Table V

The Effect of Incubating Sub-cellular Particles on the Liberation of Hestrin Positive Materials.

Activity expressed as % of control			
Controls	Incubated		Incubated
Total Activity	Supernatant	Debris	Total %
100	64	44	108
	62	43	105
	58	45	103

As shown with the bio-assay, there was an increase in material in the supernatant fluid of the order of 3 to 8%.

This experiment also provided more evidence for the parallelism between results obtained from both colorimetric and bio-assay estimations.

J. Chromatographic Distribution of Material from P_2 Fractions with ACh-like Activity.

The distribution of the ACh-like materials before and after incubation at 37°C was the next step in determining the chemical nature of the materials released or retained within the sub-cellular particles. Trichloroacetic acid extracts of normal and incubated sub-cellular particles were chromatographed and developed in a butanol-water system for 16 - 18 hours.

The results of these experiments are shown in Table VI.

From these results it would appear that most (about 80%) of the ACh-like activity was contributed by the betaine-CoA esters. The activity contributed by ACh was 10% while the pooled PCh and BuCh provided the remaining 10%.

In the incubated fraction, very little ACh, PCh and BuCh was found in the residual tissue debris. Experiments performed by other workers in our laboratory have shown that choline esters provide a small fraction of about 10% of the total ACh-like activity found in nervous tissue after extraction with TCA.

These results have revealed that incubation of sub-cellular particles isolated from brain tissue led to the release of

Table VI

Chromatographic Distribution of Substances with ACh-like Activity From Normal and Incubated Sub-Cellular Particles.

	mμgm ACh-Cl activity/P_2 fraction of brain tissue.			
	ACh R_F 0.05-0.15	PCh and BuCh R_F 0.15-0.30	Betaine-CoA Esters R_F 0.45-0.65	Total Activity
Control	256	208	1682	1890
	208	---	1484	1692
	214	164	1541	1919
	287	---	1486	1773
Incubated-Supernatant Fluid	142	---	744	886
	186	126	808	1034
	164	---	872	1036
Incubated-Tissue Residue	42	---	639	681
	43	44	718	762
	48	---	723	761

materials with ACh-like activity into the incubated supernatant fluid.

Since this liberation of materials occurred in distilled water without the influence of any agents, the next logical step in this investigation was to determine what effect the addition of various pharmacological agents to these sub-cellular particles would have on the release or retention of these materials with ACh-like activity. Since other workers (61) have shown that the acetylcholine content of the brain may be a reflection of the physiological state of the animal, it was of interest to determine what affects narcotic and convulsant stimulating drugs may have on the metabolism of these substances within the particles.

CHAPTER 2

EXPERIMENTAL

Effect of Incubation of Narcotic Agents on the Release of Materials with Acetylcholine-like Activity from Brain Sub-Cellular Particles.

(i) Magnesium and Bromide.

Magnesium and Bromide ions, employed as magnesium chloride and sodium bromide, were the first two agents which possessed narcotic properties to be investigated in this work. Essentially, a P_2 fraction isolated from 4 rat brains was prepared as a suspension in 30 ml of glass distilled water. From this suspension 10 aliquots each of 2.5 ml were prepared and $MgCl_2$ or NaBr, in varying concentrations were added to the experimental samples to a final volume of 5 ml. Eight samples served as experimentals, while the other two containing only the P_2 suspensions served as controls. With the magnesium chloride, a stock solution of 1 mg % (107 mM) was prepared and progressively diluted as required. Sodium bromide solutions were prepared by diluting a 20 mM stock solution.

After an incubation period of 15 minutes at 37°C, the particle suspension was centrifuged at 105,000 g for 30 minutes to sediment the sub-cellular particles. From each tube, 2.5 ml of the supernatant was treated with the Hestrin reagents (see p. 63) to estimate colorimetrically the amount of carboxyl ester liberated from the particles

into the incubation medium. The results from these experiments can be seen in Tables VII and VIII. Since the amounts of liberated carboxyl esters estimated colorimetrically are very small (changes occurring in fractions of a μmole), for comparison purposes it was more convenient to express these results as per cent change of the experimental over that of the control.

The Hestrin method provided results which could be obtained from each experimental run in several hours. These experiments were repeated from three to six times until a reproducible pattern was obtained. This allowed a prediction of the effect at the particular concentration during the incubation of the sub-cellular particles with the various agents. The same experiment was then repeated in which the supernatant obtained from each sample was assayed for ACh-like activity with the frog rectus preparation.

The results obtained from these experiments are reported in Table VIII and display a pattern similar to that observed from samples analyzed colorimetrically for Hestrin positive materials as can be seen in Figures 2 and 3.

Table VII

The Effect of Magnesium Chloride and Sodium Bromide on the Release of Hestrin Positive Materials from Sub-Cellular Particles Incubated at 37°C for 15 Minutes.

mM $MgCl_2$	Number of Experiments	% of Control	mM NaBr	Number of Experiments	% of Control
0.01	3	95	1.0	4	92
0.10	5	91	5.0	4	83
1.07	6	92	10.0	4	65
10.7	6	84	20.0	3	58
107	6	73			

Table VIII

The Effect of Magnesium Chloride and Sodium Bromide on the Release of Materials with ACh-like Activity from Sub-Cellular Particles Incubated at 37°C for 15 Minutes.

mM $MgCl_2$	ACh-Cl activity mμgm/sample	% of Control	mM NaBr	ACh-cl activity mμgm/sample	% of Control
Control	504	----	Control	352	----
0.01	418	83	1.0	332	94
0.10	395	79	5.0	310	88
1.00	428	86	10.0	284	80
10.7	365	65	20.0	238	65
107.0	225	46			

Figure 2

EFFECT OF MAGNESIUM IONS ON THE LIBERATION OF MATERIAL WITH ACETYLCHOLINE- LIKE ACTIVITY FROM BRAIN SUBCELLULAR PARTICLES.

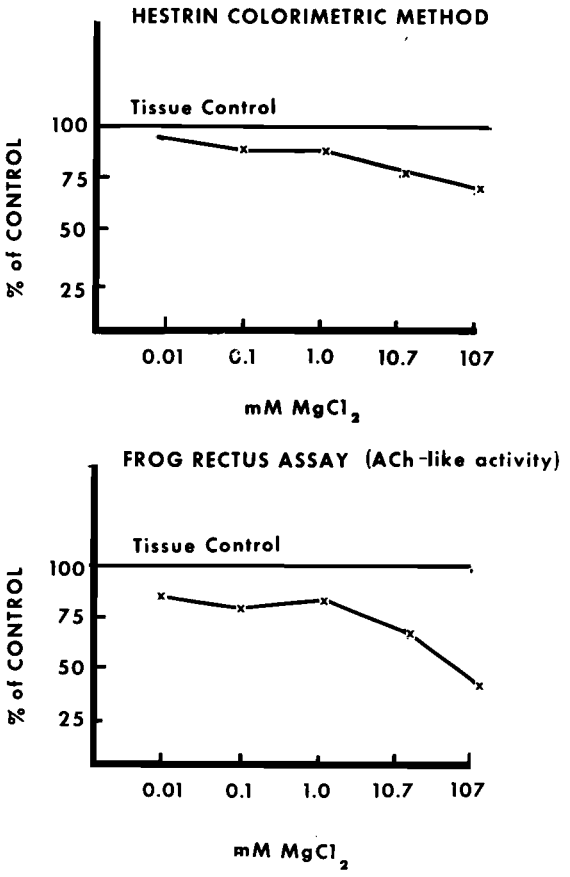
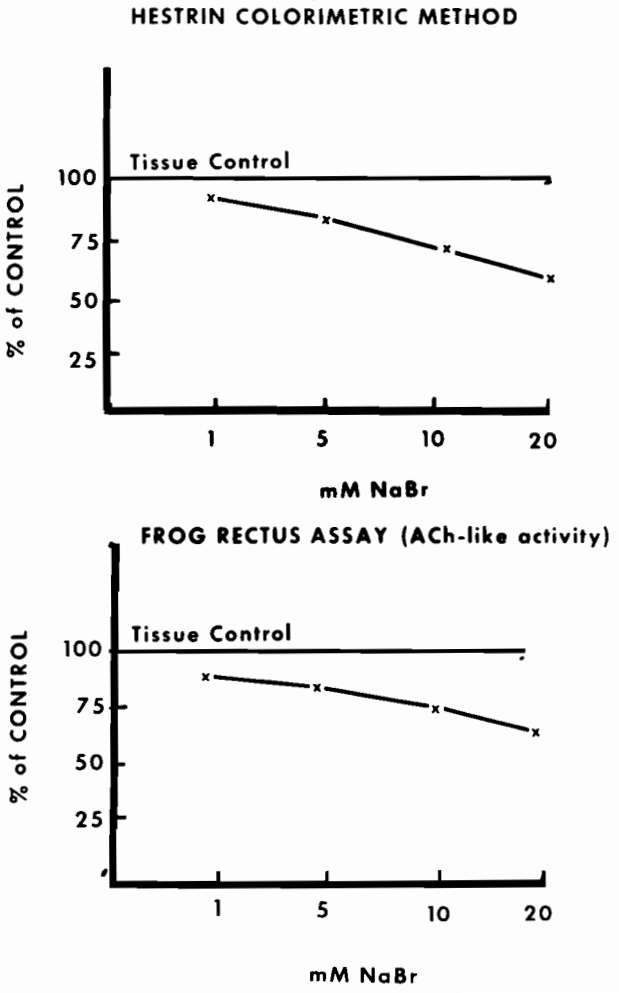


Figure 3

EFFECT OF BROMIDES ON THE LIBERATION OF MATERIAL WITH ACETYLCHOLINE- LIKE ACTIVITY FROM BRAIN SUB-PARTICLES.



From these results, it can be observed that both magnesium and bromide ions prevent the release of materials with ACh-like activity from within the sub-cellular particles into the incubation medium.

Considering magnesium first, the physiological concentration of magnesium in body fluids is 2.3 mM and, from the results drawn above, this concentration will still inhibit the liberation of ACh-like substances from sub-cellular particles. This failure to release these materials would appear to occur at both low and high magnesium concentrations.

These findings concerning magnesium are similar to those discussed by del Castillo and Katz (41, 43) who found that the application of high concentrations of magnesium ions to the motor end plate decreased the quantal release of ACh liberated during stimulation. Results by Hutter and Kostial (24) have revealed that perfusion with Locke solution high in magnesium (10 - 15 mM) resulted in a 50% fall in the amount of ACh liberated by the superior cervical ganglion upon stimulation of its pre-ganglionic nerve ending.

The concentration of bromides used ranged from 1 - 20 mM, the upper limit appearing rather high. However, the effective concentration in producing state of narcosis appears to be within that range (137).

(ii) Pentobarbital

The procedure employed was identical to that used to investigate the effect of magnesium and bromide ions on the sub-cellular particles. However, in these experiments a P_2 fraction was prepared from 3 brains instead of 4 because fewer samples were required in each experiment for this investigation. The particles were suspended in 21 ml distilled water and 8 aliquots of 2.5 ml were prepared, 6 of these being incubated with pentobarbital in concentrations ranging from 0.1 to 1.0 mM.

The supernatant fluid in the first set of experiments was analyzed with the Hestrin colorimetric procedure with the hope of establishing a definite pattern. Later it was supplanted by bioassay for ACh-like activity in the supernatant fluid after incubation.

The results from these colorimetric experiments are seen in Table IX, the bioassay studies in Table X.

Table IX

Effect of Na-Pentobarbital on the Release of Hestrin Positive Substances from Sub-Cellular Particles Incubated for 15 Minutes at 37°C.

mM Na-Pentobarbital	Number of Expts.	% of Control
Control	4	100
0.1	4	90
1.0	4	78
5.0	4	73
10.0	3	72
15.0	4	48

Table X

Effect of Na-Pentobarbital on the Release of Substances with ACh-like Activity from Sub-Cellular Particles Incubated for 15 Minutes at 37°C.

mM Na-Pentobarbital	Amount of ACh-Cl Activity released into supernatant mμgm ACh-Cl/sample.			% of Control.
	1	2	3	
Control	350	359	330	100
0.1	307	334	319	92
1.0	256	298	304	83
10.0	243	248	252	73

Thus, it can be seen from these two sets of results in Tables IX and X that, like magnesium and bromine, pentobarbital also reduced the release of substances with ACh-like activity from the sub-cellular particles when incubated in its presence in distilled water.

Although some workers have found that the average brain concentration of pentobarbital in cats under anesthesia is 0.2 mM (137), it is altogether possible that a localized concentration occurs in some target area where the narcotic concentration is much higher and this might account for the high levels of materials with ACh-like activity found in brain during pentobarbital narcosis.

During the extraction procedure with TCA

and ether, most of the pentobarbital was precipitated out of solution and the amount remaining had no effect on either the frog rectus preparation or the Hestrin reaction.

The reduction of "free" materials with ACh-like activity supports the results of other investigators who showed that pentobarbital anesthesia depressed the brain content of "free" ACh while simultaneously it elevated the so-called "bound" fraction.

Results obtained by other workers in our laboratory have confirmed the increase in materials with ACh-like activity in the bound fraction of brain during pentobarbital narcosis. Further investigations showed that the increase in activity was due entirely to an increased content of betaine-CoA esters, more specifically to an increase of acetyl-l-carnitine-CoA.

In an attempt to extend these investigations further, extracts prepared from sub-cellular particles after incubation with varying concentrations of sodium pentobarbital were chromatographed and developed in the butanol-water system. The bands were cut, eluted by shaking with methanol, evaporated to dryness and assayed for ACh-like activity with the frog rectus abdominis preparation. The results can be seen in Table XI.

Table XI

The Effect of Na-Pentobarbital on the Distribution of Materials with ACh-Like Activity from Sub-Cellular Particles.

Each value represents an average of three experiments and is expressed in mμgm ACh-Cl/gm equivalent of P_2 brain tissue assayed on the frog rectus preparation.

	Choline Esters R_F 0.05 - 0.25		Betaine-CoA Esters R_F 0.45 - 0.65	
mM Pento-barbital.	Incubation Supernatant	Tissue Residue	Incubation Supernatant	Tissue Residue
Control	128	60	841	435
0.1	125	64	712	485
1.0	62	60	685	655
10.0	64	60	525	750

From these results quoted, it can be seen that the increase in the ACh-like activity of the betaine-CoA esters during the incubation with pentobarbital contributed the principal increase in ACh-like activity recovered in the particle debris. Similarly, a concommitant decrease in the free ACh-like material in the supernatant was due almost entirely to the betaine-CoA esters; ACh itself contributing somewhat to this decrease of active material released.

(iii) Ether.

Ether is a representative of a group of narcotic agents known as the volatile anesthetics and experiments were

carried out to determine the effect of this agent on the release of materials with ACh-like activity from these sub-cellular particles. Ether is soluble in water to the extent of 12% by weight. Accordingly, ether saturated water was diluted from 1 to 20 to 1 to 100 resulting in 0.12 to 0.60 mg % solutions. These diluted ether solutions were incubated with sub-cellular particles placed in tubes and sealed with parafilm at 37°C for 15 minutes. Total ACh-like activity was estimated in both supernatant and tissue residue, after incubation with ether. The results are illustrated in Table XII and are an average of three experiments.

Table XII

Effect of Ether on the Liberation of ACh-like Substances from Brain Sub-Cellular Particles Incubated at 37°C for 15 minutes.

Activity is expressed as mμgm ACh-like Activity/P_2 fraction of brain tissue assayed on the frog rectus preparation.		
% Concentration Ether in Water.	Incubation Supernatant	Tissue Residue
Control	945	456
0.12	826	634
0.24	878	610
0.60	1035	354

The amount of ACh-like activity increased about 30% in the particles during incubation with low concentrations of

ether. As the ether concentration was increased, the ACh-like content in the particles decreased while a concommitant increase was found in the supernatant. This observation is valid because ether extraction is a common method for the extraction of "bound" material with ACh-like activity from nervous tissue.

Other work in our laboratory has demonstrated that, like pentobarbital, ether can increase the betaine-CoA ester content of narcotized brain (139). To illustrate these findings more clearly, chromatographic separation of extracts after incubation with various concentrations of ether was undertaken in an effort to show the distribution of ACh-like materials. Results from these experiments can be found on Table XIII. Each figure represents an average of three trials.

TABLE XIII

Effect of Ether on the Distribution of Materials with ACh-like Activity from Sub-Cellular Particles.

Activity expressed as mμgm ACh-like activity/P_2 fraction of brain tissue assayed on the frog rectus abdominis preparation.				
	Choline Esters R_F 0.05 - 0.25		Betaine Esters R_F 0.45 - 0.65	
mg% Ether in water.	Incubation Supernatant	Tissue Residue	Incubation Supernatant	Tissue Residue
Control	84	40	542	356
0.12	84	46	466	510
0.24	68	44	512	482
0.60	88	--	624	454

From these results it would appear that most of the fall in ACh-like activity within the particles was due to a decreased liberation of the betaine-CoA esters and the retention of these substances within the particles themselves.

(iv) Ethanol.

Ethanol acts by producing depression of the central nervous system, and in high enough concentrations can produce narcosis. Consequently, ethanol was incubated with particles and ACh-like activity was determined after incubation. As described above for the other narcotic agents studied, chromatographic separation in butanol-water of the incubation supernatants and tissue residue was performed for the distribution of the various substances with ACh-like activity. These results can been seen in Tables XIV and XV.

Table XIV

Effect of Ethanol on the Liberation of Materials with ACh-like Activity from Sub-Cellular Particles Incubated at 37°C for 15 Minutes.

Activity expressed as mμgm ACh-Cl activity per P_2 fraction of brain tissue as assayed on the frog rectus preparation.		
mM Ethanol	Incubation Supernatant	Tissue Residue
Control	938	560
21	754	768
42	140	1417
84	358	1225

Table XV

Chromatographic Distribution of Material with ACh-like Activity After Incubation with Ethanol at 37°C for 15 Minutes.

Activity expressed as mμgm ACh-Cl activity per P_2 fraction of brain tissue as assayed on the frog rectus preparation.				
mM Ethanol	Choline Esters R_F 0.05 - 0.25		Betaine-CoA Esters R_F 0.45 - 0.65	
	Supernatant	Debris	Supernatant	Debris
Control	142	42	855	544
21	140	46	727	626
42	134	--	820	606
84	142	--	984	452

Like ether, ethanol inhibits the release of material with ACh-like activity from the particles into the incubation medium with consequent retention of this material within the particles themselves. A concentration of 21 mM ethanol corresponds to 200 mg % which is about the concentration of ethanol in the blood of individuals under complete alcoholic intoxication. High (84 mM) concentrations, liberate the ACh-like material from the particles and this may explain why ethanol extraction has been employed in the extraction of ACh-like substances from nervous tissue.

(v) Nitrous Oxide.

Nitrous Oxide (N_2O) is representative of a class of inert gases capable of producing a state of narcosis in animals. The gas was prepared by heating ammonium nitrite, and the N_2O so liberated, was collected by bubbling it into cold distilled water. Nitrous

oxide is soluble in water at that temperature in the ratio of 35:1. In the present experiment, this H_2O:N_2O mixture was diluted 10 times and incubated with sub-cellular particles at 37°C for 15 minutes. The amount of materials with ACh-like activity released in the incubation medium was assayed on the frog rectus preparation and is illustrated in Table XVI.

Table XVI

The Effect of Nitrous Oxide on the Release of ACh-like Substances from Sub-Cellular Particles Incubated at 37°C for 15 Minutes.

Activity expressed as mμgm ACh-Cl activity per P_2 fraction of brain tissue as assayed on the frog rectus preparation, and represents an average of three experiments.		
Concentration N_2O/H_2O (v/v)	Incubation Supernatant	Tissue Residue
Control	774	332
35:1	765	442
3.5:1	654	482

From the results quoted in Table XVI, it can be seen that saturation of water with nitrous oxide resulted in the retention of materials with ACh-like activity within the particles.

In the experiments described above, six different types of narcotic agents were studied, a cation, an anion, a representative of the barbiturates, a volatile anesthetic, an aliphatic alcohol, and finally an inert gas.

All the substances used in this investigation can in vivo produce a state of narcosis. From the results demonstrated in this Chapter, it has been revealed that they are all capable of depressing the liberation of substances with ACh-like activity from brain sub-cellular particles incubated at 37°C. Furthermore, several of these narcotic agents investigated showed that they lead to an accumulation of active substances within the particles and results from chromatography experiments have shown that the increased ACh-like activity in the "bound" fraction was attributed to the betaine-CoA esters.

CHAPTER 3

Effect of Incubation of Convulsant Agents on the Content of Materials With Acetylcholine-like Activity in Sub-Cellular Particles.

Since all the narcotic agents investigated produced a similar *in vitro* effect, it was thought that convulsant drugs or chemical agents when incubated with the particles might have a property common to all the convulsant agents. Consequently, a representative group of convulsant drugs differing in chemical nature was selected and their effect on the release of material with ACh-like activity in brain sub-cellular particles was investigated.

(i) Picrotoxin.

The experimental procedure was similar to that on p. 79 employed in studying the effects of magnesium and bromides on the release of materials with ACh-like activity from brain sub-cellular particles. A P_2 fraction was prepared from four brains, divided into 10 aliquots, two of which served as controls while the remaining 8 were incubated with various concentrations of picrotoxin for 15 minutes at 37°C and the supernatant fluids were assayed for total ACh-like activity. Because picrotoxin interfered with the Hestrin reaction, the method was abandoned after a few trials. Results on the bioassay of materials with ACh-like activity after incubation can be seen in Table XVII.

Table XVII

The Effect of Picrotoxin on the Release on Material with ACh-like Activity from Sub-Cellular Particles Incubated at 37°C.

mM Picrotoxin	mμg ACh-Cl activity liberated in each sample. Frog rectus assay	% of Control
Control	277	100
1.0	248	89
0.5	245	88
0.1	304	110
0.01	402	144

Each value expressed in Table XVII represents an average of three experimental runs.

As the concentration of picrotoxin in the incubation fluid diminished the amount of ACh-like substances liberated from the particles increased. The chromatographic distribution of the materials with ACh-like activity in the presence of 0.01 mM Picrotoxin was next investigated and the results obtained are presented in Table XVIII.

Table XVIII

The Distribution of Materials with ACh-like Activity During Incubation of 0.01 mM Picrotoxin in a Suspension of Sub-Cellular Particles at 37°C and Chromatographed in a Butanol-Water System.

Activity expressed as mμgm ACh-Cl Activity/P_2 fraction of brain tissue assayed on the frog rectus preparation.				
	Choline Esters R_F 0.05 - 0.25		Betaine-CoA Esters R_F 0.45 - 0.65	
mM Picrotoxin	Incubation Supernatant	Tissue Residue	Incubation Supernatant	Tissue Residue
Control	88	84	732	676
0.01	108	74	968	484

The increase in ACh-like activity was mainly attributed to changes in the Betaine-CoA ester content. The amount liberated into the incubation fluid increased. This is a reflection of the diminution of these substances within the particles themselves.

(ii) Strychnine.

Although this agent is a spinal convulsant, it also was incubated with sub-cellular particles. Since it did not interfere with the Hestrin reaction, experiments were carried out in which varying concentrations of strychnine from 1 to 100 μmoles were incubated with sub-cellular particles. Results appear to indicate that both materials with Hestrin positive and ACh-like activity were liberated as indicated in Tables XIX and XX. The pattern was reproducible from

both Hestrin and Bioassay methods of estimations as shown in Figure 4.

Table XIX

Effect of Strychnine on the Liberation of Hestrin Positive Materials in the Supernatant Fluid after Incubation with Sub-Cellular Particles at 37° C

μmoles Strychnine	Number of Experiments	% of Control
Control	4	100
1	3	100
10	3	108
50	3	111
100	3	119

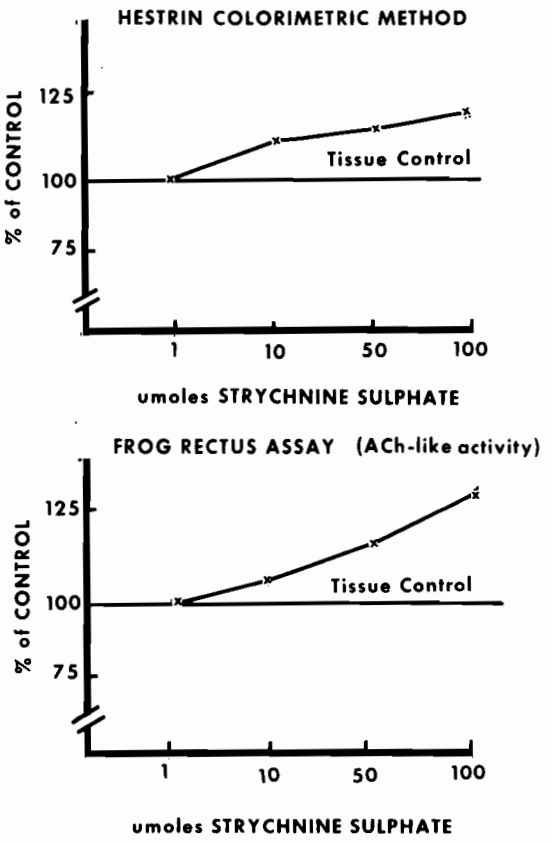
Table XX

Effect of Strychnine on the Liberation of Materials with ACh-like Activity into the Supernatant Fluid from Sub-Cellular Particles Incubated at 37°C

μmoles Strychnine	Equivalent ACh-Cl liberated. Frog rectus assay	% of Change
Control	366	100
10	376	103
50	415	114
100	456	133

Figure 4

EFFECT OF STRYCHNINE ON THE LIBERATION OF MATERIALS WITH ACETYLCHOLINE- LIKE ACTIVITY FROM BRAIN SUB-CELLULAR PARTICLES.



Results in Table XIX represent averages of values from three experiments. From these results, it can be observed that although strychnine is a spinal convulsant, nevertheless, upon incubation with sub-cellular particles isolated from brain, μmolar amounts cause the liberation of material with ACh-like activity into the incubation medium.

(iii) Metrazol.

Although the administration of metrazol to an animal can produce violent convulsions, the addition of this agent in vitro either to respiring brain slices or to brain homogenates does not alter the oxidative metabolism and glycolysis at pharmacological concentrations. Since those investigations could not demonstrate any action for metrazol in vitro in our work, this drug was incubated with sub-cellular particles in the same manner described for picrotoxin, and the ACh-like content was estimated. Six trials were attempted on both Hestrin and biological assay methods of estimation. No changes between the experimental and the control were observed in the ACh-like content or in the Hestrin positive material released in the surrounding medium.

Studies (191) on the metabolism of metrazol have demonstrated its conversion to a derivative (conjugation with a glucuronide) in the liver and it may be that this compound or some other derivative is the active substance which produces the convulsive activity in vivo.

(iv) Camphor.

This convulsant agent is lipid soluble and, for our purposes, it first had to be dissolved in an oil. A solution of camphor in olive oil was prepared and aliquots of this were added to suspensions of sub-cellular particles.

Preliminary results revealed that, although olive oil interfered with both Hestrin and bioassay determinations, the former method (highly coloured blank) could nevertheless be used. Experiments similar to the procedure for picrotoxin were performed in which the material was estimated by the Hestrin procedure. Results from these experiments are recorded in Table XXI.

Table XXI

Effect of Camphor on the Liberation of Hestrin Positive Materials into the Supernatant Fluid after Incubation with Sub-Cellular Particles at 37°C.

mg % Camphor	Number of Trials	% of Control
Control	4	----
0.10	3	109
0.25	3	113
0.50	3	119
0.75	4	126

In addition to the tissue control, camphor concentrations were prepared with samples containing identical amounts of camphor in olive oil and this served as control samples for that particular incubated concentration.

It appears that camphor, like picrotoxin and strychnine, promotes the liberation of material with ACh-like activity from the sub-cellular particle. Since it proved impossible to employ the bioassay technique, the Hestrin method of analysis showed that similar pattern of release was observed as that described earlier for strychnine. These results for camphor can be seen in Table XIX.

(v) d-Tubocurarine and Decamethonium.

Although d-Tubocurarine and decamethonium (D_{10}) are neuromuscular blocking agents, their injection into the brains of animals has produced convulsive activity (123). The experiments to be described were carried out using minute quantities of these drugs which were incubated with sub-cellular particles. Because these two agents block the action of ACh on the frog rectus preparation, the Hestrin colorimetric method of analysis was required for this experiment.

Table XXII

The Effect of d-Tubocurarine and Decamethonium on the Liberation of Hestrin Positive Materials During the Incubation of Sub-Cellular Particles at 37°C for 15 Minutes.

d-Tubocurarine used $\mu g/P_2$ fraction	% of Control	Decamethonium μmoles/sample	% of Control
Control	100	Control	100
3	208	1	110
30	206	10	122
60	206	100	135
75	206		

It can be seen that both these neuromuscular blocking agents, when incubated with nerve ending particles have the property of liberating materials which give a positive Hestrin reaction. From our previous studies these results (see p. 98) might be correlated with an increase in ACh-like activity.

(vi) Semi-Carbazide.

Semi-carbazide was originally believed to produce its pharmacological action as an inhibitor of the enzyme, glutamine synthetase. This inhibitory action prevented the formation of gamma-amino-butyric acid (GABA), the inhibitory substance which results in an overbalance of excitatory impulses. However, more recent investigations (196) have disproved these earlier findings. In line

with our studies on the other drugs of varying chemical classification, sub-cellular particles from brain were incubated in the presence of semi-carbazide. After incubation, the ACh-like activity of the incubation medium was estimated. Results from this study are shown in Table XXIII.

Table XXIII

Effect of Semi-Carbazide on the Liberation of Materials with ACh-like Activity into the Supernatant Fluid from Sub-Cellular Particles Incubated at 37°C.

All values are expressed as mμg ACh-Cl activity per P_2 fraction as assayed on the frog rectus muscle. Results are an average of three experiments.

mM Semi-carbazide	Tissue Residue	Incubation Supernatant
Control	644	710
0.1	638	705
1.0	602	784
5.0	573	792
10.0	506	874

From these results, it can be seen that the liberation of materials with ACh-like activity is facilitated at a concentration of about 1 mM semi-carbazide and increases progressively, although at a high concentration of 10 mM, the amount liberated is only about 20% above that of the control sample. It would appear, therefore, that very large amounts of semi-carbazide are necessary for the

liberation of these ACh-like bioactive substances from within the sub-cellular particles.

(vii) Calcium.

Although calcium has never been employed as a convulsant agent, intraventricular infusion of high concentrations of this cation into brain has resulted in convulsive activity (197). The procedure for the investigation of the effect of calcium on the release of material with ACh-like activity from brain sub-cellular particles was similar to those studies described earlier for magnesium and bromide ions. The results obtained are recorded in Tables XXIV and XXV.

Table XXIV

Effect of Calcium Ions on the Liberation of Materials with Hestrin Positive Activity into Supernatant Fluid from Sub-Cellular Particles Incubated at 37°C.

mM $CaCl_2$	Number of Experiments	% of Control
Control	6	100
0.009	4	100
0.09	4	96
9.09	6	116
90.9	6	76

TABLE XXV

Effect of Calcium Ions on the Liberation of Materials with ACh-like Activity into the Supernatant Fluid from Sub-Cellular Particles at 37°C.

All values are expressed as mμg ACh-Cl activity per sample as assayed on the frog rectus preparation. Results are an average of the experiments.

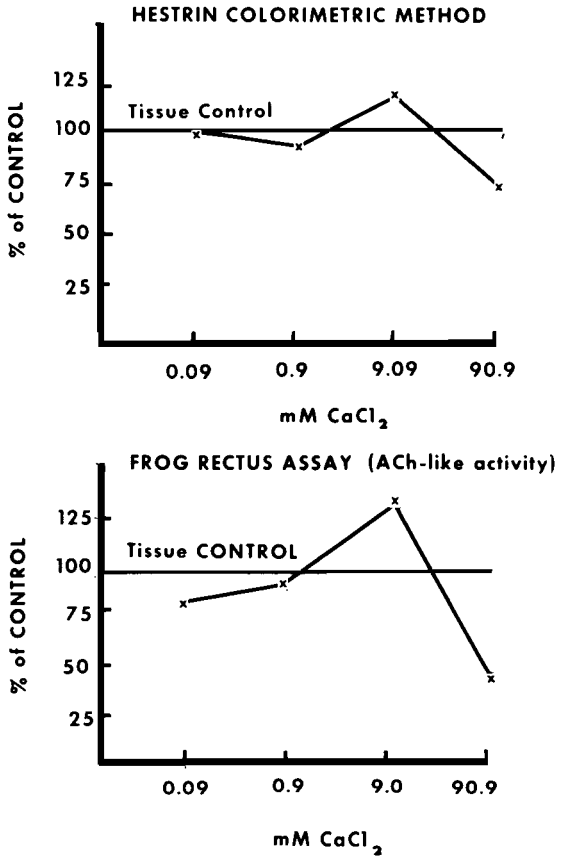
mM $CaCl_2$	mμg ACh-Cl activity per sample	% of Control
Control	251	----
0.09	181	73
0.90	210	84
9.09	322	128
90.9	124	49

These results reveal the influence of ionized calcium on the liberation of materials with ACh-like activity from sub-cellular particles. It causes an increase of the free material appearing in the incubation medium. These findings again illustrate a definite parallel pattern between the two methods of estimation used in these studies as can be seen from Figure 5.

Since the physiological intracellular concentration of calcium is 1.3 mM, a rise to 10 mM results in a greater liberation of materials with ACh-like activity. Above this concentration, there occurs a retention of these substances in the particles. Similarly, at calcium concentrations lower than physiological, the same phenomenon occurs.

Figure 5

EFFECT OF CALCIUM IONS ON THE LIBERATION OF MATERIALS WITH ACETYLCHOLINE- LIKE ACTIVITY FROM BRAIN SUB-CELLULAR PARTICLES.



Previous investigators have shown that increased calcium ion concentrations can promote the liberation of ACh from nervous tissue such as the motor nerve terminals (44) and the superior cervical ganglion (24). The amount of calcium employed by these workers to liberate ACh were within the range used in the present set of experiments. Alternatively, a fall in the concentration of ionized calcium is compatible with tetany, in which spontaneous discharges of the neurons occur.

A survey of the type of convulsive agents employed in this study reveals substances differing widely in chemical nature. These agents are all capable of producing convulsions in animals. If we associate this phenomenon with the findings of Richter and Crossland (61) that the functional state of an animal is inversely proportional to the total brain ACh content, then the fall in total brain ACh-like activity observed by others during convulsions can be reflected in the low residual ACh-like activity found in the sub-cellular particles subsequent to their incubation with all but one of the convulsant drugs studied above.

CHAPTER 4

The Effect of Several Physical States on the Acetylcholine-like Activity of Sub-Cellular Particles.

There are several physical or environmental states which can produce convulsive activity. Since all the convulsive pharmacological agents tested above possessed the common property of lowering the ACh-like content of the sub-cellular particles and increasing it in the supernatant fluid, the results suggested that physical conditions which elicit convulsions in vivo might produce the same effects when sub-cellular particles were subjected to these conditions.

(i) Temperature.

In these experiments, brain sub-cellular particles were incubated at various temperatures ranging from $0^{o}C$ to $43^{o}C$ and the materials with ACh-like activity liberated into the surrounding medium was estimated with the frog rectus preparation. Aliquots of suspensions of sub-cellular particles were incubated for 30 minutes instead of the usual 15 minutes because a more concise picture could be obtained with a longer incubation time.

Sub-cellular particles were prepared from 4 rat brains and made up to a total volume of 102 ml and then divided into 10 aliquots of 10 ml. In each case duplicate samples were incubated at the same temperature. After incubation, centrifugation and evaporation to dryness, each sample was made up to 1 ml with Locke solution

and assayed for ACh-like activity with the frog rectus preparation.

The results from these experiments can be seen in Table XXVI. As can be observed, there is an increased release of material with ACh-like activity steadily from 0°C to 43°C. At this final temperature, a tremendous liberation of material with ACh-like activity occurred in some experiments.

Such findings illustrated in Table XXVI and Figure 6, can be interpreted along with the data of other investigators who observed that heating brain extracts to 70°C for a period of three minutes resulted in the liberation of the total ACh activity from the tissue (54).

To confirm these findings further, these experiments were repeated and the materials liberated were estimated by the Hestrin colorimetric procedure. The results illustrated in Table XXVII represent an average of three experiments and clearly demonstrate the influence of temperature in elevating the amount of carboxyl derivatives released into the supernatant fluid. When chromatographic separation was performed on the material released into the incubation fluid, it was observed that increasing temperature caused an elevation in the amount of material with ACh-like activity released with a concomitant fall in that present in the tissue residue. See Table XXVIII.

Figure 6

EFFECT OF TEMPERATURE ON THE LIBERATION OF MATERIALS WITH ACH-LIKE ACTIVITY FROM SUB-CELLULAR PARTICLES.

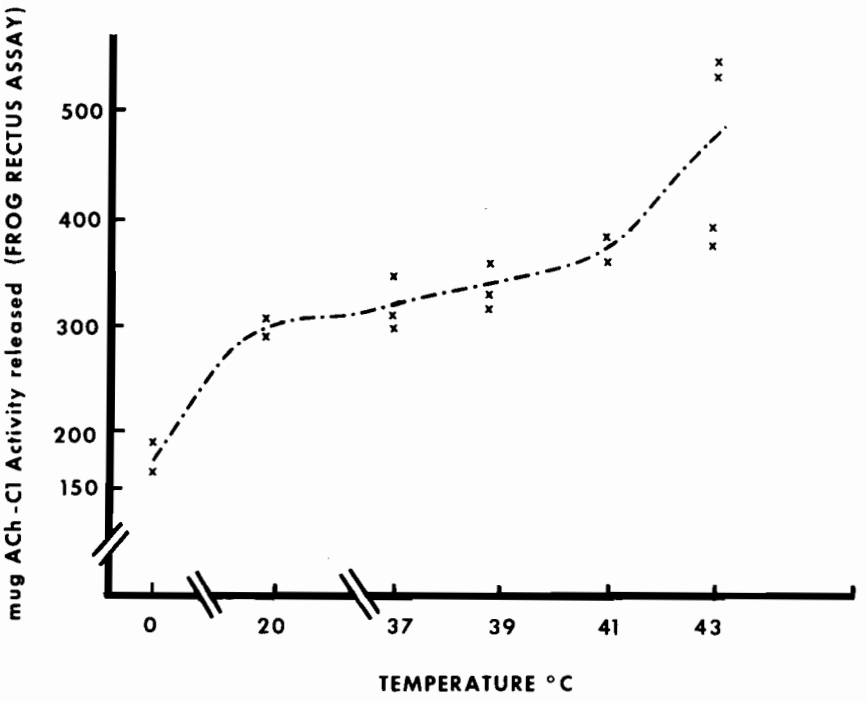


Table XXVI

The Effect of Temperature Change on the Liberation of Material with ACh-like Activity from Incubated Brain Sub-Cellular Particles.

Temperature °C	Number of Experiments	mμgm ACh-Cl Activity/Sample (frog rectus assay)
0	2	160
20	2	305
37	3	314
39	3	340
41	2	374
43	4	455

Table XXVII

Effect of Temperature Change on the Liberation of Hestrin Positive Substances from Sub-Cellular Particles

Temperature °C	Number of Experiments	% increase over that at 0°C
0 (in ice)	4	--
20	4	14
37	6	25
39	6	27
41	6	32
43	4	36

The bioactive substances liberated into the surrounding medium, along with the TCA extractable materials remaining within the particles, were chromatographed in the butanol-water system and the distribution of choline esters and betaine-CoA esters was determined.

TABLE XXVIII

Influence of Temperature Changes on the Distribution of Material with ACh-like Activity Chromatographed in the Butanol-Water System.

	mμg ACh-Cl Activity/P_2 fraction (frog rectus assay)				
	R_F	37° C		43° C	
Incubation Supernatant	0.05 - 0.15	120		140	
	0.15 - 0.30	55		80	
	0.45 - 0.65	254		565	
	sub-total		429		785
Tissue Residue	0.05 - 0.15	125		140	
	0.15 - 0.30				
	0.30 - 0.65	996		668	
	sub-total		1121		808
Total ACh-Cl activity/P_2 fraction			1550		1593

(ii) Sonic Stimulation.

In these experiments, sub-cellular particles were subjected to sonic stimulation from a sonic oscillator (Ultrasonic power disintegrator). A P_2 fraction was prepared from 5 rat

brains suspended in 50 ml of distilled water, and divided into 5 aliquots. Four samples were then separately placed in specially designed heavy-walled glass cups in an ice-bath, placed under the oscillator and sonicated at a frequency of 18,000-20,000 cycles per second for periods ranging from 15 seconds to 3 minutes. One sample receiving no stimulation served as control. After sonication was completed the suspension was centrifuged at 105,000 g for 30 minutes and the ACh-like activity of the supernatant was estimated with the frog rectus preparation. It was interesting to note that sonication for 2 minutes or longer disrupted all the particles such that on ultracentrifugation all that appeared was the clear supernatant.

The results of these experiments are illustrated in Table XXVII. They indicate the tremendous effect of sonication as a means of disrupting small structures within the cell. Because of this disruption, the amount of ACh-like activity recovered in the surrounding medium after a three minute sonication period was about 90-100% of the amount of ACh-like material normally found in a P_2 fraction. When this material was chromatographed in a butanol-water system however, all of the material was recovered in the ACh band (R_F 0.05 - 0.15). It appeared that sonication broke sub-cellular particles, but did not disrupt all the bondings and therefore it appeared that large complex molecules were being chromatographed, and these ran to R_F 0.1 band of the chromatogram. TCA breaks this complex down further and

this TCA released material can be recovered in the R_F 0.45 - 0.65.

TABLE XXIX

The Effect of Auditory Stimulation on the Liberation of Materials With ACh-like Activity from Sub-Cellular Particles, at a Frequency of 18,000 - 20,000 Cycles per Second.

Stimulation Time (Sec.)	Number of Experiments	mμg ACh-Cl Activity/ P_2 fraction. Frog rectus assay
0 (control)	3	634
15	3	924
30	2	1103
45	2	1420
60	3	1444
120	1	1582
180	1	1564

It has been demonstrated by other investigators (188) that auditory stimulation can produce convulsive activity in a special strain of mice and in certain groups of human epileptics. The ringing of church bells can trigger an epileptic seizure because of the particular sonic frequency originating from the bells. This seizure may be due to a particular sound frequency bursting the (probably defective) sub-cellular particles, releasing the ACh-like materials and thereby precipitating the convulsive activity.

CHAPTER 5

Other Studies on Sub-Cellular Particles

(i) Sodium and Potassium

Since the investigations with magnesium and calcium produced definite results when these cations each were incubated separately with sub-cellular particles, the next logical step was to study Na^+ and K^+ ions in the same system. Consequently, each of these cations was incubated with sub-cellular particles in the identical manner as were magnesium and calcium. Incubation was carried out at 37°C for 15 minutes. The difficulty with these experiments was that they involved the use of the Hestrin colorimetric method only because these cations, in high concentrations, interfered with the bioassay preparation.

The results from these experiments can be seen in Table XXX and an illustration of the type of pattern observed on incubation of the sub-cellular particles with these two cations can be seen in Figure 7.

These results indicate that at physiological concentrations, sodium ions promote the release of Hestrin positive material from the particles. At the same concentration range, potassium ions depresses the liberation of these materials.

Figure 7

THE EFFECT OF SODIUM AND POTASSIUM IONS ON THE LIBERATION OF MATERIALS WITH ACETYLCHOLINE-LIKE ACTIVITY FROM BRAIN SUB-CELLULAR PARTICLES.

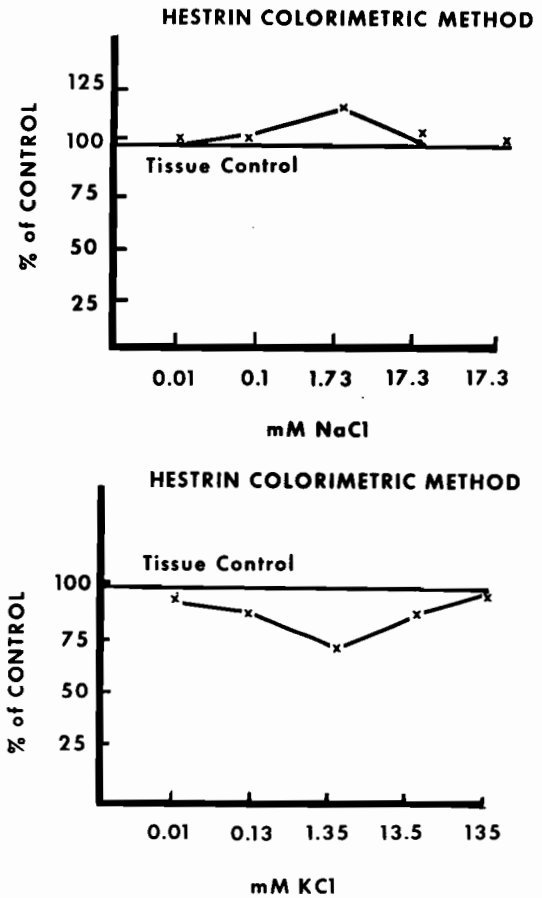


Table XXX

The Effect of Sodium and Potassium Ions on the Liberation of Hestrin Positive Reacting Substances from Brain Sub-Cellular Particles.

mM NaCl	Number of Experiments	% of Control	mM KCl	Number of Experiments	% of Control
Control	6	100	Control	6	100
0.1	4	100	0.01	4	98
0.17	6	103	0.13	4	94
1.73	6	114	1.35	6	74
17.3	6	100	13.50	6	96
173.0	4	100	135.0	6	100

If we can correlate the parallelism of the Hestrin method with the bioassay results from all the previous studies carried out, then within the same concentration range sodium ions would be expected to promote the release of materials with ACh-like activity, while KCl depresses it.

Although 135 mM KCl produced no effect on the liberation of ACh-like substances from the particles, the addition of 1.3 mM $CaCl_2$ increased the liberation of material 30% above that of the tissue control. Other studies have shown that, with the ganglia perfused with KCl, the addition of calcium ions markedly increased the rate of liberation of ACh (23).

Since sub-cellular particles had been incubated with each of the four major cations present in intra- and extracellular fluids, it was decided to prepare a Krebs' Ringer medium and then by varying its composition, study the effect of incubating sub-cellular particles within this medium.

Results from these experiments can be seen in Table XXXI.

Table XXXI

Effect of Varying the Composition of Cations in Krebs'-Ringer-Bicarbonate Medium on the Content of Hestrin Positive Reacting Substances Liberated from Sub-Cellular Particles Suspended in this Medium.

Medium Krebs'-Ringer Bicarbonate (KRB	Number of Experiments	% of Control
KRB (control)	6	100
KRB + 10 x Mg^{*+}	3	88
KRB + 1/10 K^+, 10 x Mg^{++}	3	85
KRB + 1/10 K^+, 10 x Ca^{++}	3	120
KRB + 10 x Ca^{++}	3	114
KRB + 10 x K^+, 10 x Ca^{++}	2	116
KRB + 10 x K^+	2	105

*1/10 or 10 refers to the additional use of 1/10 or 10 times the physiological concentration in Krebs'.

Krebs' medium was prepared minus the particular cation investigated which was then added as 1/10th or 10 times the physiological concentration. In some experiments the composition of two cations in the Krebs' solution was varied by addition of particular amounts of the various salts to be investigated.

These results appear to indicate that, in a Krebs'-Ringer medium, alteration of the composition of the various cations can selectively produce changes in the amount of Hestrin positive reacting substances released into the incubation medium containing sub-cellular particles.

From previous knowledge regarding parallel findings between Hestrin and bioassayed results, similar changes in materials with ACh-like activity liberated or retained within the particles can be assumed to take place. Bioassay of these samples was not attempted because of the high salt content.

(ii) Neostigmine

Neostigmine, the anti-cholinesterase drug, was incubated in the presence of sub-cellular particles and the amount of material with ACh-like activity liberated from the particles into the supernatant fluid in the presence of various concentrations of neostigmine bromide was estimated on the frog rectus muscle preparation. The experimental procedure involved homogenization of 6 rat brains in cold 0.32 M sucrose and then division of the resultant homogenate into six

aliquots. Neostigmine bromide was added to 5 tubes to give final concentrations in the suspension from 0.1 to 1.0 mM. Then a P_2 fraction was isolated from each aliquot and diluted to 10 ml with distilled water. The sub-cellular particle suspensions were incubated without the addition of any other anticholinesterase agents for a 15 minute period at 37°C , then centrifuged at 100,000 g for 30 minutes and the ACh-like activity was estimated from each supernatant on the frog rectus preparation. One sample contained 0.4 mM eserine sulphate which prevented any hydrolysis of the ACh-like substances and thus served as a control.

The results from these experiments can be seen in Table XXXII and represent an average of three trials.

From these findings, it is seen that this pharmacological agent has a dual action on nervous tissue, firstly as a reversible anticholinesterase agent and secondly, promoting the liberation of materials with ACh-like activity from sub-cellular particles within the tissue. The increase in ACh-like activity in the supernatant was not due entirely to any increased anticholinesterase activity because incubating the particles with 0.4 mM eserine sulphate (a concentration which affords maximal protection) resulted in the recovery of less ACh-like activity in the supernatant fluid than that found upon incubation with high amounts of neostigmine.

Table XXXII

Effect of Neostigmine Bromide on the Liberation of Material with ACh-like Activity from Sub-Cellular Particles.

μM Neostigmine Bromide	Frog Rectus Assay mM ACh-like Activity/ P_2 fraction
Control	355
100	308
200	367
400	380
600	410
1000	464

The neostigmine in the extract did not sensitize the frog rectus muscle because samples were assayed against standard ACh-Cl containing varying amounts of neostigmine bromide. With standard ACh samples containing neostigmine, no difference in the heights of contraction of muscles was observed in these samples as compared to those samples containing equal amounts of ACh-Cl alone.

(ii) Cobra Venom (Naja Naja)

Heated cobra venom was initially prepared to a concentration of 300 μg/ml in distilled water and then boiled for 15 minutes at 100°C as specified by Braganca (198). This solution when cooled and diluted was ready for use. Various amounts ranging from

3 to 300 μg/ml of venom suspension were incubated with sub-cellular particles at 37°C for 15 minutes. Results from these incubation studies appear in Table XXXIII.

Table XXXIII

Effect of Heated Cobra Venom on the ACh-like Content of Sub-Cellular Particles Incubated at 37°C for 15 Minutes.

Activity expressed as mμg ACh-Cl activity/P_2 fraction and represents an average of three experiments.		
	Frog Rectus Assay	
μg/ml Cobra Venom	Incubation Supernatant	Tissue Residue
Control	768	954
3	895	684
30	1244	454
300	1346	355

Table XXXIV

Effect of Heated Cobra Venom on the Distribution of ACh-like Materials from Sub-Cellular Particles Chromatographed and Developed in the Butanol-Water System

Activity expressed as mμg ACh-Cl activity/P_2 fraction and represents an average of three experiments.				
μg/ml Cobra venom	R_F 0.05 - 0.25 Choline Esters		R_F 0.45 - 0.65 Betaine-CoA Esters	
	Incubation Supernatant	Tissue Residue	Incubation Supernatant	Tissue Residue
Control	35	34	285	648
30	40	33	394	584
300	44	38	562	432

These results reveal the marked influence of cobra venom to liberate materials with ACh-like activity from sub-cellular particles. This increase in free activity was due almost entirely to a liberation of the betaine-CoA esters on the basis of chromatographic analysis.

(iv) Acetylcholine.

Acetylcholine (ACh) was incubated with brain sub-cellular particles in order to observe the influence of this substance itself on these particles. A P_2 suspension from 5 rat brains was divided into 5 aliquots of 10 ml each and to 4 of these samples, 10 mμg to 1 μg of ACh-Cl was added. The samples were incubated at 37°C for 15 minutes, centrifuged at 105,000 g for 30 minutes and the supernatant fluid was assayed for total ACh-like activity on the frog rectus preparation. The results are presented in Table XXXV.

Table XXXV

Influence of ACh-Cl on the Content of Materials with ACh-like Activity in Brain Sub-Cellular Particles Incubated at 37°C for 15 Minutes.

mμg ACh-Cl added	mμg recovery ACh-Cl	mμg ACh-like Activity/P_2 fraction. Frog Rectus Assay
Control	----	655
10	----	659
100	----	740
200	----	782
500	472	896
1000	954	1216

In column #2 of Table XXXV, results are presented in which 500 and 1000 mμg ACh-Cl were incubated in distilled water and then subjected to the same conditions as was the experimental samples. The theoretical ACh-Cl activity if no synthesis or destruction of the active materials takes place, should include the endogeneous ACh-like activity plus the added ACh-Cl. Since the recovery of added ACh-Cl was quite high (95%), it is unlikely that the fall in total ACh-like activity observed was due to an increased hydrolysis of the esters. It appears that concentrations of 200 mμg ACh-Cl in our samples began to block the release of materials with ACh-like activity. From our previous studies, this could be equated with a state of narcosis in vivo. This would support the findings of Feldberg and Sherwood (123) that intraventricular injection of ACh into the unanesthetized cat led to a condition or state of "catatonia".

CHAPTER 6

(i) Electrical Stimulation of Rat Brain Cortex Slices.

Since experiments have illustrated that electrical stimulation has no effect on the metabolism of sub-cellular particles, minces or homogenates (79), it was decided to study the effect of electrical pulses on the ACh metabolism in rat brain cortex slices. As described under Methods, (p. 65) these slices were stimulated electrically for varying periods of time ranging from 5 to 60 minutes and both the amounts of materials with ACh-like activity in the tissue and in the suspension medium were estimated with the frog rectus preparation. Since the prime motive of these experiments was to determine the effect of alternating electrical pulses on the liberation of the ACh-like bioactive substances from the brain slice, no eserine sulphate was added to this incubation medium thus preventing any large scale synthesis of bioactive substances.

The first phase of this study involved the determination of the normal content of materials with ACh-like activity in rat brain cortex slices. The results from these investigations are shown in Table XXXVI. The values quoted in this table are activities equivalent to mμg ACh-Cl per gram of brain tissue (wet weight).

From these results, it can be seen that about 80% of the ACh-like activity is contributed by the betaine-CoA ester

fraction. The propionyl- and butyrylcholine ester bands were not estimated because their content was usually very low.

Table XXXVI

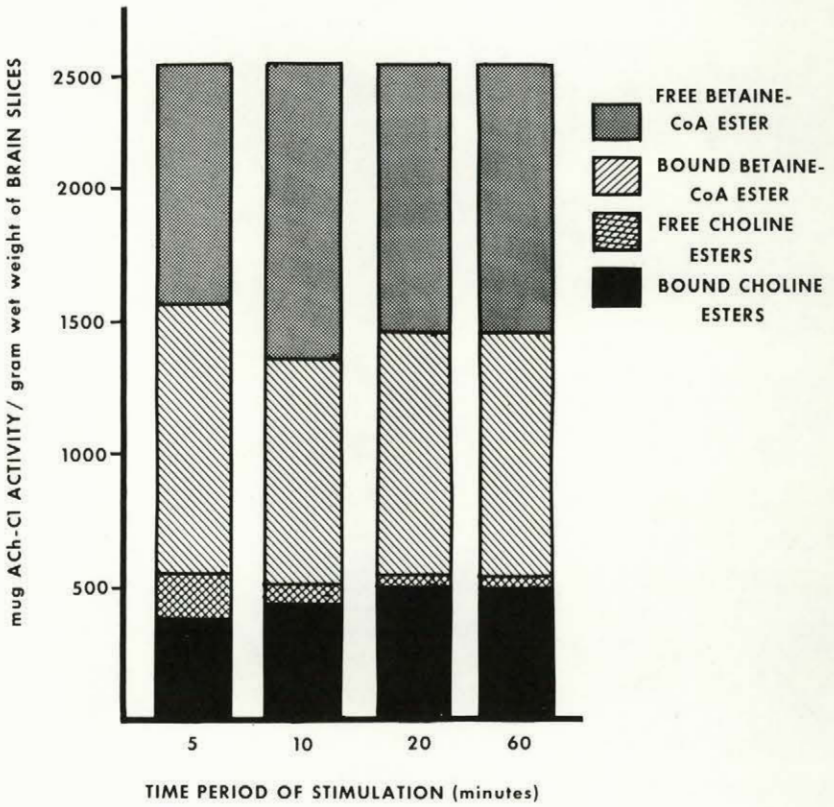
Distribution of Materials with ACh-like Activity from Normal Rat Brain Slices Developed Chromatographically in the Butanol-Water System.

	Frog Rectus Assay	
ACh band R_F 0.05 - 0.15	Betaine-CoA Ester band R_F 0.45 - 0.65	Total Biological Activity
345	2225	2570
360	2160	2520
334	1935	2269
455	2410	2865
520	2355	2875
482	2110	2592

The distribution of the material with ACh-like activity in rat brain cortex slices during electrical stimulation is summarized in Figure 8. From this illustration, it would appear that the betaine-CoA ester fraction is liberated to the extent of 40% of the total ACh-like activity contained in the tissue slice at the height of stimulation. After a five minute period of electrical stimulation there is a 30% increase in free material in the suspension medium, increasing approximately to 40% in a ten minute stimulation period. With further electrical stimulation, no change occurs in the amount of betaine-CoA esters appearing in the suspension medium.

Figure 8

THE INFLUENCE OF ELECTRICAL STIMULATION ON THE LIBERATION OF MATERIALS WITH ACh-LIKE ACTIVITY FROM RAT BRAIN CORTEX SLICES.



In the ACh fraction, which normally contributes about 20% of the total biological activity, a five minute stimulation period provides an increase of free ACh to a value of 20%. Stimulation for longer periods results in a small increase of both free and bound material recovered from the R_F 0.1 band of the chromatogram, because it would appear that resynthesis begins to keep pace with the release.

Finally, these results indicate that it is the betaine-CoA ester content which is the rapidly changing component of the ACh-like activity during electrical stimulation of brain tissue. The ACh content itself appears to change slightly, but accounts only for small changes during electrical stimulation.

DISCUSSION

The underlying ideas which led to the initiation of this project was derived from several sources of information. The first idea was developed by Richter and Crossland (61) that nervous activity was dependent on and associated with the concentration of ACh in brain. These authors conceived the theory that the ACh level *in vivo* should vary with the physiological activity of the brain. Therefore, any external factor or influence which resulted in a depression of activity in the animal, might be expected to decrease the liberation and destruction of bound ACh and consequently allow the reserve of bound ACh to reach a maximal high level. As a result they concluded that the bound ACh should be maximal during a reduced state of activity of the brain such as that induced by sleep, anesthesia or narcosis. While in a state of maximal activity caused by the over-stimulating influence of convulsants or electroshock, the ACh content should be lower than normal.

Secondly, an initial suggestion by de Robertis and Bennett (40) that ACh might be held in vesicles or sub-cellular particles visible under the electron microscope led Whittaker (48) to isolate these particles from brain homogenates using the technique of differential density gradient centrifugation in sucrose. These experiments revealed that most of the ACh activity was recovered in a fraction

which was mainly mitochondrial in nature and which also contained the synaptic vesicles. Further investigations by Whittaker (48) and other workers illustrated the presence of other substances with implied neurohumor activity which were obtained in highest yield in this fraction e.g. nor-adrenaline (199), ATP (200), 5-hydroxytryptamine (48),(201). Whittaker had suggested (191) that these sub-cellular particles isolated from brain homogenates could provide a useful preparation for the investigation of the mechanism of transmitter release, and for the study of the mode of action of drugs and toxins on nerve endings.

Thirdly, evidence has been obtained by Hosein et al. (85) using the parallel techniques of paper chromatography and chemical identification, that the transmitter substances within the central nervous system belong to a group of substances, namely ACh, PCh, and BuCh and the CoA esters of GBB, crotonbetaine, l-carnitine and acetyl-l-carnitine. These workers illustrated that the choline esters contributed about 20% of the total ACh-Cl activity extractable from brain whereas the betaine-CoA esters provided the remainder of the activity.

Thus, against the background of these previous three developments, the project was undertaken. The aim was to investigate the possible effects of the incubation of narcotic and convulsant drugs, and conditions conducive to the liberation or retention of materials with ACh-like activity from sub-cellular particles isolated from brain

homogenates. In this way it was hoped to define more clearly the relationship between the pharmacological actions of these drugs and the changes in the level of materials with ACh-like activity in brain.

In the preliminary experiments, sub-cellular particles were incubated with glass distilled water and the substances liberated into the incubation supernatant were assayed on the frog rectus preparation. The data from these experiments illustrated that incubation for a period of 15 minutes at 37°C led to the liberation of 50-55% of the total activity of the particles into the incubation supernatant fluid. These data do not agree with those of Whittaker in which the incubation of sub-cellular particles in distilled water for a period of one hour caused total liberation of material assayed as ACh into the incubation medium. However, these results of Whittaker also do not agree with those of Hosein and Proulx (88) who incubated sub-cellular particles under the same conditions as did Whittaker, but found only 55% of the total material with ACh-like activity in the incubation supernatant. Hosein and Proulx (88) described three forms of bound active substances within the particles, (i) material with ACh-like activity which remains in the P_2 fraction after water treatment (designated firmly bound ACh-like substances), (ii) another material with ACh-like activity which is released by water, and is pharmacologically active, and (iii) a third form which is released by water, but is pharmacologically inactive. In our experiments it would appear that the 15 minute incubation experiments

liberated all of the material with ACh-like activity described as type (ii). The bioactive materials from the incubation supernatant and tissue residue were subjected to paper chromatography and developed in the butanol-water system. The materials from the bands were assayed for ACh-like activity on the frog rectus preparation. The results illustrated that about 80% of the total ACh-like activity was contributed by the betaine-CoA ester fraction, while the choline esters furnished about 15 - 20% of the total ACh-Cl activity. These findings confirmed the results obtained by Hosein and Proulx (88) in experiments involving the incubation of sub-cellular particles in distilled water medium. Furthermore, chemical analysis of the active material liberated from the particles illustrated that the activity was due to the presence of acetyl-l-carnitine. Since the incubation of sub-cellular particles from brain homogenates in distilled water led to the liberation of material with ACh-like activity into the incubation medium, the question arose whether various agents or drugs could increase the removal from or retention of materials with ACh-like activity within sub-cellular particles. Clues of such possibilities had been provided by Whittaker (48) when he illustrated that various substances added to sub-cellular particles suspended in cold distilled water and incubated for periods from 5 to 60 minutes, produced a liberation of 2 - 100% ACh above that of the control value.

Since Richter and Crossland (61) showed that narcotics increased the content of material with ACh-like activity in the

brains of animals, the influence of these substances on the level of materials with ACh-like activity produced by their incubation with sub-cellular particles was investigated. The anesthetic agents were selected because of their widely varying chemical natures and because it was desirous to see if a property common to all narcotic agents could be found, thus providing a possible answer to the pharmacological actions of a large number of chemical compounds. From the experimental data provided in Chapter 2, it appeared that these compounds, at their pharmacological concentrations, inhibited the release of materials with ACh-like activity from within the particles.

In considering magnesium, the first narcotic agent investigated in our work, an extensive literature has been devoted to its property of preventing the liberation of ACh from nervous tissue (44). Accordingly, is it not possible for this cation to act primarily by preventing the liberation of materials with ACh-like activity from sub-cellular particles from nervous tissue? del Castillo and Katz (41, 42, 43) have presented evidence that this cation, when applied in high concentrations, decreased the quantal release of ACh from the motor nerve terminal. Similarly, the addition of high concentrations, 10 - 15 mM $MgCl_2$ to Locke perfusion medium prevented the liberation of ACh from the perfused cervical sympathetic ganglion of the cat. On the other hand, bromides, whose mechanism of pharmacologic action has remained obscure, like magnesium, can prevent the liberation of material with ACh-like activity

when incubated with sub-cellular particles. It is therefore possible to assume that their mechanisms of action are similar although no work, to the knowledge of this investigator, has been described on the effect of bromides on ACh metabolism. Nevertheless, this substance can prevent the liberation of bioactive materials from brain sub-cellular particles thus providing a clue to its mechanism of action.

The prevention of the liberation of materials with ACh-like activity from sub-cellular particles by pentobarbital resulted in a reapproval of the results obtained by earlier investigators. The depression of ACh synthesis in brain slices by pentobarbital was illustrated by McLennan and Elliott (93) and Johnson and Quastel (94). In part, this depression of synthesis may actually be a failure to liberate the bound materials with ACh-like activity from the particles into the incubation medium.

Tobias, Lipton and Lepinat (90) were the first group of workers to find that the total ACh content of rat brain is increased during pentobarbital anesthesia. These observations are in accord with those of Richter and Crossland (61) and were later extended by Elliott et al. (35). The results have also been supported by Crossland and Merrick (91) and also by Wajda (202). It is probable that the increased material with total ACh-like activity was also due to the failure to liberate the bioactive materials from the sub-cellular particles within this tissue. Crossland (203) found that, during anesthesia, ACh is present in some form which is not easily releasible by nervous stimulation.

He further showed that during the induction of anesthesia an accumulation of ACh did not occur; it did occur during a later phase in the anesthesia. Our data illustrates that as the narcotic concentrations of the samples were increased, the amount of material with ACh-like activity within the tissue residue increased. However, there are exceptions to these rules. In particular, the two aliphatic organic compounds, ether and ethanol, when incubated at concentrations above those necessary to produce narcosis, produced a liberation of active materials into the incubation supernatant fluid. It is, however, noteworthy that these two organic compounds have found extensive use in the extraction of materials with ACh-like activity from nervous tissue. Hence, it is not surprising to observe the tremendous liberation of materials with ACh-like activity on incubation of these sub-cellular particles in the presence of large amounts of these organic solvents. It is perhaps possible to visualize these organic solvents, in large quantities, dissolving the sub-cellular particle membrane, thus liberating the content of the particles into the supernatant fluid. According to T. A. B. Harris (204):

"Since, in vitro, narcotics in a sufficient concentration produced the rapid release of ACh, the generalized convulsions which occasionally occur during deep blood-borne anesthesia--the so-called ether convulsions--those produced when a dangerously high concentration of a local anesthetic is achieved in the circulating blood--may also be attributed to an excessive release and accumulation of ACh at central synapses produced in each instance by the presence of high concentrations of the narcotic agent in the cells of the brain. Generalized convulsions have been reported from time to time with all anesthetics in common clinical use: potent members of the methane series, whose physical properties permit them to be concentrated in the brain, are

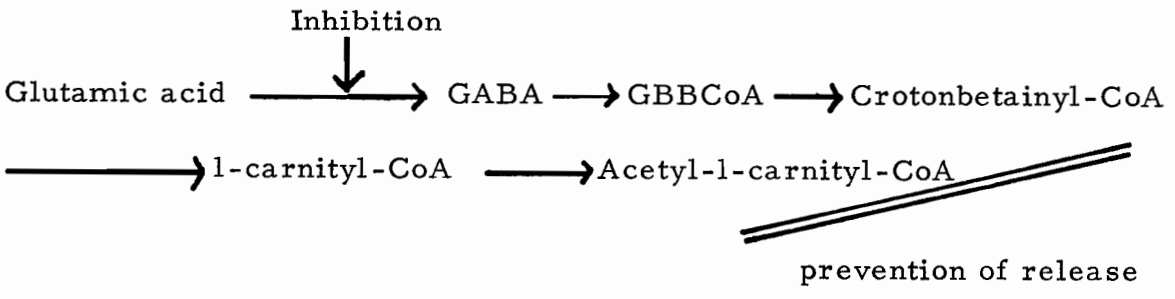
unsuitable for clinical anesthetic practice because of their convulsant action."

As to the nature of the changes of the materials with ACh-like activity within the tissue residue and incubation supernatant fluid, paper chromatograms prepared from extracts of these two fractions and developed in the butanol-water system illustrated that the fall or increase in the ACh-like activity according to physiological state was almost entirely due to changes in the amount of material recovered from the R_F 0.5 -0.7 band of the chromatogram; the betaine-CoA ester fraction.

Hosein and Koh (unpublished) have illustrated both by chemical identification and by colorimetric determinations that only one compound with ACh-like activity could be found in the brains of narcotized animals - the CoA ester derivative of acetyl-l-carnitine. These authors postulated a scheme for the synthesis of this compound which can be seen in Figure 9. The narcotic agent would therefore appear to act primarily by preventing the liberation of this bioactive material from the tissue.

Figure 9

Scheme for the Production of Narcosis



In our experiments, it is possible that the conversion of GBB to acetyl-l-carnitine occurred during the incubation of the particles with the narcotic agents, although it was impossible to ascertain whether this conversion was completed, due to the difficulty of obtaining pure tetra-chloroaurate crystals prepared from extracts of the tissue residues. However, from the results obtained, it is reasonable to assume that these narcotic agents could act on the sub-cellular particles preventing the liberation of materials with ACh-like activity. It is the opinion of this author that these narcotic agents act primarily on the membranes of the sub-cellular particles decreasing their permeability and blocking the liberation of these quaternary ammonium compounds into free solution.

One can rule out the possibility of an increased synthesis of bioactive materials rather than a failure to liberate these active substances on the basis of two sources of information. First, the hypotonic conditions do not provide a medium for synthesis and, secondly, the findings by Bernheim and Bernheim (205) indicate that a number of narcotics do not augment the in vitro synthesis of ACh by brain tissue. Narcotic agents are poor anticholinesterases, thus ruling out the further possibility of an increased content of bioactive material due to the inhibition of the hydrolyzing enzyme system.

Thus it can be concluded from the data presented in Chapter 2 that all the six chemically unrelated anesthetic agents

studied, in their pharmacological concentrations, can produce similar effects. When incubated with sub-cellular particles they cause a retention of materials with ACh-like activity, resulting in a failure to release normal amounts of bioactive materials and causing an increase of this material within the tissue itself. The failure to liberate bioactive materials from the sub-cellular particles within nervous tissue can be equated with a decrease in neuronal activity with the eventual production of the state of narcosis.

Since the incubation of sub-cellular particles with narcotic agents produced the results described above, it seemed logical according to Richter and Crossland's (61) findings that the addition of convulsant agents could produce the opposite picture when applied to a similar system. Accordingly, as with the narcotic agents, a representative group of convulsant agents were incubated with sub-cellular particles and their content of materials with ACh-like activity was estimated on the frog rectus preparation and colorimetrically, the Hestrin method was used for acyl-esters determinations.

From the data described in Chapter 3, all of the convulsant drugs used at pharmacological concentrations with the exception of metrazol, caused the liberation of materials with ACh-like activity and the concomitant loss of these bioactive substances from the sub-cellular particles.

Studies have shown that metrazol does not alter

the metabolism of isolated brain tissue except at high concentrations when some inhibition of respiration and glycolysis occurs. Furthermore, investigations (195) have revealed that this chemical agent undergoes conjugation in the liver to form a glucuronide, and it is possible that this is the active material which is responsible for the convulsive activity.

McLennan and Elliott (93) illustrated that the synthesis of ACh by brain slices incubated in an eserine-Locke-glucose-bicarbonate medium, was stimulated by low (0.02 mM) and depressed by high picrotoxin concentrations. Our results tend in part to confirm these results in that a concentration of 0.01 mM picrotoxin, incubated with sub-cellular particles, increased the liberation of materials with ACh-like activity into the supernatant fluid. Increasing the concentration of picrotoxin to 0.1 mM resulted in a fall in the amount of bioactive materials liberated. McLennan and Elliott (93) also recorded no change in the "bound" ACh in tissue slices after incubation with picrotoxin, or for that matter, with any other convulsant agent. This was probably due to rapid resynthesis to the normal level. Richter and Crossland (61) have demonstrated that the rate of resynthesis of ACh in vivo after the initial convulsion is very rapid, of the order of 7 μgm/gm/min. With slices respiring in Locke-glucose-bicarbonate medium at pH 7.4 at 37°C it is not surprising that McLennan and Elliott (93) found no change in the bound ACh level in the tissue slice after incubation with convulsant agents. Our experiments were carried out in an

non-aerated hypotonic distilled water medium and hence rapid resynthesis of ACh was not likely to occur.

Cortell et al. (206) have reported that prolonged strychnine convulsions elevated total brain ACh in the frog. With the rabbit, these workers found that strychnine and metrazol did not increase the ACh concentration in the brains of these animals. Tobias et al. (91) measured the effect of picrotoxin or strychnine administration on brain ACh content in rats and frogs during the convulsive activity. The findings of Tobias et al. (91) on the action of strychnine on frog brain ACh, where a decrease was observed, was contradictory to the results obtained by Cortell et al. (206) who found an increase. On the other hand, strychnine convulsions produced no effect on rabbit brain ACh as found by Cortell et al. (206). This, however, has been contradicted by Fegler et al. (207) who observed a decrease. Thus, the literature on strychnine and metrazol convulsive activity is indeed very confusing. Our findings indicated that as the amount of strychnine increased from 0.01 to 0.1 mM in our incubation supernatant, the amount of material with ACh-like activity liberated from the particles increased. Thus, it would appear that, although strychnine is a spinal convulsant, nevertheless it can liberate material with ACh-like activity from brain sub-cellular particles. Its action in vivo would perhaps be dependent upon the species of animal employed in the experiment and whether or not it could pass the blood-brain barrier of the particular species, thereby

producing the convulsive activity.

A related situation exists from studies with the neuromuscular and ganglionic blocking agents, d-Tubocurarine and Decamethonium. Feldberg and Sherwood (123) implanted a permanent cannula into the skull of a cat for subsequent injection of drugs into the lateral ventricle of the unanesthetized animal. They found that these ganglionic blocking agents, when injected into the lateral ventricle of the cat, produced convulsive activity. In our experiments, we found that the action of ACh-Cl on the frog rectus preparation was inhibited by these blocking agents, hence only results from the Hestrin colorimetric methods of estimation were presented. By this method of analysis we illustrated an increased release of Hestrin positive material into the supernatant fluid during incubation of particles with d-Tubocurarine and Decamethonium. From parallel results obtained in previous studies where both methods of analysis were employed it is possible to assume that an increase of material with ACh-like activity in the supernatant fluid, occurred during incubation of sub-cellular particles with either d-Tubocurarine or Decamethonium. Because these substances do not penetrate the blood-brain barrier after in vivo administration, convulsive activity is rarely seen.

Although it is difficult to consider ionic calcium as a convulsive agent, studies (197) have illustrated that the injection of ionized calcium intraventricularly in the cat has resulted, like strychnine

or d-Tubocurarine, in convulsive activity. Hutter and Kostial (24) have shown that concentrations of calcium ions five to ten times that of the physiological concentration when perfused through the cervical sympathetic ganglion resulted in the liberation of ACh. In our experiments, calcium ions at similar concentration levels liberated material with ACh-like activity from the particles into the incubation medium. At this point an assessment of information gathered from studies on the two cations, magnesium and calcium, provided an interesting piece of information--namely, there existed an antagonism between these two intracellular cations, as can be seen by comparing the results given in Figures 2 and 5.

The information presented by del Castillo and Katz (44) can best describe this mutual antagonism. These workers revealed:

"There is convincing evidence that calcium and magnesium ions exert a direct and specific action on ACh release. It has been known for years that magnesium blocks neuromuscular transmission..... It is of special interest that calcium opposes the effect of magnesium and relieves the block. Withdrawal of calcium, on the other hand, acts like the addition of magnesium and produces junctional blockade through a lowering of the ACh output.

"Over a wide range of concentrations, a balance between the two effects can be obtained suggesting a direct 'competitive' antagonism between the calcium and magnesium ions at some critical phase of the presynaptic events. There are indications that at other types of nerve endings at which a different transmitter is released, calcium and magnesium may have mutually antagonistic effects of the same kind and that this antagonism may be a general synaptic phenomenon."

From a comparison of Figures 2 and 5, one can

readily observe that this mutual antagonism is due primarily to the different abilities these two cations possess to liberate or retain materials with ACh-like activity within the sub-cellular particles isolated from brain homogenates.

From results obtained in these investigations, sodium and potassium ions also can effect the liberation from or retention of bioactive materials within sub-cellular particles.

Birks et al. (208) have shown that sodium ions are essential for the synthesis of ACh by the superior cervical ganglion of the cat, and that prolonged stimulation thereof in the presence of insufficient sodium ions results in a tiny release of ACh into the perfusion fluid due to insufficient synthesis. Our experiments in part support the findings of these workers in that an increased liberation of materials with ACh-like activity from the particles incubated with sodium ions at a concentration approaching that of the intracellular physiological concentration occurred. However, the amounts of bioactive materials liberated into the incubation supernatant appeared to be rather small, and from these results it was difficult to attribute to sodium an essential role in the liberation of bioactive substances from nervous tissue. Hutter and Kostial (24) have reported that in the perfusion of the cervical sympathetic ganglion, so long as the perfusion fluid contained 30% of the normal sodium concentration, no change in the amount of ACh released on preganglionic stimulation could be detected. These authors illustrated that further

lowering of the sodium concentration decreased the output of ACh abruptly, presumably due to a block of conduction in the preganglionic nerve endings. Hence, sodium can affect the mobility of bioactive materials from nervous tissue, but only over a wide range of concentration.

Potassium ions in amounts approaching low intracellular concentrations caused retention of the bioactive substances within the sub-cellular particles and at physiological concentrations had no effect on the liberation of these substances. However, in experiments where physiological amounts of calcium and potassium were incubated with particles, there resulted a 30% increase in liberation of materials with ACh-like activity. These results lend support to studies by other workers (23), who reported that the perfusion of the cervical sympathetic ganglion with high amounts of potassium containing physiological concentrations of calcium ions, increased the rate of liberation of ACh over the amount obtained when only the potassium ions alone were added to the perfusion fluid.

Quastel et al. (209) have reported that increased K^+/Ca^{++} ratios produces an increase in the rate of liberation of ACh from rat brain cortex slices. A glance at Table XXV illustrates some confirmation of these findings--a high potassium ion concentration had little effect on the liberation of bioactive materials from brain sub-cellular particles. Increasing,or alternatively,decreasing the calcium ion concentrations of the Krebs' medium however, tended, in either instance, to increase the liberation of bioactive materials recovered in the

incubation supernatant. However, it can be observed that increasing the K^+/Ca^{++} ratio (low Ca^{++}, high K^+) produced the largest release of Hestrin positive materials thus supporting the findings of Quastel et al. (209).

From our results, it appears that changes in concentration of basic cations can drastically affect the liberation of materials with ACh-like activity from sub-cellular particles. Sodium and calcium ions at intracellular physiological concentrations tend to liberate bioactive substances, while potassium and magnesium depress this liberation. Depolarization, in which the distributions of the basic cations are altered in such a way that sodium and potassium ions are exchanged, along with the inward movement of calcium ions, would result in an intracellular ionic medium conducive to the liberation of bioactive materials from the sub-cellular particles within the neuron. On repolarization, the cations revert to the original amounts present in the neuron before depolarization and this again represents a condition that depresses the release of bioactive substances from the sub-cellular particles within the neuron.

Several compounds were investigated which displayed neither narcotic nor convulsive properties, but which nevertheless had been shown to affect the metabolism of materials with ACh-like activity in nervous tissue.

From the data presented in Chapter 4, it can be seen that incubation of sub-cellular particles with heated cobra venom

produced an increase in the supernatant fluid of materials with ACh-like activity, with a concomitant fall in the amount of bioactive materials within the tissue residues. The main toxicological effect of cobra venom is respiratory arrest. Although these venoms can produce a curare-like action, unlike curare, evidence in the literature on any convulsant action has not been found. In fact, cobra venom has been known to possess anesthetic properties (210). It is difficult to correlate these findings with the results obtained in our study of this toxic agent, in which a liberation of "bound" to "free" bioactive materials was found to occur on addition of cobra venom to a suspension of brain sub-cellular particles. However, similar findings were reported by Braganca and Quastel (176). These workers illustrated that rat brain cortex slices respiring in a 27 mM potassium-Locke-eserine medium, on the addition of 30 μg/ml heated cobra venom, increased their rate of synthesis of free ACh and at the same time produced a fall in bound ACh within the tissue. It is possible that cobra venom does not enter into the brains of animals. The venom contains a host of enzymes, mainly phospholipases and lecithinases, capable of destroying cell walls. In our experiment, like the one of Braganca and Quastel (176), these enzymes most probably hydrolyzed cell wall linkages of the sub-cellular particles thus liberating their contents into the intracellular medium within the slices.

Unrelated to the study presented in the above

paragraph was the influence of neostigmine on the liberation of bioactive substances from sub-cellular particles. It provides a secondary pharmacological action for neostigmine, besides its value as an anticholinesterase agent. This drug has found widespread use in the treatment of myasthenia gravis--a condition involving continuous flaccid paralysis and lethargy of the muscles of the body presumably due to insufficient liberation of transmitter substances at the neuromuscular junction. The administration of neostigmine relieves this flaccidity of the musculature by its anticholinesterase properties and at the same time, from these studies, it appears to promote the liberation of bioactive materials from the sub-cellular particles within the neuron.

Our investigations showed that both low and high concentrations of ACh itself, when incubated with sub-cellular particles, depressed the liberation of materials with ACh-like activity. Feldberg and Sherwood (123) demonstrated that, on intraventricular injection of ACh into the lateral ventricle of the cat, it produced a state of catatonia or stupor--a schizophrenic-like condition, in which the animal did not respond to the environment around him.

Since ACh has been widely accepted as the chemical mediator of neurohumoral transmission, this role is difficult to visualize in view of the fact that ACh fails to liberate materials with similar biological activity from nervous tissue both from in vivo and in vitro studies.

Not investigated, due to their extremely toxic nature and our limited safety facilities available, were the bacteriological toxins-botulinum and tetanus. Studies (28,29) have shown that botulinum toxin depressed the liberation of ACh from the neuromuscular junction, producing motor paralysis and an anesthetic-like condition. Tetanus toxin (211) produced in animals effects opposite to those of botulinum, an increase in free ACh and the production in vivo of convulsive activity. Although experiments in which these toxins were incubated with sub-cellular particles were not attempted, it is nevertheless the opinion of this author, based on the results of our experiments, that it is safe to speculate that the addition of botulinum toxin to brain sub-cellular particles would probably depress the liberation of bioactive substances much as do the anesthetics, while tetanus toxin would probably facilitate the liberation of these bioactive materials from the sub-cellular particles.

In the final group of experiments performed, sub-cellular particles from brain homogenates were subjected to conditions which in vivo would elicit convulsive activity. The effect of high temperature or fever in animals, especially in young children, has resulted in the production of convulsive activity. When sub-cellular particles were incubated at 43°C, which corresponds to 107°F, some experiments revealed a tremendous liberation of bioactive substances into the incubation medium. Although these temperatures appear to be rather high, it is altogether possible that the internal brain temperatures during high fever

could reach such a level. In young children, perhaps their sub-cellular particles may yet be fragile that a particular external stimulus could cause them to burst, releasing their contents and thus producing the seizure activity. Heat has been employed to liberate ACh from nervous tissue (54, 55), and hence there is a possibility of this situation occurring within the sub-cellular particles.

Another condition investigated was the influence of sonication on sub-cellular particles. It has been illustrated that this form of stimulation can produce convulsive activity in epileptics (185) and in a special strain of mice sensitive to this type of stimuli. Our findings showed that sonication of sub-cellular particles resulted in the liberation of bioactive materials into the incubation supernatant fluid. Almost no tissue residue was found after one minute of sonication as this probably disrupted all the tissue including the sub-cellular particles, liberating their content of bioactive materials.

It was impossible to study the influence of electrical stimulation on sub-cellular particles as electrical pulses produce metabolic changes in intact tissue only. However we obtained results which indicated that alternating electrical pulses liberate materials with ACh-like activity from rat brain cortex slices. Previous workers (180) have described these findings. Richter and Crossland (61) have shown that electrical stimulation of rats for a period of 1 to 3 seconds, resulted in a loss of the total ACh-like activity in the brain.

In most instances in which either convulsant agents of convulsion-causing conditions were applied to sub-cellular particles, an increase in the liberation into the supernatant fluid occurred of bioactive materials with ACh-like activity, which on chromatography in the butanol-water system were recovered in the R_F 0.45 - 0.65 band, the betaine-CoA ester fraction. Similarly, extraction of the bioactive substances in the tissue residue showed that the fall in bioactive material was due almost entirely to changes in the betaine-CoA ester fraction.

From these results on the basis of chromatographic analysis, it can be concluded that the active components released during convulsive activity are mainly the betaine-CoA esters. They can be equated with the so-called easily releasible "ACh fraction", although some conditions such as sonication and high temperatures appeared to liberate the more "firmly bound ACh fraction".

The convulsant drugs which act to liberate the bioactive substances can conveniently be placed within three groups:

(i) Those acting on the sub-cellular particles by altering the composition of the medium within the particles, and thus promoting the outward liberation of bioactive materials. Examples of these influences are electrical stimulation, and cationic changes such as excess calcium, or the potassium/calcium ratio factor:

(ii) Those having a direct effect on the membrane wall by the

interaction of the convulsant drug with various side-chains. Examples are strychnine, picrotoxin, d-Tubocurarine, Decamethonium, and camphor:

(iii) Finally, those which can disrupt the sub-cellular particle membrane wall releasing all of its contents. Examples are sonication, increased temperature and treatment with organic solvents such as ether and ethanol. This final set of agents and conditions results in nearly total liberation of all bioactive materials presumably because they caused disruption of the particles due to harsh treatment. However, it can be seen from the results that in any one of these three groups presented above, the majority of the material with ACh-like activity released consisted of the betaine-CoA esters. It is concluded that the action of convulsants involves the liberation of the betaine-CoA esters from sub-cellular particles, which in turn are responsible for the convulsive activity.

This investigation was designed with the purpose of using isolated brain sub-cellular particles as a pharmacological tool as had been suggested by Whittaker (191). From the results obtained in these studies, it is the feeling of this author that Whittaker devised an ingenious method of approach to a problem. The use of sub-cellular particles provides a method for the understanding of how narcotic and convulsant drugs act on nervous tissue. It further extends the

findings of Richter and Crossland (61) about ACh metabolism at a sub-cellular level - convulsants act by liberating endogeneous materials with ACh-like activity from brain sub-cellular particles and conversely narcotic agents by preventing the release of these bioactive substances from the same particles.

The utilization of brain sub-cellular particles assisted in explaining the reports by del Castillo and Katz (44) on the influence of calcium and magnesium ions at the neuromuscular junction, and Hutter and Kostial's (24) findings on the influence of calcium and magnesium on ACh metabolism in autonomic ganglia. It is proposed that the antagonism of these two cations observed at these sites is due to their opposite abilities to either liberate or retain materials with ACh-like activity within the sub-cellular particles present in these structures.

These isolated sub-cellular particles represent a transmitter-rich fraction, for it has been illustrated (44, 199, 200, 201) that not only are materials with ACh-like activity present in highest amounts in these particles, but also other substances with known transmitter functions have been found there (e.g. nor-adrenaline, 5-hydroxyptamine, ATP). Since the incubation of narcotic and convulsant agents with brain sub-cellular particles produced effects on the metabolism of materials with ACh-like activity, it is likely that other drugs or agents which also produce changes in vivo in the content of these other biologically active compounds might produce their in vivo effect in a manner similar

to those described for narcotic and convulsant drugs *in vitro*. This method may also be of advantage in testing the pharmacological potency of drugs by determining which concentration is necessary to alter the metabolism of the sub-cellular particles. It is thus felt that the results described in this thesis are only the beginning of the applicability of a powerful method of testing pharmacological activity.

SUMMARY

1. The incubation for a period of 15 minutes of sub-cellular particles from brain homogenates in distilled water resulted in the liberation of materials with ACh-like activity into the incubation fluid. About 55% of the total bioactive materials measured either by biological assay on the frog rectus preparation or colorimetrically using the Hestrin method, was estimated to be liberated from the particles in P_2 fractions. Paper chromatography of TCA extracts prepared from both the incubation supernatant and tissue residue in a butanol-water system showed that 80% of the ACh-Cl equivalent activity was recovered in the R_F 0.45 - 0.65 band of the chromatogram--the betaine-CoA ester fraction.

2. The addition of members of representative groups of narcotic agents - ether, ethanol, magnesium, sodium bromide, sodium pentobarbital and nitrous oxide--to brain sub-cellular particles suspended in distilled water at 37°C reduced the amount of bioactive substances with ACh-like activity in the incubation fluid when compared to the control and, at the same time, increased its content in the tissue residue. Paper chromatography of extracts prepared from both the incubation medium and the tissue residue, after treatment with TCA and ether showed that changes in activity were due to variations in the amounts of betaine-CoA esters.

3. Various convulsant agents such as picrotoxin, strychnine, camphor, d-Tubocurarine, calcium and semi-carbazide were also incubated with brain sub-cellular particles. The results illustrated that all these convulsant drugs liberated materials with ACh-like activity from the sub-cellular particles. In experiments where the ACh-Cl equivalent activity of the tissue residue was estimated, there was a fall in the content of bioactive materials with ACh-like activity. On paper chromatography of extracts in the butanol-water system, the changes in activity were shown to be due to variations of the materials in the R_F 0.45 - 0.65 band of the chromatogram; the betaine-CoA ester band.

4. Physical conditions approximating those required to produce convulsive activity were applied to brain sub-cellular particles. The effect of an increase in temperature from 37°C to 43°C was a liberation from the particles of bioactive materials, which on paper chromatographic analysis were shown to be due to the betaine-CoA esters.

Sonication of brain sub-cellular particles resulted in almost complete liberation of all the bioactive substances from the particles.

5. Application of alternating electrical pulses, for 5 - 60 minute periods, to brain cortex slices respiring in a Locke-glucose-phosphate medium (pH 7.4) resulted in a liberation of materials with ACh-like activity into the incubation medium and an equivalent fall in ACh-Cl equivalent activity in the tissue slices. TCA extracts of the two fractions,

when chromatographed and developed in a butanol-water system, showed that changes in the ACh-Cl activity were due to the betaine-CoA ester fraction.

6. The addition of cobra venom to brain sub-cellular particles produced effects similar to those induced by convulsant agents - a liberation of bioactive substances with ACh-like activity, and a corresponding decrease in the tissue residue of these substances, which on chromatographic analysis in the butanol-water system were recovered in the R_F 0.45 - 0.65 band of the chromatogram, the betaine-CoA ester fraction.

7. Sodium and potassium ions were incubated with brain sub-cellular particles. At their intracellular concentrations sodium ions appeared to liberate a small amount of Hestrin positive materials, while potassium ions produced no change from that observed with the tissue control. Because of the interference of these cations with the biological assay preparation, these experiments were carried out using only the Hestrin method of estimation.

8. On incubation of brain sub-cellular particles in Krebs' medium, altering its composition produced changes in the amount of materials released when estimated by the Hestrin colorimetric method.

9. Acetylcholine-Cl in concentrations ranging from 10 mμg to 1 μgm, added to sub-cellular particles, failed to liberate bioactive materials and instead produced a decrease in the amount of bioactive materials

recovered in the incubation medium.

10. Neostigmine-bromide in concentrations ranging from 0.1 to 1.0 mM per P_2 fraction, when added to sub-cellular particles, progressively increased the liberation of materials with ACh-like activity into the incubation fluid.

CLAIMS TO ORIGINAL RESEARCH

1. Various narcotic agents when incubated with brain sub-cellular particles were shown to block the release of materials with ACh-like activity into the incubation medium, and at the same time to cause an increase in the ACh-like activity within the sub-cellular particles. This increased ACh-like activity within the sub-cellular particles was due primarily to an accumulation of the betaine-CoA esters.

2. The addition of convulsant drugs to brain sub-cellular particles stimulated the liberation of materials with ACh-like activity into the incubation medium, and chromatographic analysis of this material showed the activity to be due almost entirely to the presence of the betaine-CoA esters.

3. Physical conditions which can induce convulsive activity in vivo e.g. temperature rise and auditory stimulation did liberate materials with ACh-like activity from brain sub-cellular particles.

4. The antagonism between calcium and magnesium ions at the neuromuscular junction was likely due to their effects on the release from or retention of materials with ACh-like activity within the sub-cellular particles.

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