THE ISOLATION OF ACETYL-L-CARNITYL CoA FROM THE BRAIN AND HEART OF NARCOTIZED ANIMALS AND THE INFLUENCE OF VARIOUS NARCOTICS ON THE SUBCELLULAR DISTRIBUTION OF ITS ACETYLCHOLINE-LIKE ACTIVITY IN BRAIN

by

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ABBREVIATIONS

TCA	Trichloroacetic acid
ACh	Acetylcholine
PCh	Propionylcholine
BuCh	Butyrylcholine
ATP	Adenosine triphosphate
CoA	Coenzyme A
GBB	Gamma-butyrobetaine
GBBM	Methyl ester of gamma-butyrobetaine
GBBE	Ethyl ester of gamma-butyrobetaine
AC	Acetyl-l-carnitine
Crotonbetaine M	Methyl ester of crotonbetaine
Crotonbetaine E	Ethyl ester of crotonbetaine
Carnitine M	Methyl ester of carnitine
Carnitine E	Ethyl ester of carnitine
Acetylcarnitine M	Methyl ester of acetylcarnitine
GABA	Gamma-aminobutyric acid

GENERAL INTRODUCTION

Development of the Concept of Humoral Transmission

The stimulation of nervous tissue leads first to excitation at the point of application, and from here the excitation is propagated to an effector organ which responds to the transmitter excitation. Prior to the first decade of 1900, there were only hypotheses which sought to suggest the intimate mechanism by which nervous excitation produces a functional change in an organ. Elliott (1), observing that the effect of stimulation of sympathetic nerves and of adrenaline was identical, proposed the hypothesis that stimulation of sympathetic nerves caused liberation of adrenaline and that it was the adrenaline which was responsible for the end effect of nervous stimulation. The question of whether nervous stimulation directly affected the organs involved was tested experimentally by Howell (2) who indicated that stimulation of the vagus led to a liberation of potassium, this substance in turn causing the resulting phenomenon. Although Howell's assumption was not corroborated, he was the first to investigate experimentally the possible mechanism of the inhibitory function of a nerve. In the same year, Dixon (3) cited evidence for the presence of a substance in heart tissue, "pro-inhibitin", which was converted to "inhibitin" on vagal stimulation. This substance, when combined with heart muscle, brought about cardiac arrest.

Loewi (4) proved beyond doubt the correctness of the fundamental idea of Elliott (1) and Howell (2) with direct

experimental evidence for the humoral or chemical transmission of excitatory or inhibitory process, from the endings of the vagus and of sympathetic nerve fibres to the heart muscle of the frog. In this classical investigation, Loewi isolated two hearts, one with its vagus, the other without it. Both hearts were filled with a small amount of Ringer solution. When the vagus was repeatedly stimulated, the Ringer solution acquired material from that heart, which, when transferred to the other heart, caused it to react as if its own vagus was stimulated. The conclusion was obvious that the vagus did not act directly on the heart musculature, for it was not being directly inhibited by the stimulation of the vagus. The stimulation of the nerve caused it to produce a chemical substance which brought about the inhibition of heart action. Loewi named this substance Vagusstoff. This experiment, firmly establishing the concept of humoral transmission of nervous impulse, was quickly and universally accepted. It must be remembered, however, that Loewi's results applied only to the postganglionic fibres of the autonomic nervous system.

Dale et al. in 1936 sought to extend the concept of humoral transmission of excitation from motor nerves to voluntary muscles. When the cat's tongue, cat's and dog's gastrocnemius and the hind leg muscles of the frog were electrically stimulated via their respective nerves, evidence was obtained (5) that a substance resembling acetylcholine (ACh) was released, because it caused the eserinized leech dorsal muscle to contract and this effect was potentiated by eserine and abolished

by atropine on a suitable test object such as the blood pressure of the cat. Further evidence that nerves acted by liberating ACh was demonstrated when direct stimulation of the cat's gastrocnemius with its nerve supply intact caused release of ACh but, when the muscle was completely denervated, no ACh appeared in response to effective stimulation. Conversely, injection of small doses of ACh into empty arteries elicited rapid contraction of normal mammalian muscle (6).

Brown (7) has obtained records of action potentials from normal mammalian muscles which indicate that the quick contraction evoked by close arterial injection of ACh is a brief asychronous tetanus. Implicit in the discussion of Brown et al. (6) is that ACh is released from the nerve on arrival of the impulse, which, having acted on motor end plate, is quickly destroyed by an esterase. Eserine, an anti-esterase, potentiates this response. Bacq and Brown (8) have examined some eserine substitutes and found that their potentiation of muscle response was proportional to their inhibition of cholinesterase.

By analogy to the experiment of Loewi (4), Feldberg and Gaddum (9) showed that electrical stimulation of the sympathetic cervical nerve elicited the appearance of ACh in the perfusate of the cat superior cervical ganglion. When the preganglionic nerve was not stimulated, no ACh could be found in the perfusate. These series of experiments established that the mechanism of transmission was humoral in nature from nerve to cardiac muscle, striated muscle and ganglia.

The possible function of ACh as a central synaptic transmitter was first envisaged by Dale and was the outcome of experiments which suggested ACh as the mediator of excitation from motor nerve to skeletal muscle and for synaptic transmission to the sympathetic ganglia. Direct evidence is usually based on the release of ACh from nervous tissue of central origin. Thus, ACh was recovered in the cerebrospinal fluid when the central end of the cut sciatic nerve was stimulated (10), and after epileptic seizures (11). MacIntosh and Oborin (12) showed that free ACh release from the cerebral cortex was inhibited during narcosis. The presence of cholinergic nerves in the central nervous system was demonstrated by responses which simulated the activity of these nerves, for example, application of ACh intraventricularly caused cardiac arrest, a phenomenon similar to stimulation of the central end of the cut vagus (13), and local application of eserine on motor areas of cerebral cortex of cats evoked muscular effects (14). Acetylcholine applied locally on the eserinized cortex or injected into the vertebral or carotid artery produced alterations in behaviour and the conscious state (15) which suggested that some neurones in the central nervous system were indeed cholinoceptive. The distribution of ACh (16), its synthesizing enzyme, choline acetylase (17), and the cholinesterase system (18) in different parts of the cat brain exhibited a reasonable degree of correlation and lent support for the physiological role of ACh at certain central synapses. This correlation also supported the suggestion of Feldberg and Vogt (17) that there may be two types of neurones in the central nervous system, one

rich in the ACh system and the other lacking in it. Further, they suggested that these two systems may be correlated with a cholinergic and a non-cholinergic mode of synaptic transmission respectively. Finally, because nerve endings contain the richest source of ACh (19,20,21), this provided indirect evidence for the role of ACh as a neurohumoral transmitter at certain central synapses.

INTRODUCTION

A. Acetylcholine, Discovery and Physiological Properties

Interest in ACh as a parasympathetic transmitter was centred principally on its intense physiological effects. ACh was chemically synthesized (22) in 1867, before its occurrence in plant or animal tissues was known. Thus, Hunt in 1899 (23) found that the injection of aqueous suprarenal extract, from which epinephrine had been removed with benzoic acid, caused a fall of blood pressure. This effect was due in part to choline, which was isolated and identified as the platinum salt. Hunt further obtained evidence for the presence of a more active depressor substance in the suprarenal gland extract, which yielded choline on chemical manipulation (24) and suggested that the original substance could be an ester of choline. As a direct outcome of these experiments, Hunt and Taveau (25) discovered the intense depressor activity of ACh. They attributed the fall of blood pressure to its cardiac origin and showed that it was prevented by small amounts of atropine. Ewins (26) isolated ACh from ergot and Dale (27) showed that the depressor effect of ACh was a peripheral vasodilation

effect, which was abolished by atropine. Dale suggested the evanescent action of ACh was due to the rapid hydrolysis of the ester. Eserine was found by Hunt (28) to potentiate the pharmacological effect of ACh. Hitherto, it had been believed that the mechanism of atropine action was concerned with the paralysis of the vagus. Against this view, Loewi (4) was able to demonstrate that atropine did not paralyse the vagus, but that it acted as an antagonist to the Vagusstoff by preventing the effect of endogenous Vagusstoff on the heart.

Loewi and Navratil (29) extended Hunt's evidence that eserine potentiated the effect of ACh, and that it did not potentiate the effects of choline. They observed that, with eserine (physostigmine) and ergotamine pretreatment, the heart was sensitized, not only for the effect of the vagal stimulation, but also for that of Vagusstoff. Further, concentrations of eserine and ergotamine which potentiated <u>in vivo</u> effects of Vagusstoff and of ACh, inhibited <u>in vitro</u> the enzymatic hydrolysis of both these substances by the esterase in heart extracts. On this basis, the action of the alkaloids was regarded to be that of an antiesterase. In the same year, these authors (30) compared the properties of Vagusstoff with ACh and showed that both were destroyed by aqueous extracts of heart, liver or intestine tissue. Hydrolysis did not occur if the heart extract was inactivated by heating at 56°C or by exposure to fluorescence and ultraviolet light. The cleavage of both substances proceeded at pH 6.0 to 7.5 and increased with alkaline pH. Reacetylaction of the choline remaining in the Vagusstoff

after hydrolysis by the esterase in heart extracts, restored the biological activity. Loewi, therefore, concluded that Vagusstoff was a choline ester.

B. Nature of Acetylcholine in Brain and in Cardiac Tissue

Early studies by Mann et al. (31), Trethewie (32) and Corteggiani (33) revealed that a considerable portion of ACh in brain tissue is saline insoluble because it is bound to some tissue component. This material is known as bound ACh and in vivo it is physiologically inactive. Chang and Gaddum (34) observed that alcoholic extracts of rat brain were less active than TCA extracts and Barsoum (35) found that the deficit in activity could be recovered if the alcoholic extract was treated with TCA. The results of Loewi and Hellauer (36) supported those of Chang and Gaddum (34) in that alcoholic extracts of the frog central nervous system contained only about half the ACh activity of TCA extracts.

Mann et al. (31) suggested that the binding material in bound ACh was some protein constituent of the cell, but later (37) indicated that it was the ACh synthesizing enzyme. Loewi et al. (38) suggested that ACh was bound both to lipid and protein components. More recently, Braganca and Quastel (39) have suggested that bound ACh could be held within the mitochondria.

In 1938 (31) it was shown by Mann et al. that free ACh could be released from the bound form by treatment with denaturing agents, such as chloroform or acidification. Elliott et al. (40) have taken the view that any free ACh found is largely

an extraction artifact, varying in amounts with the physical factors in the homogenization. In agreement with the observation of Mann et al. (31), addition of ACh caused no rapid increase in the rate of synthesis of bound ACh; Brodkin and Elliott (41), however, have shown that there is some binding of the ACh added to tissue suspensions. The latter authors also showed that convulsant and narcotic drugs exerted no effects on the binding of ACh by tissue suspensions.

Hobbiger and Werner (42) concluded that an equilibrium existed between free and bound ACh, an observation first noted by Mann, Tennenbaum and Quastel (31). In addition, they (42) suggested that a third unknown form of ACh is present in brain tissue. Birks and MacIntosh (43) have introduced the terms "stationary ACh", "available ACh" and "depot ACh" to describe the various storage forms of ACh in sympathetic ganglia.

In 1925, Witanowski (44) showed that a substance with the pharmacological properties of a choline ester could be obtained from the frog heart by mincing the tissue in alcohol. Subsequently, Chang and Gaddum (34) showed that dog auricle contained 1.3 ug. and ventricle 0.2 ug. ACh per gm.of tissue. The relatively higher concentration of ACh in auricles was confirmed by Englehardt (45). Abdon and Hammarskjold (46,47) reported the presence in skeletal muscle and in rabbit heart of a labile complex of ACh, which they termed ACh precursor. This precursor, which was most stable at pH 3-4, could be broken down to physiologically active, free ACh by treatment with acid or alkali at a pH removed from 3-4. It was, therefore, different

from the ACh complex of Mann et al. (31), which was destroyed on standing at pH 3 for 30 minutes, but which resembled the complex studied by Corteggiani (33), which was soluble in acetone. Later work by Abdon and co-workers (48) showed that the amount of ACh precursor in rabbit heart decreased after prolonged stimulation and that administration of choline led to its restoration by resynthesis (49). This suggested the possibility that the precursor was the immediate source of the vagal transmitter, in accordance with the classical experiment of Loewi (4). Eserine and atropine have no effect on the breakdown or resysthesis of the precursor (50,51).

Tucek and Vlk (52) established that the distribution of ACh in the heart auricles of mammals corresponded to a gradient similar to that of autonomic activity, the highest concentration being in the region of the sino-auricular node and the lowest in the left ventricle. The distribution of the choline acetylase system corresponded to autonomic activity, whereas that of cholinesterase did not.

Abdon (51), studying the metabolism of the ACh precursor in isolated hearts, spoke of the general appearance of tissue ACh in many places where it could not have the function of a humoral transmitter and concluded that the breakdown and formation of the precursor belonged to the metabolism of the cardiac muscle. Thus, in contrast to the ACh of nervous origin liberated on vagal stimulation (4), it is also alleged that ACh is synthesized by the cardiac cell. This could probably take

place in the cell membrane corresponding to the "endogenous ACh" of Burn et al. (53,54) and to the "herzeigene" ACh of Rothschuh (55), responsible for the spontaneous contraction of the heart. The "herzeigene" ACh of Rothshuh is said to exist in the form of Pro-ACh which liberates free, physiologically active ACh on treatment with TCA.

Beznak (56) studied the chromatographic behaviour of material believed to be ACh in TCA extract of heart tissue. He found a fast moving component on the Augustinsson system (acetic acid-n-butanol-ethanol-water) of R_F 0.75 which resembled the F component of Banister et al. (57) which had R_F 0.65 (butanol-water system) and also the R_F 0.80 component of Augustinsson et al. (58,59) in spleen extracts.

It seems fair to state that TCA extracts of heart tissue contain ACh, both of neural and muscle origin. While the ACh content of heart tissue during increased nervous activity, i.e. in vagal stimulation, has been discussed (48), the ACh content during decreased activity, i.e. narcosis, has not been investigated.

C. The Distribution of Acetylcholine in Subcellular Particles

From a consideration of the nature of bound ACh, it is not surprising that the possibility that ACh is stored within a tissue organelle has been a matter of much speculation. DeRobertis and Bennett (60), Palay (61) and Palay and Palade (62) demonstrated by electron microscopy that small organelles or vesicles are concentrated in great numbers at presynaptic

terminals of nerve fibres and are clustered especially on the membrane that is in contact with the muscle. They advanced the theory that the submicroscopic vesicular components, termed "synaptic vesicles", may be associated with the production and release of ACh or other neurohumoral transmitters. The suggestion that synaptic vesicles carry ACh is attractive, because this type of localization prevents ACh from enzymic action and, at the samt time, prevents it from exerting its intense physiological effects. Ejection and breakdown of such microcarriers would account for the quantized release of ACh at the neuromuscular junction in accord with the concept proposed by del Castillo and Katz (63). In certain synapses, the number of vesicles was reported to be increased after prolonged stimulation (64), and to be decreased after denervation (64,65,66). The suggestion has indeed been put forward that the vesicles represent "quantal units of ACh" (67). The presynaptic vesicle theory may need reevaluation from the finding of structures resembling the synaptic vesicles of DeRobertis and Bennett (60), Palay (61) and Palay and Palade (62) in the postsynaptic areas of nerve junction. For example, vesicles are found in postsynaptic regions of the giant nerve fibres of the earthworm Eisenia foetida (68). Vesicles have also been found in the dendrites of Pacinian corpuscles (69) and on both sides of the presumed synaptic junction in Meissner's corpuscle in human fingers (70).

While there is no direct evidence that vesicles at the neuromuscular function do contain ACh, there is evidence

showing that certain vesicles of the central nervous system do (21,71). In these vesicles this ACh is inactive and may be equated with bound ACh.

Of significant importance is the harvesting of subcellular fractions which contain vesicles by differential centrifugation of brain homogenates. Preliminary work to separate and collect the subcellular components in brain homogenate and to gather information concerning the distribution of ACh and the choline acetylase system was initiated by Hebb and Whittaker (20) in 1958. In the following year, Whittaker published an account (21) in which he described the separation of rabbit and guinea pig brain homogenates in 0.32 M sucrose by means of differential centrifugation into various subcellular fractions, namely P_1 (nuclear fraction), P_2 (crude mitochondrial fraction), P_3 (microsomal fraction) and S (cytoplasmic supernatant). The microsomal and cytoplasmic together were designated S_2. In confirmation with the previous finding (20) that bound ACh was located in particles which have sedimentation properties broadly similar to that of mitochondria, most of the ACh activity was found in the P_2 fraction. The ACh activity in P_1 and S_2 were regarded as due to particle contamination from the P_2 fraction.

According to Whittaker (21), and to Kurokawa *et al.* (72), the distribution of ACh in brain homogenates is as follows:

Subcellular Particle Fraction

	ACh in Brain	
	Whittaker (21)	Kurokawa et al. (72)
P_1 (nuclear)	22%	0.16 µg.ACh/gm. brain
P_2 (crude mitochondrial)	60	1.39
P_3 (microsomal) + S (cytoplasmic supernatant) = S_2	18	0.76

The release of ACh from the P_2 fraction can be partially effected by procedures such as freezing and thawing, dilution from hypertonic to isotonic solution and prolonged storage at 0° to 10°C in isotonic solution. The ACh released by this fashion was termed by Whittaker "loosely" bound ACh and may be imagined as ACh simply occluded within the vesicle. The total release of all endogenous ACh can be effected by ether, TCA or chloroform extraction. This type of ACh is the "firmly" bound ACh which was thought to be bound to the matrix of the vesicle. The observation may also suggest that the partial release of the ester occurs because some particles may be more labile than others and these may represent the aging part of the total population of similar particles.

Further fractionation of P_2 by means of equilibrium centrifugation in 0.32 M, 0.80 M and 1.2 M sucrose gradient layers, yielded three sub-fractions termed A, B and C. Histological examination and biochemical evidence were used to interpret the nature of these fractions. Sub-fraction A was tentatively identified as enucleated cells derived from glial tissue; B represented synaptic vesicles and C was mitochondria. The

B sub-fraction was found to contain most of the extractable ACh. Hosein and Proulx (73) were able to confirm this localization of ACh activity. Gray and Whittaker (19) and DeRobertis et al. (74) claimed that the B fraction was mainly pinched off nerve endings packed with synaptic vesicles. The difficulties involved in isolating a "pure" subcellular component was indicated by DeRobertis et al. (71) who, using five sucrose density gradient layers, separated P_2 into five fractions which they termed A, B, C, D and E. The content of bound ACh was highest in the C fraction, which they claimed to be pinched off nerve endings packed with synaptic vesicles. There was less ACh in the other fractions. Whittaker, Michaelson and Kirkland (75) have now separated crude mitochondrial fractions into seven subfractions designated 0, D, E, F, G, H and I, of which sub-fraction D has highest ACh activity and consists almost entirely of particles about 50 Å in diameter, identical in appearance with synaptic vesicles.

D. Bioassay of Acetylcholine

Biological preparations for the determination of ACh were employed long before the discovery of this substance as a normal constituent of body tissues. The action of pure ACh on the frog rectus abdominis preparation was first described by Riessner (76) and on the eserinized dorsal muscle of the leech by Fuehner (77). The behaviour of ACh on biological preparations was widely studied but had not been used in the testing of tissue extracts, although Witanowski (44) used the frog heart to demonstrate that heart tissue contained a substance with pharmacological properties of a choline ester. The determination

of ACh in horse spleen extract, thus showing ACh to be normal constituent of animal tissues, by Dale and Dudley (78), gave impetus for the use of these biological preparations in testing for ACh in tissue extracts. Thus Minz (79) recommended the use of the eserinized dorsal muscle of the leech for the assay of extracts for ACh. This preparation subsequently found its way to Dale's laboratories.

In 1933, Chang and Gaddum (34) published a paper in which they described the behaviour of ACh and of tissue extracts on several biological preparations namely the frog's heart, rabbit's intestine, rabbit's blood pressure, cat's denervated gastrocnemius, the normal and eserinized frog rectus abdominis and the eserinized dorsal muscle of the leech. All these preparations are suitable for use in the assay for ACh in various tissues, although each has its own individual merit. Thus an extract which showed ACh-like activity on the frog's heart, the rabbit's intestine and blood pressure was thought to be due entirely to ACh if the activity was abolished by small doses of atropine. The frog rectus abdominis provided a sensitive and specific test object for ACh in tissue extracts; its real value lies in the fact that eserine specifically sensitized this preparation to ACh. The normal dorsal muscle of the leech did not prove such a satisfactory test object as the rectus, but when treated with eserine this preparation reacted to ACh only. Chang and Gaddum (34) also demonstrated that extracts could be compared quantitatively with pure ACh with reasonable accuracy by giving the two solutions in alternate doses. The quantitative amount of activity measured on the rabbit's intestine and blood

pressure which are sensitive to other substances, for example, adenosine and its derivatives, could be checked by the rectus abdominis and leech muscle assay.

The main contribution of the work of Chang and Gaddum is their establishment of the basis of pharmacological identification of ACh present in tissue extracts. They showed that choline, propionylcholine, butyrylcholine, valerylcholine, glycollylcholine, carbaminoylcholine had different relative potency in terms of ACh on the rabbit's intestine and blood pressure, the normal and eserinized frog rectus abdominis and the eserinized leech dorsal muscle and logically drew the conclusion that parallel quantitative assay will distinguish ACh from any other choline esters in tissue extracts. The sample of pyruvylcholine which could not be differentiated on these preparations was probably impure ACh (80). Gill and Parson have since shown that pure authentic pyruvylcholine can be differentiated by parallel quantitative assay (81).

E. Colorimetric Determination of Acetylcholine

In spite of the interest in ACh, methods for the colorimetric determination of this substance in tissue extracts have been few. In the past, the method for the production of colour by means of complexing with Reinecke Salt (82) has lacked both sensitivity and specificity. The method of Hestrin (83), based on the formation of brown hydroxamate when ACh is allowed to react with hydroxylamine under alkaline conditions, also lacks specificity. However, ACh may be determined by this method in tissue extracts in the presence of large excesses of acetate and

choline (85). Acetylcholine in brain has been measured colorimetrically in this fashion.

Mitchell and Clark (84) determined ACh in nerve tissue extracts using the bromophenol blue dye. These authors developed this bromophenol blue method to estimate quaternary ammonium compounds; tertiary nitrogen compounds do not give this reaction (85,86). This method has the advantage that the dye complex formed in the original biological media can be quantitatively extracted with dichloroethane without interference from the unreacted dye or from any of the naturally occurring organic bases. This method gives the following relative sensitivity of quaternary ammonium compounds, from which it is noted that ACh gives more absorbance than neostigmine.

The Bromophenol Blue Reaction of Mitchell and Clark (85)

Compounds which give a blue dichloroethane-soluble complex	Concentration/ml. reaction
ACh	0.2 µg.
Tetraethylammonium	0.3
Neostigmine	0.4
d-Tubocurarine	0.4

Nowell and Wilson (87) modified the original procedure of Clark and Mitchell's bromophenol blue method (85) for the demonstration of quaternary ammonium compounds in extracts of foetal whale thymus glands. This method again depends on the formation of a blue chloroform-soluble complex, when certain quaternary ammonium compounds are allowed to react under alkaline

conditions with bromophenol blue. Thus determined, the relative sensitivity of a number of these coumpounds given below shows an inverse absorbance relationship to the method of Mitchell and Clark (85). With their modification they found that acetylcarnitine was 10 times more sensitive than ACh.

The Bromophenol Blue Reaction of Nowell and Wilson (87)

Compounds which give a blue chloroform-soluble complex	Concentration/ml. reaction
Choline Chloride	10 mg.
Gallamine Triethiodide	8
Succinyl Dicholine Chloride	1
Acetylcholine Chloride	100 µg.
d-Tubocurarine Chloride	20
Decamethonium Iodide	10
O- Acetylcarnitine	10
Benzoyl Choline Chloride	10
n- Propionyl Choline Perchlorate	10
n- Butyryl Choline Chloride	5
n- Valeryl Choline Iodide	5
Pyridostigmine Bromide	2.5
Win 8077	2.5
Neostigmine Methylsulphate	2.5

F. Other Biologically Active Quaternary Ammonium Compounds

Carnitine was discovered in meat extracts independently in 1905 by Gulewitsch and Krimberg (88) and by Kutscher (89). The latter author gave it the name of novaine. Carnitine and novaine were soon recognized by Krimberg as one and the same

substance (90). Its structural formula as γ-trimethyl-β-hydroxy butyrobetaine first postulated by Krimberg (91) was not confirmed until 1927 when Tomita and Sendju (92), through correct chemical synthesis, demonstrated the structure proposed by Krimberg was correct and also established the laevo-rotatory property of natural carnitine. In the initial report of Gulewitsch and Krimberg, concerning the discovery of carnitine (88), the authors had indicated that carnitine was optically active and in fact was laevo-rotatory. This was determined by the orientation of the asymmetric β carbon atom in the molecule.

In the following year after his discovery of carnitine, Krimberg (93) indicated that carnitine was probably a homologue of gamma-butyrobetaine (GBB). Gamma-butyrobetaine has a wide distribution in the animal world; it is present in the muscles of cold blooded animals, namely sea anemones (94), the eel (95) and the python (96). Without prior knowledge of its structure, Brieger (97) indicated that GBB was the toxic factor in rotting horse meat and dogs which ate this meat had symptoms which included salivation, dilation of the pupils, cramps, excessive tear secretion, contractions of the blood vessels and paralysis of the nerves. Takeda (98) isolated a base from the urine of dogs which had been poisoned with phosphorus compounds and demonstrated its identity as GBB. Engeland and Kutscher (99) then demonstrated the relation of the base with the protein molecule, in that they stated that the precursor of GBB was GABA, which in turn could arise from glutamic acid:

Protein⟶ glutamic-acid⟶ GABA⟶GBB

Later, Engeland (100) found GBB in the urine of rabbits after administration of carnitine and Reinwein and Thielmann (101) found it in the urine of patients with pernicious anaemia. Earlier studies by Krimberg and Linneweh failed to find its presence in meat extract. With the advent of ion exchange resins, GBB was separated from other quaternary ammonium compounds in meat extract and identified by Friedman et al. (102). It is of interest to note that choline itself is completely absorbed on the resin (Dowex 50) and cannot be eluted. Hosein and Proulx (103) have isolated it in aqueous extract of brain tissue obtained from animals killed during convulsions, but not from normal animals.

Linneweh (104,105) isolated crotonbetaine from animal tissues. On administration to dogs, GBB, crotonbetaine and carnitine were found to be interconvertible. On basis of this, he postulated this pathway:

GBB→ crotonbetaine→ carnitine→ breakdown products

Strack and Forsterling (106,107) also isolated crotonbetaine from mammalian muscles and prepared some of its esters. Hosein, Proulx and Ara (108) obtained evidence that the naturally occurring form of these betaines are the CoA esters.

The distribution of carnitine in its free and bound form is summarized by Deltour (109). It is present in the rat brain in the order of 60 µg. per gm. wet weight; in the rat heart 50-230 µg. per gm. wet weight and in the rat muscle 310 µg. per gm. wet weight. The highest content is in meat extract, which can contain up to 0.2% of it.

Physiological Properties of Quaternary Ammonium Compounds

The discovery of carnitine brought forth many attempts to establish its pharmacological properties. Kutscher and Lohmann (110) first established the weak activity of carnitine on blood pressure, heart and intestine preparations. Administration of carnitine in large doses to dogs, cats and frogs produced no striking effects except strong peristalsis (111). Korchov (112) and Bickel and Korchov (113) all reported that carnitine acted as a stimulant to intestinal secretions and to the motor functions of intestines. Linneweh (105), studying the effects of administration of GBB, crotonbetaine and carnitine to animals, found carnitine devoid of toxicity, but GBB and crotonbetaine had weak curare-like action. Injection of GBB and crotonbetaine into mice caused increased respiration, profuse salivation, red-tears secretion, dilation of pupils, convulsion, constriction of blood vessels, paralysis of motor nerve endings, diuresis, diarrhea and cardiac arrest in diastole.

Carnitine was also found in the grey matter of the central nervous system. Intravenous injection of carnitine, in high doses, showed no central effect. It is likely that carnitine did not pass through the blood-brain barrier. Intraventricular administration circumvents the blood-brain barrier. Thus Purpura *et al.* (114) showed that carnitine and γ-aminobutyrobetaine, when administered intraventricularly, exerted strong synaptic action. Both these substances were found to be selective blockers of inhibitory synapses.

Of special interest is the activity of acetyl-l-carnitine (AC) on isolated organs. This substance was briefly mentioned by Engeland (115) in 1905. Krimberg and Wittandt (116) acetylated natural carnitine and extensively described the chemical properties of its derivatives. On the basis of the similarity of structure of AC to ACh, these authors indicated that it was not a priori to suggest that AC could be formed in animal tissue and to play a role in physiological events. Furthermore, this possibility was likely on account of the high concentration of l-carnitine in muscle tissue and its easy acetylation. The physiological role of AC was realized by Weger in 1936 (117), who showed for the first time one of the biological functions of carnitine. The test object employed by Weger was the frog heart, on which he showed carnitine itself in large doses caused an irreversible damage to the heart, resulting in negative chronotropy which was only partially inhibited by atropine. Acetyl-l-carnitine, however, in small doses had a negative inotropic and chronotropic effect on the heart, which was enhanced by eserine and abolished completely by atropine. The activity of AC was destroyed after incubation with a heart tissue extract. This substance had, like ACh, only a slight alkali resistance.

Strack and Forsterling (118) could not repeat the work of Weger. They found that AC was inactive on the frog heart, the frog rectus abdominis, leech dorsal muscle and mouse intestine preparations. They came to the conclusion that the ACh-like effects of AC were caused by contamination of preparations of

natural carnitine with choline and, after acetylation with ACh. This conclusion is open to doubt. Strack and Forsterling assumed their preparation was a mixture of choline and carnitine (by their inference, Weger's preparation was also a mixture of choline and carnitine) and tried to obtain pure carnitine by repeated recrystallization in absolute ethanol. The recrystallizable material, presumably carnitine, when acetylated, was found to be devoid of activity. These authors, by their own assumption, should have had choline as the non-recrystallizable material, since choline is easily soluble in ethanol. They did not attempt to acetylate the available choline in ethanol solution and hence demonstrate its pronounced biological activity. Their conclusion was, therefore, based on an incomplete experiment.

It was not surprising, in view of this contradiction, that Dallemagne et al. (119) undertook to study the effects of l-carnitine, its methyl and ethyl esters, AC and the dihydrochloride of the disulphide of β-mercapto -γ-butyrobetaine on different elements of the cholinergic system. They demonstrated that AC had between 1/4 to 1/10 the activity of ACh on the frog rectus. The methyl ester of l-carnitine was, in fact, more active than ACh on the frog rectus; while the ethyl ester was less active than the methyl ester and ACh. Acetyl-l-carnitine, the methyl and ethyl 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).

Gamma-butyrobetaine itself is pharmacologically inert, but its aliphatic esters are parasympathomimetic agents with activity less than that of ACh. Hosein and McLennan (120) have shown that the methyl ester of GBB can duplicate in mice the effects described by Linneweh for GBB, in comparison with GBB itself, was effective at a smaller dosage. This substance was found by these authors to possess most of the physiological properties ascribed to ACh; with the exception that it was not hydrolysed by cholinesterase. When assayed on the cat's blood pressure preparation, the synthetic aliphatic methyl and ethyl esters of GBB caused a fall in the blood pressure, but they were 45 times less active than ACh. Similar results were obtained in other preparations such as isolated frog heart and the frog rectus abdominis (121). On these preparations, Strack and Forsterling have shown that the methyl ester of GBB was 1/12 and 1/4 the activity of ACh respectively (122). Hosein et al. (123) found the ethyl ester of GBB resembled ACh in its pharmacological action, in that it depressed the blood pressure of the cat, stimulated the perfused cervical ganglion in the cat and inhibited the isolated perfused frog heart and mammalian neuromuscular transmission. This synthetic ester was not affected by esterases commonly found in tissues and therefore can exert an unhindered action (123).

In association with other betaines which occur in muscle tissue, crotonbetaine is found, although in smaller quantities (104,105,106,107). Crotonbetaine itself exhibits weak curare-like effect (105), but its esters are parasympathomimetic

agents. Strack et al. (106,107) have shown that the methyl ester of crotonbetaine acts like ACh on isolated organs, the mouse intestine, frog rectus abdominis, frog heart and leech dorsal muscle. Although the ethyl ester acted similar to ACh on the frog rectus abdominis and the leech dorsal muscle, its effects were less marked on the mouse intestine and on the frog heart. In addition, the ethyl ester of crotonbetaine antagonized the action of ACh on the frog heart. The esterification of crotonbetaine by a methyl group strengthened its effect on the mouse intestine 5,000 fold; the rectus 10,000 fold and the frog heart 50,000 fold. The enhancement of activity of ethyl esterification was less pronounced. Later, Strack and Forsterling (122) demonstrated that the methyl ester of crotonbetaine had 1/15 and 1/4 the activity of ACh on the frog heart and the sensitized frog rectus preparations respectively. Burgen and Hobbiger (124) have confirmed and extended these observations for the methyl ester, demonstrating its action on numerous biological preparations. Both its muscarinic and nicotinic types of action were reaffirmed and they varied from 1/15 to 10 times that of ACh according to the test preparation used. The ester was not split by cholinesterase and hence had a more prolonged action than ACh on unsensitized test preparations.

G. The Naturally Occurring Betaine Esters

In 1953, Banister et al.(57) demonstrated chromatographically the presence of ACh and propionylcholine (PCh) and a faster running component (butanol-water system) in alcoholic extracts of ox spleen. This faster running component was termed

component F and accounted for 26% of the total activity. The location of ACh-like activity in this R_F 0.7 region was also shown by Beznak in TCA extracts of rat heart tissue (56). In another chromatographic solvent system, namely, the Augustinsson system, where the normal position of pure ACh is R_F 0.45, Levy and Pierron (125) showed that ACh-like activity could be recovered in the R_F 0.7 region from acidified alcohol and cadmium hydroxide extract of ox brain. These authors also showed that acidified alcohol and cadmium hydroxide used to deproteinise the brain tissue did not alter the mobility of ACh.

Hosein (126) detected a component analogous to component F of Banister et al. in the debris fraction of extracts of normal rat brains and he added the observation that this material could be identified as the betaine esters, since chromatographic analysis of acid hydrolysate of this material revealed the presence of GBB and carnitine. Subsequently, Hosein and Proulx (103) and Hosein (127) chemically identified GBB, crotonbetaine and carnitine in the acid hydrolysate of the F component derived from extracts of brains from normal and convulsed rats.

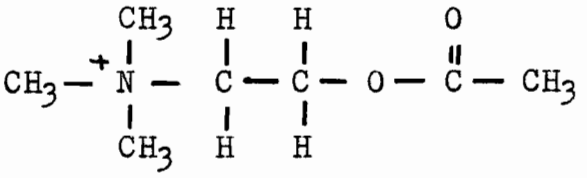
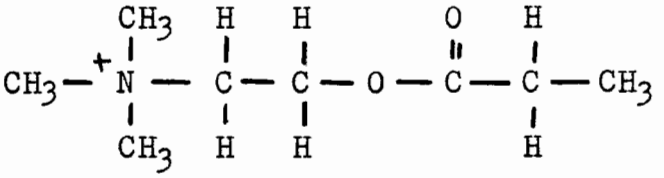
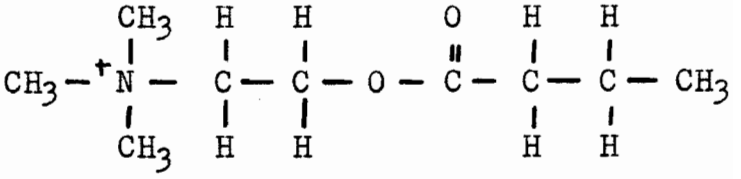
Hosein, Proulx and Ara (108) have described the chromatographic separation and identification of the components of normal rat brain extracts. They demonstrated that the ACh-like activity was separable into 4 distinct bands. The components with R_F lower than 0.50 in this system, after conversion to tetrachloraurate derivatives, were chemically identified as ACh, PCh and butyrylcholine (BuCh) corresponding to R_F 0.05-0.15,

R_F 0.15-0.25 and R_F 0.25-0.35 respectively. The band with higher R_F (0.50-0.70) was shown, in a similar manner, to consist of GBB, crotonbetaine and carnitine. This component on acid hydrolysis yielded phosphate, β-alanine, ribose and adenine, which are moieties of the CoA esters of GBB, crotonbetaine and carnitine. The correct identification was enhanced by the observation that these betaines probably occurred normally in the tissue in their conjugated forms, since their appearance was elicited only by Reinecke Salt precipitation at alkaline pH (128). More recent work has revealed the presence of AC in the R_F 0.50-0.70 band (129). The unhydrolysed material from this band demonstrated ultraviolet absorbance at 232 and 260 mμ; alkaline treatment decreased the absorbance at 232 mμ, but did not alter that at 260 (Hosein et al., unpublished data).

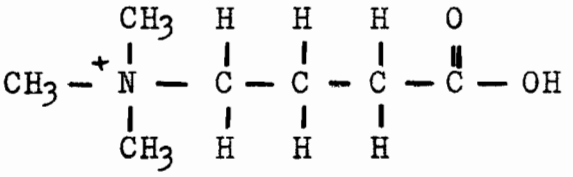
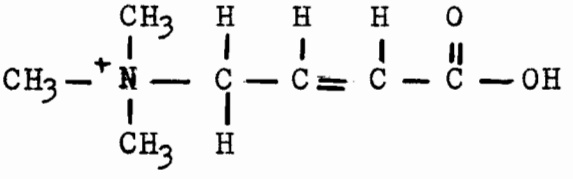
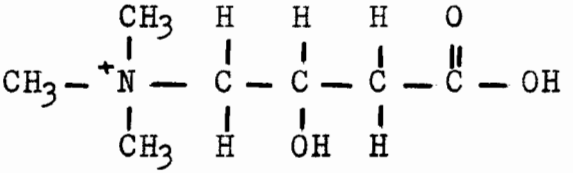
These substances in the band of the chromatogram with R_F 0.50-0.70 were, therefore, identified as the betaine CoA esters and contributed to 75% of the total ACh-like activity found in TCA extracts of normal rat brain (108). Gamma-butyrobetainyl CoA has been enzymically synthesized and shown to have an R_F of 0.55 in the butanol-water system (130). It is active on the frog rectus abdominis preparation. Hosein and Smoly (unpublished data) have enzymically synthesized acetyl-l-carnityl CoA. Preliminary results show this substance also has an R_F 0.50 in the butanol-water system and is active on the frog rectus abdominis preparation.

The chemical structures presented below are the choline esters and the synthetic and naturally occurring betaine esters which have been described in the discussion above.

Choline Esters

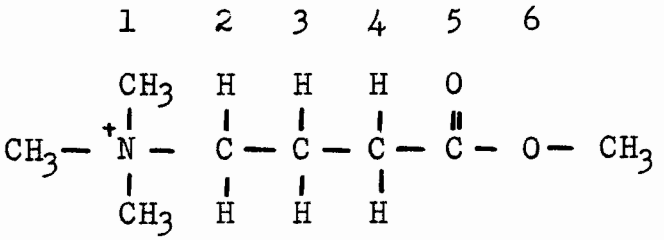
Substances	Positions of atoms 1 2 3 4 5 6
ACh	
PCh	
BuCh	

Unconjugated Betaines

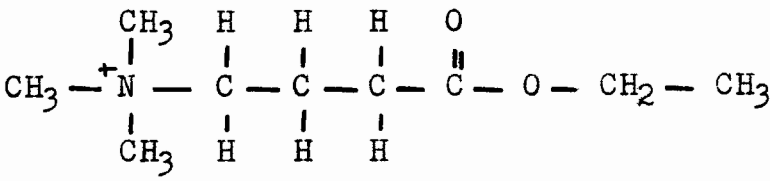
GBB (gamma-butyrobetaine)	
crotonbetaine	
carnitine	

Synthetic Aliphatic Betaine Esters

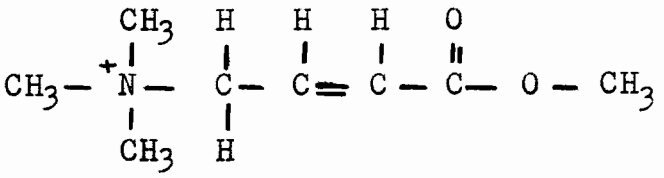
GBBM
(Methyl ester of gamma-butyrobetaine)



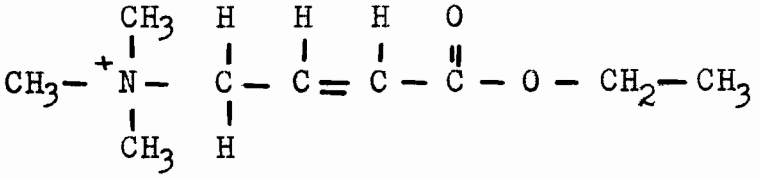
GBBE
(Ethyl ester of gamma-butyrobetaine)



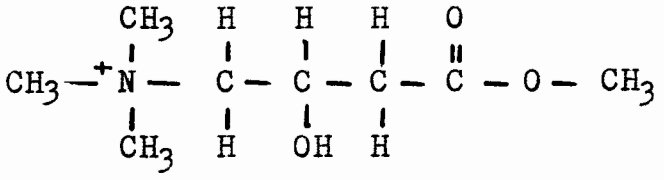
crotonbetaine M
(Methyl ester of crotonbetaine)



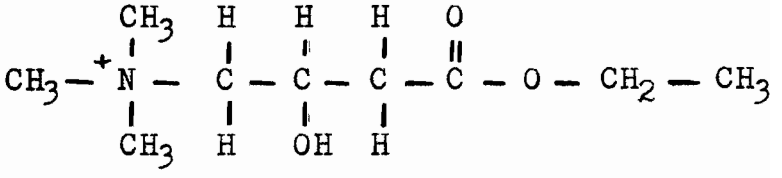
crotonbetaine E
(Ethyl ester of crotonbetaine)



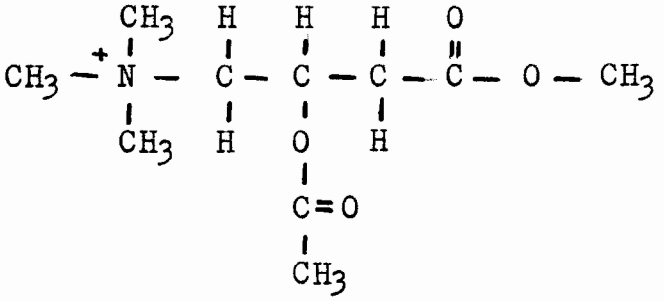
carnitine M
(Methyl ester of carnitine)



carnitine E
(Ethyl ester of carnitine)

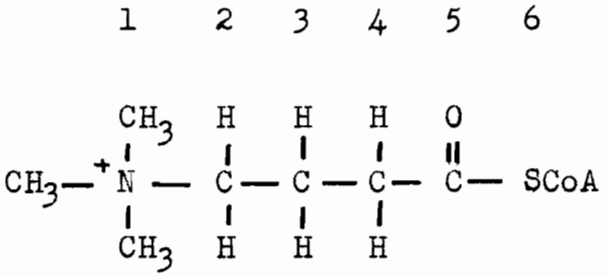


acetylcarnitine M
(Methyl ester of acetylcarnitine)

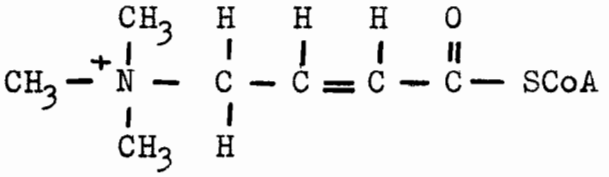


Naturally Occurring Betaine CoA Esters and Acetyl-l-carnitine

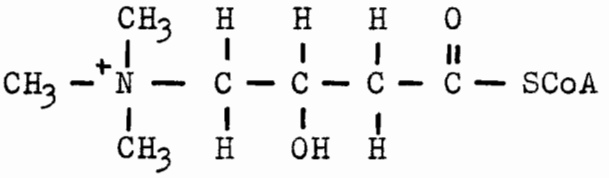
GBB CoA ester



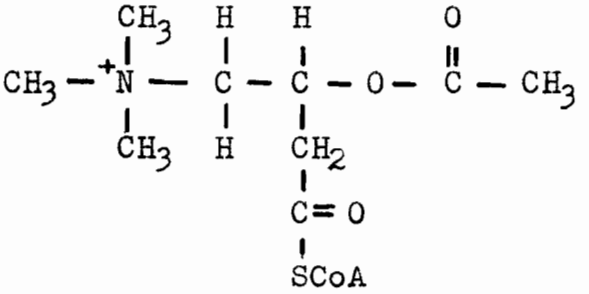
crotonbetainyl CoA ester



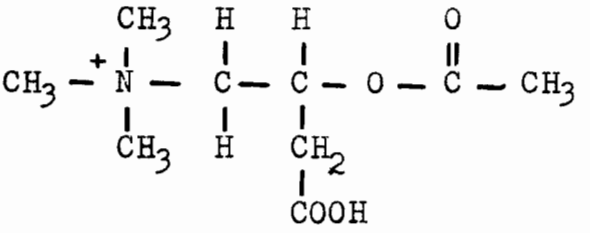
carnityl CoA ester



acetyl-l-carnityl CoA ester



AC (acetyl-l-carnitine)



It is well established that for a substance to have ACh activity it must possess both the quaternary ammonium and carbonyl radicals (131).

Pfeiffer (132) suggested from a study of the pharmacological actions of congeners of ACh, that the ester end was mainly responsible for muscarinic activity while the onium head represented predominantly nicotinic activity. The data were derived from the anaesthetized cat or dog where the depressor response was recorded as muscarinic and the pressor response after atropine was the nicotinic potency.

The naturally occurring betaine CoA esters all possess both of these radicals. In fact, AC and acetyl-l-carnityl CoA duplicate the structure of ACh from atoms 1 to 6. There is evidence that the two linkages in acetyl-l-carnityl CoA might contribute to the overall activity of this molecule on the frog rectus (133). Measured from atoms 1 to 6, AC, acetyl-l-carnityl CoA and ACh have identical bond length.

Careful examination of the structure of ACh on one hand and the carboxyl aliphatic and CoA ester of the betaines on the other shows that the polarity of the ester linkage is reversed in the latter. Hunt and Renshaw (134) have studied the effect of reversing the polarity of the ester linkage in ACh homologues. They prepared aliphatic esters of glycine betaine and showed that these substances were physiologically active with marked "muscarinic" action but with less pronounced "nicotinic" action. On the other hand, Strack and Forsterling

(122) showed that the methyl ester of β-homobetaine had 0.4 and 0.9 the activity of ACh on molar basis on the frog heart and the sensitized frog rectus respectively.

H. A Criticism of Acetylcholine Identification

Since the observation of Schafer and Moore (135) that an extract of brain tissue, made with boiling water or saline solution, when injected into the veins of animals, caused a fall in blood pressure, there has been a controversy about the nature of the substance in these extracts. Mott and Halliburton (136) suggested the substance was choline, but Vincent and Sheen (137) provided evidence that it was not choline.

Later investigations (138,139,140) have shown that there are several depressor substances in the brain. In fact, Major and Weber (139), after comparing the chemical and pharmacological behaviour of brain extracts and pure ACh, concluded that the depressor activity of brain extracts was not due to histamine, choline or ACh.

The basis for the establishment of ACh as the parasympathetic transmitter has been furthered mainly by:

1. Maximal nicotinic and muscarinic potency of ACh.
2. Bioassay of rat brain extracts by the use of the frog rectus abdominis preparation, Richter and Crossland (141).
3. Bioassay on various test objects in comparison to pure ACh, Chang and Gaddum (34).

4. Demonstration of ACh activity in horse spleen extracts, Dale and Dudley (78).
5. Extraction of ACh from mammalian brain tissue synthesized in vitro, Stedman and Stedman (142).

1. Maximal nicotinic and muscarinic potency of ACh.

The prior knowledge of the potent nicotinic and muscarinic activity of ACh, even before this substance was found in the tissues, was made use of to fit the proposed ACh theory of transmission. However, the biological activity of ACh is overshadowed by that of PCh (34).

2. Bioassay of rat brain extracts by the use of the frog rectus abdominis preparation, Richter and Crossland (141).

This investigation by Richter and Crossland is a good example in which ACh has been identified in extracts or perfusates on the basis of bioassay upon a single test object. Based on this procedure there is a definite uncertainty of whether the substance is really ACh. The product of the choline acetylase system is predetermined by the nature of the substrates. Korey, de Braganza and Nachmansohn (143) have shown that purified choline acetylase can utilize propionic acid to synthesize an active choline ester and Gardiner and Whittaker (144) have provided evidence that this product is PCh. As to the possible occurrence of bioactive non-choline esters in nervous tissue, Nachmansohn, Hestrin and Voripaieff (145) provided the first hint. These authors reported the biosynthesis of a bio-active substance by a brain ACh-synthesizing enzyme, in the absence

of choline as a substrate. The product which was, therefore, not a choline acetate ester, was termed the enzymically formed factor. The results of Middleton and Middleton (146) showed that this factor had less activity (relative to ACh) on the frog rectus than on the cat's blood pressure and the frog heart. The depressor activity of this factor on the cat's blood pressure and the frog heart was abolished by atropine. Hosein and Smoly (147) have synthesized AC, using the Feldberg's ACh synthesizing system, but employing the substrates carnitine and acetate. These results indicate the nature of the substrate can predetermine the path of the acetylating system and that bioassay based on a single test object cannot determine the nature of the product, much less the active substance(s) in tissue extracts.

3. Bioassay on various test objects in comparison to pure ACh, Chang and Gaddum (34).

The general principles regarding biological demonstration of ACh have been discussed by Chang and Gaddum (34). These authors compared the relative potencies of several pure synthetic choline esters on different biological test objects. These choline esters were found to have different potencies (relative to ACh) on the normal and eserinized frog rectus abdominis, the eserinized dorsal muscle of the leech, the rabbit intestine and the rabbit's blood pressure preparations. Chang and Gaddum drew the conclusion that the biologically active material in tissue extracts could be considered as ACh if, using several different biological test objects, the same quantitative

results in terms of ACh should be obtained in each case. This conclusion can be accepted only with reservations, because Chang and Gaddum never demonstrated that parallel quantitative assay could differentiate between several bioactive substances in admixture with ACh (which could be present in tissue extracts) and pure ACh.

Gardiner and Whittaker (144) have chemically identified PCh in spleen extract. Dale and Dudley (78) extracted ACh activity from the horse spleen. The biologically active materials in the spleen extracts assayed identically on 3 test preparations (rabbit jejunum, cat's blood pressure and cat denervated gastrocnemius). The active material was logically but erroneously considered as entirely ACh, to the exclusion of PCh. This example shows that in experiments where differential assay results of biological activities of tissue extracts may agree, no reliable conclusion can be drawn. Conversely, in experiments in which differential assay results differ, several active substances in the extracts are indicated. Although Chang and Gaddum (34) presented no data, they concluded that, on basis of parallel bioassay, ACh alone contributed to the biological activity of tissue extracts. This conclusion is erroneous. For, as pointed out above, parallel quantitative assay on as many as 3 different test objects failed to differentiate PCh in admixture with ACh in spleen extracts. Gardiner and Whittaker (144) successfully used the guinea pig ileum and the frog rectus abdominis preparations to differentiate pharmacologically between PCh and ACh. Unless one uses the

distinguishing test object, i.e. the guinea pig ileum, the presence of a proportion of PCh in admixture with ACh in spleen extract might easily escape detection. This obstacle can no longer be considered as a minor objection ever since Hoesin et al. (108) chemically identified several choline and betaine esters in brain extracts. Traditionally, the ACh activity of brain extracts is considered identical on parallel bioassay. However, there are reports in the literature, in which the use of distinguishing test objects such as the guinea pig ileum and the frog heart has shown this identical relation does not hold. These data are summarized.

TABLE I

Total ACh of Sciatic Nerve Tissue Extracts Assayed on the Frog Rectus Abdominis and the Guinea Pig Ileum (Carlini and Green) (148).

µg. ACh/gm and S.D.

Assay on frog rectus abdominis (r)	Assay on guinea pig ileum (i)	Ratio r/i calculated by the writer
2.55 ± 0.43	1.57 ± 0.41	1.6/1.0

S.D. Standard Deviation. This abbreviation is used in all subsequent tables.

From Table I, it is seen that the frog rectus preparation assayed the biological activity of the sciatic nerve extract at a value 60% higher than by the guinea pig ileum preparation. Carlini and Green also noted that to prevent the action of the extract on the frog rectus, a greater concentration of

d-tubocurarine was required than for known ACh. They concluded that extracts of sciatic nerve exhibited ACh-like activity that was only partly attributable to ACh.

TABLE II

Total ACh of Rat Brain Extract, µg./gm, under various Physiological Conditions (Richter and Crossland) (141)

Assay Preparation / Condition	Frog Rectus (r)	Leech Dorsal Muscle (d)	Cat Blood Pressure (p)	Ratio r/d/p calculated by the writer
Normal	1.27	1.55	2.0	0.6/0.8/1.0
Anaesthesia	1.7	2.7	2.5	0.7/1.1/1.0
Sleep	1.43	2.4	-	0.7/1.2/ -
Convulsion	0.57	1.4	1.5	0.4/0.9/1.0

From Table II, it is observed that in each case the tissue extracts prepared from brains taken from various physiological conditions did not assay identically. In the absence of really distinguishing test objects, the deviation is small, but still apparent.

Tables IIIa and b describe the inequiactivity of rat cerebral cortex extracts assayed on the Venus heart and on the frog heart. These distinguishing test objects clearly show the difference in potencies (relative to ACh) in each sample of tissue extract. In Table IIIa, the ratios range from 2.7 to 1 to 13.0 to 1. In Table IIIb, where free ACh was defined as ACh

TABLE IIIa

Total ACh of Rat Cerebral Cortex under Normal and Anoxic Conditions (Welsh) (149)

µg. ACh/gm tissue

Condition	Rat no.	Assay on Venus heart(a)	Assay on Frog heart(b)	Ratio a/b calculated by the writer
Normal	19	2.8	1.00	2.8/1.0
	24	1.6	0.50	3.2/1.0
	27	1.4	0.25	5.6/1.0
	29	0.9	0.30	3.0/1.0
	32	1.2	0.25	4.8/1.0
Anoxic	18	2.0	0.80	2.5/1.0
	25	1.3	0.10	13.0/1.0
	28	0.7	0.12	5.8/1.0
	30	0.4	0.15	2.7/1.0
	31	1.0	0.25	4.0/1.0

TABLE IIIb

Free ACh of Rat Cerebral Cortex under normal and Anaesthetized Conditions (Welsh) (149)

µg. ACh/gm tissue
No. of Experiments in Parentheses

Condition	Assay on Venus heart(a)	Assay on Frog heart(b)	Ratio a/b calculated by the writer
Normal	0.36 (9)	0.26 (9)	1.4/1.0
Anaesthesia	0.43 (3)	0.33 (3)	1.3/1.0

activity extracted by cold Ringer solution, the Venus heart assayed the biological activity of the extract consistently higher than by the frog heart. It would appear that rat cerebral cortex contained several bioactive substances.

TABLE IV

ACh Activity of Rat Heart Tissue Extracts under Normal and Thiamine deficient Conditions Assayed on the Frog Rectus Abdominis and the Guinea Pig Ileum Preparations. (Beznak) (56).

µg. ACh/gm tissue

Condition	Assay on Frog Rectus Abdominis(r)	Assay on Guinea Pig Ileum (i)	Ratio r/i calculated by the writer
Thiamine deficient	1.87	1.29	1.4/1.0
Pair fed	1.05	0.95	1.0/1.0
Normal	0.44	0.57	0.7/1.0

From Table IV, the frog rectus abdominis and the guinea pig ileum preparations assayed differently the biological activity of heart tissue extracts from normal and thiamine deficient rats. It would appear that other bioactive substances besides ACh are present in the rat heart.

The guinea pig ileum and the frog heart preparations seem to be distinguishing test objects for brain extracts. Thus Holtz and Schumann (150) and Ambache (151) have provided evidence for the inequiactivity of brain extracts assayed on these 2 preparations. Ryall (152) found that extract from P_2 fraction

of brain homogenates assayed identically on 4 test objects, but not on the toad atrium. Finally Crossland, Pappius and Elliott (153) found that TCA extracts of frozen brain tissue gave identical results on the eserinized frog rectus and the eserinized dorsal leech muscle. However, the leech dorsal muscle gave variably higher values of acid saline extracts; the average of 14 comparisons differed by 25% and the difference was statistically significant ($p<0.01$).

As discussed above, random selection of test objects may obscure the main objective of differential assay. From this point of view the statements (no data were given) of Feldberg (154), Feldberg and Gaddum (9) and MacIntosh (16), regarding the identical differential assay results of tissue extracts and of perfusates may have no significance at all.

The work of Chang and Gaddum (34) also provided 2 specific tests for the identification of ACh.

i. Production of a vasodepressor action which is abolished by atropine.
ii. Production of a contraction of the isolated frog rectus abdominis which is enhanced by eserine.

It must be realized that these pharmacological tests are not applicable for identification of ACh in tissue extracts. As early as 1936, Weger (117) showed that they applied to AC just as well as to ACh. Also Nachmansohn's enzymically formed factor which was not a choline ester depressed the amplitude of the frog heart beat and the cat's blood pressure, effects which

were abolished by atropine.

4. Demonstration of ACh activity in horse spleen extracts, Dale and Dudley (78).

Contrary to common belief, Dale and Dudley (78) did not chemically identify ACh in spleen extracts. These authors demonstrated the likely presence of ACh. Their pharmacological identification of ACh in spleen extracts has been shown to be erroneous, as described in the above discussion.

5. Extraction of ACh from mammalian brain tissue synthesized in vitro, Stedman and Stedman (142).

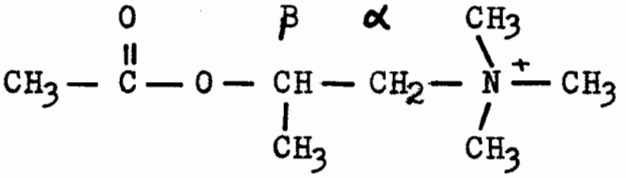
The greatest drawback in the ACh theory is the decided lack of chemical identification. Although Stedman and Stedman (142) chemically identified ACh in ox brain, close examination of their results reveals certain discrepancies. These authors obtained 1400 mg. of chloroplatinate derivative. On conversion to its tetrachloroaurate and its subsequent identification as ACh, 99% by weight of the original chloroplatinate was lost. Since the loss was almost total, it is likely other active materials were discarded.

There is confusion in the literature regarding ACh identification. Bacq and Mazza (155) isolated ACh and carnitine from squid nerve. Bischoff, Grab and Kapfhammer (82) believe they have isolated choline and ACh from skeletal muscle with their methods, while Strack et al. (156) express the view that the substances, isolated as the reineckate salts and claimed by the first named authors as choline or ACh, might be

the reineckate of carnitine whose gold content is almost similar to that of choline and ACh.

Structure activity studies provide serious discrepancies of the ACh theory. In a recent review, Pfeiffer (132) has cast doubt on the significance of ACh as the parasympathetic transmitter. He contends that there are a number of parasympathetic transmitters. Pfeiffer's arguments are summarized here.

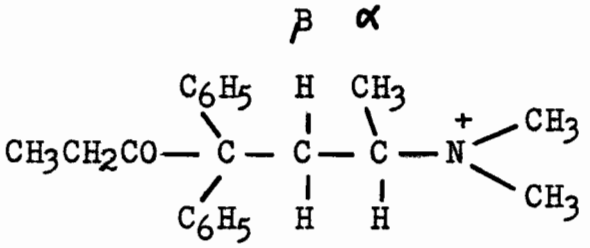
i. Cholinesterase 11 will hydrolyse many choline esters, while cholinesterase 1 will hydrolyse most specifically acetyl-β-methylcholine.



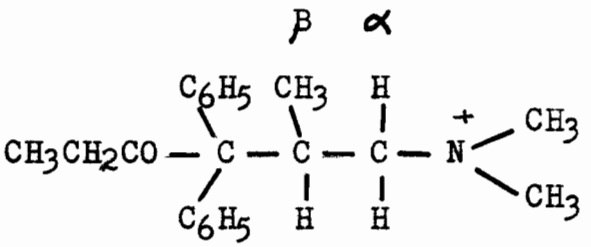
Acetyl-β-methylcholine

Substrate specificity of cholinesterase 1 on acetyl-β-methylcholine suggests that one of the parasympathetic transmitters should occur with a β carbon substitution.

ii. Greig and Howell (157) studied the cholinesterase inhibition produced by the d- and l- isomers of the analgesic drugs methadone and isomethadone.



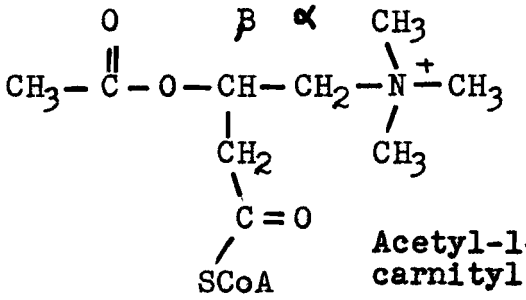
Methadone



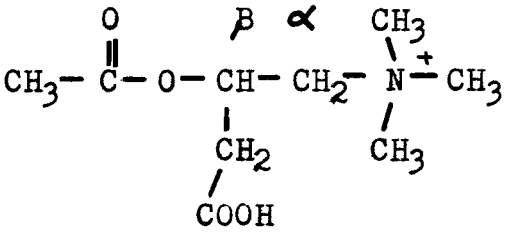
Isomethadone

Methadone has an α carbon substitution, while isomethadone a β carbon substitution. β carbon substitution confers optical isomerism. These authors found that l-isomethadone is about 30 times more potent than the d- isomer in inhibiting cholinesterase 1, and that the l-isomer of methadone is only twice as potent as the d- isomer. This again indicated the need for a β carbon substituent, rendering the β carbon asymmetric, on the parasympathetic neurotransmitter which should be hydrolysable by cholinesterase 1.

This condition cannot be fulfilled by ACh. It should be pointed out that the β carbon substituent on AC or acetyl-l-carnityl CoA molecule fits this hypothesis admirably.



Acetyl-l-carnityl CoA



Acetyl-l-carnitine

iii. Finally, if in cerebral tissue the pharmacological effect of epinephrine may be that of modulation of the ACh effect to produce a transient degree of atropine-like action, then the β-hydroxy group on the epinephrine molecule should be mirrored by a β-hydroxy or isosteric group on the parasympathetic transmitter(s) structure (132). It should be pointed out that the β hydroxy group is lacking in the ACh molecule, but is present in the l-carnityl CoA molecule.

I. The Mechanism of Narcosis

Theories of Narcosis

(i) Lipoid Solubility Theory

Claude Bernard (158) observed that some drugs, either in large doses or acting for a long time, caused a reversible depression of all living cells. Such drugs he termed narcotics and the process, narcosis. The investigation of the phenomenon of narcosis, therefore, had begun much earlier than 1846, when Morton introduced ether into surgical practice. In the following year, Bibra and Harless, studying the correlation between the effects of ether and ethylchloride on mammals and amphibians on one hand, and the fat content of brain on the other, concluded that these agents were solvents for ordinary fat and fat-like substances. These latter substances, they believed, were dissolved out of the brain and deposited in the liver, causing narcosis. Although their conclusion was not borne out experimentally, their work called attention to the solubility of narcotics in lipoids and served as the precursor of the lipoid solubility theory.

The definite relationship between solubility in lipoid material and substances which have narcotic action was established by Hans Meyer (159). Meyer expressed his theory in the form of three postulates.

1. Alle chemisch zunächst indifferenten Stoffe, die für Fett und fettähnliche Körper löslich sind, müssen auf lebendes Protoplasma, sofern sie darin sich verbreiten können, narkotisch wirken.

2. Die Wirking wird an denjenigen Zellen am ersten und stärksten hervortreten müssen, in deren chemischem Bau jene fettahnlichen Stoffe vorwalten und wohl besonders wesentliche Träger der Zellfunktion sind: in erster linie also an dem Nervenzellen.

3. Die verhaltnissmässige Wirkungsstärke solcher Narcotica muss abhangig sein von ihrer mechanischem Affinität zu fettähnlichen Substanzen einerseits, zuden übrigen Körperbestandtheilen, d.i. hauptsachlich Wasser anderseits; mithin von dem Theilungscöefficienten, der ihre Vertheilung in einem gemisch von Wasser und fettahnlichen Substanzen bestimmt.

Independently but simultaneously, Overton (160) came to the same conclusion as a result of his work on cellular permeability. The findings of these two workers were formulated into the Meyer-Overton Law, which had the tenet that fat solubility was the chief characteristic of effective narcotic substances. They established that there was a relation between narcotic potency of a given substance and its partition coefficient, $\frac{\text{Solubility in fat}}{\text{Solubility in water}}$ usually determined in olive oil. The law implied that, the higher the partition coefficient, the more potent was the narcotic and, as put forward by K.H. Meyer (161), equal depths of narcosis were attained by different substances when they were present at the same molecular concentrations in the lipoids.

Winterstein provided data that the parallelism between narcotic strength and the partition coefficient appeared to be a fixed relationship. This strongly supported the lipoid solubility theory, but he pointed out that it was a theory of narcotics rather than narcosis and the many discrepancies made it hard to accept it, e.g. narcotic action could be exerted on acetone extracted yeast which were quite free from lipoids (162).

Bancroft et al.(163) held the view that the lipoid solubility theory only defined the physical properties a narcotic must have and considered it a theory of transport, not of narcosis. He drew attention to the typical actions of narcotics on enzymes, in which lipoid was not involved.

It was known that magnesium ion and chloral hydrate were capable of inducing narcosis in animals, but they have negligible or no solubility in oil. Their narcotic action, therefore, cannot be explained by the theory. Conversely, many substances have more affinity for oil than water and yet cannot induce narcosis.

K.H. Meyer (164) later indicated that this theory took into account observed experimental facts, but agreed with Verworn (165) and others that it did not explain the mode of action of narcotics.

(ii) The Adsorption Theory of Narcosis

Traube (166) in 1904 observed that substances which lowered the surface tension of water passed more easily through cell membranes than substances which did not exert this effect. He also found that the ability of the members of a homologous series of alcohols to penetrate the cell or to lower surface tension at air-water interface increased in series, as 1:3:9. Since it was already known that these members exhibited narcotic action in a like manner, he suggested that a relation existed between narcotic strength and ability to lower the surface tension of water.

Meyer criticised this rule, since there was no distinct relationship between capillary activity and narcotic strength of most narcotic drugs. Traube was finally forced to the conclusion that, while strongly water-soluble narcotics depended on capillary activity for their penetration into cells, the "true" narcotics depended more on the presence of lipoids. However, Traube's rule paved the way for the adsorption theory.

From earlier work, Lillie (167) considered that narcotics were adsorbed on cell surfaces. Warburg (168) found that narcotics of various types would depress the oxidation of many kinds of living cells and that the effect was reversible and proportional to the drug concentration. With a respiration model which consisted of animal charcoal, Warburg observed that oxalic acid, when adsorbed on it, resulted in its oxidation; narcotics prevented this oxidation. He then explained the depression of oxidation of cells by narcotics as due to differential adsorption of narcotics on cell membranes which displaced enzymes from vital surfaces. However, Warburg's charcoal models did not provide any evidence that there was any significant adsorption of narcotics on "vital surfaces" in the cells of living organisms.

(iii) Coagulation and Flocculation Theories

Binz stated that brain cells when exposed to high concentration of morphine would be coagulated and the early stages of this process were reversible. If the reaction was allowed to progress further it became irreversible. Bernard (158), continuing this line of study, suggested that narcosis

might be due to some kind of reversible semicoagulation of substances in sensory nerve cells. Bancroft and Richter (163) supported Bernard's hypothesis and attempted to refute criticisms of this theory by trying to explain:

i. The concentrations at which narcotics are active are much less than those required to coagulate the cell colloids.

ii. Coagulation of cell colloids represent toxic effects of a narcotic and are irreversible; whereas, narcosis is a reversible phenomenon.

Bancroft and Richter argued that the narcotic concentration of a drug in the tissue during narcosis could not be used to overthrow the theory, since there was no reason to believe that narcotics acted equally on all of any organ but, rather, many observations showed that lipoids were the site of action. More specifically, they cited other evidence which indicated that the surface of the cell was involved primarily. Henderson and Lucas (169) pointed out the weakness of Bancroft and Richter's deductions and they rejected the coagulation theory of narcosis on the grounds that its authors had not been able to demonstrate that coagulation always accompanied narcosis.

(iv) Cell Permeability Theory

Hober, Lillie and Winterstein proposed theories which have in common the postulation that narcosis is concerned with decreasing the permeability of cell membranes (167,170,171). Hober (170) appears to have been the first to observe the association between narcosis and cell membrane permeability.

Winterstein (171) noticed that narcotics reduced the permeability of frog muscle membrane to water. Lillie, as a result of experimental observations of narcotic effects on ionic permeability of Arenicola larvae (172,173), proposed the theory that narcotics act by preventing the depolarization of nerve cell membrane by abolishing the increased permeability of ions on which depolarization depended. Hober considered lipoid in cell membrane was important in controlling permeability changes; Winterstein did not think this was the case.

Quastel and Wheatley (174) agreed that the burden of evidence indicated that adsorption of narcotics at the cell surface or at intracellular structures influenced the metabolic activities of the cell. In addition, these authors observed that the nature of the lipoids present controlled the extent of the quality, amount and site of adsorption. However, there was no experimental evidence to show which specific metabolic reactions were involved, nor what connection existed between metabolic disturbances and functional activity. The cell permeability theory was not substantiated by Brooks (175) on the grounds that there was no general rule concerning the effects of narcotics on permeability but he added, as suggested by Quastel and Wheatley (174), the alternate possibility of competitive inhibition by the narcotic with an enzyme or enzymes was involved in metabolic reactions.

(v) Theories of Narcosis Based on Inhibition of Oxidation

Verworn (165) pointed out that narcosis was accompanied by decreased oxidation and proposed that oxygen defic-

iency produced the narcotic phenomenon. This theory implied that narcosis was due to incapacity of cell substances to undergo oxidation, although non-oxidative processes could proceed unhindered, thus linking narcosis with metabolism. Oxygen deprivation was, however, an effect rather than the cause of narcosis. The oxygen deprivation theory was rejected when it was shown that indole inhibits brain respiration (176), but that its administration in quantities sufficient to secure 30% to 50% inhibition of brain respiration led to convulsions rather than narcosis (177).

Larrabee, Ramos and Bulbring (178) provided evidence that narcotics depressed neuronal activity by some mechanism which did not interfere with oxygen consumption. This conclusion was drawn from data derived from parallel measurements on nerve transmission and oxygen consumption in excised superior cervical sympathetic ganglia of rabbits. The rate of oxygen consumption was determined by the rate of fall of oxygen concentration and transmission by the height of postganglionic action potential evoked by preganglionic nerve stimulation. These workers were able to show that all the narcotics tested (chloretone, pentobarbital, ether, chloroform and alcohols from methanol to octanol) except urethane, depressed synaptic transmission reversibly without slowing the resting oxygen consumption. These narcotics slowed resting oxygen consumption markedly at high concentrations. The extra oxygen consumption caused by repeated stimulation was depressed by pentobarbital or chloretone in direct proportion to the depression of activity.

The concept of mechanism of narcosis based on the inhibition of the enzyme system necessary for the oxidation of carbohydrates in brain was formulated by Quastel and Wheatley in 1932 (174). 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 (179). Quastel more recently (180) is of the view that the major effect of a narcotic is not the suppression of 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 bonds of ATP, i.e. narcotics act by suppressing the rate of oxidative development of ATP in the nerve cell. However, to achieve this in vivo required a concentration of narcotics up to 50 times higher than is required to induce narcosis (181). Quastel (182) has emphasized that, although the distribution of a narcotic is homogeneous in brain tissue, its effect is produced by its localization in certain specific sites in vivo; and in vitro the concentration of narcotic is reduced by diffusion into the suspension medium, so that a higher concentration of narcotic is needed in vitro than is required in vivo; this would be expected on account of the reversibility of narcotic action.

The suggestion has been put forward by McElroy (183) that reversible denaturation of enzyme systems controlling metabolic processes induces a state of narcosis. An equilibrium was thought to exist between native and denatured enzyme systems and that narcotics shifted the equilibrium to the

denatured form.

(vi) Pauling's Molecular Theory of General Narcosis

The rich lipid composition of the brain has led to the formulation of a number of theories of narcosis involving essentially the incorporation of narcotic molecules into the non-aqueous phase of the cell membrane and the consequential depression of neuronal function. Pauling (184) has pointed out that the brain, like other tissues of the body, consisted largely of water (about 78% water as compared with about 12% lipid and 8% of proteins) and has come forward with a concept that emphasizes the aqueous phase of the living cell. For a long time it was known that water was not a random solution of single water molecules; even in the absence of cells or proteins the state of organization of water is modified by ions and various ionizable drugs. In this theory of Pauling, narcosis is attributed to the formation in synaptic regions of the brain of minute hydrate crystals of the clathrate type (pseudo-ice formation by water in association with typical hydrocarbon narcotic agents) which, by trapping ions and charged side chains of proteins of brain tissues, increases the impedance of the encephalonic network of conductors and hence decreases the activity of the exciting mechanisms on which consciousness or loss of consciousness depends. Pauling has demonstrated the feasibility of clathrate crystal formation involving ethanol, nitrous oxide, ether and xenon, but has noted that this was not proof for the proposed theory. The concept at this time fails to account for the ultimate consequences of clathrate formation

on the ion movements which are the basis of excitation. The Pauling concept raises the possibility of actual calculations of the complex of protein-ion-drug-water, which could be the beginning of a real theory of pharmacology.

J. Pharmacological Properties of Narcotic Drugs

Mann et al. (31) were the first to show that ether at a concentration sufficient to depress in vivo respiration, was able to suppress also ACh synthesis by brain tissue homogenate. McLennan and Elliott (185) have shown that ACh synthesis by brain cortex slices was accelerated at low concentrations and inhibited by higher concentrations of many narcotic drugs. The concentration of the narcotic which produce inhibition with slices in vitro corresponded to its estimated concentration in vivo during narcosis. Johnson and Quastel (186) have shown that the narcotics (barbiturates and chloretone) did not affect the ACh synthesis taking place in extracts of acetone-dried beef brain, but they (pentobarbital and chloretone) inhibited aerobic acetylation of choline taking place in rat brain mince. These authors showed that the suppression of ATP synthesis during narcosis inhibited aerobic choline acetylation in rat brain mince.

In this investigation, the influence of various narcotic drugs in brain metabolism has been studied. A short review of the pharmacological properties of these drugs follows:

(i) Ether

Ether was first prepared by Valerus Cordus in 1540. In 1842 Crawford Long used it for the relief of pain during

surgical procedures, but it remained for Morton to demonstrate its anaesthetic effect in 1846.

Ether, like other liquid or volatile inhalation anaesthetics, is powerful enough to produce complete anaesthesia, even to respiratory arrest, with an adequate concentration of oxygen in the inhalation mixture. In low ether concentrations, analgesia or depression of sensation and consciousness is produced. As the drug concentration in the tissue is increased, the lower motor areas are depressed. This depression involves not only motor areas controlling the skeletal musculature, but also the areas and systems involved in the coordination of muscle innervated by cranial nerves, e.g. swallowing. Further increase of ether produces a gradient depression of spinal cord activity with an ascending paralysis. The muscles of the extremities, the abdomen and the thorax cease to function, in this order. Finally, the respiratory center in the medulla is so depressed that action of muscles of respiration ceases (187).

The absorption of ether in the alveoli occurs very quickly. But, due to the relatively low concentration of ether in the inspired air and the relatively high amount of ether necessary in the blood and tissues for anaesthesia, the period of induction of anaesthesia is long (188).

Studies on the trained dog indicated that, until deep anaesthesia was reached, there was no significant reduction in oxygen consumption. There was an immediate increase in blood sugar, a gradual reduction in alkali reserve and an increase in

lactate in the blood (189). Hyperglycaemia involved mobilization of liver glycogen by conversion to glucose. The effect of ether on intermediary carbohydrate metabolism and inorganic phosphate has also been reported by Henneman and Bunker (190). Watts (191) and Brewster et al. (192) reported that the release of epinephrine was largely responsible for the mobilization of liver glycogen by ether. The epinephrine content of the adrenals was decreased by ether anaesthesia. However, epinephrine mobilization was not indispensable to ether hyperglycaemia for the mobilization can be induced after excision of the adrenal glands. The increase in liberation of epinephrine was one of the factors responsible for the increase in heart rate during both the induction and the period of anaesthesia.

Ether is quite rapidly and generally distributed throughout the body (193). The elimination of ether from the body is mainly, up to 90-95%, by way of the respiratory tract. However, it may be found in the urine. There is no evidence to indicate chemical destruction in the body.

(ii) Pentobarbital

Barbital or 5,5-diethylbarbituric acid was synthesized by Emil Fischer and von Mering in 1903 and since that time many derivatives of barbituric acid have been prepared and investigated for their pharmacological effects in man and in animals. Pentobarbital is 5-ethyl, 5(1-methylbutyl) barbituric acid.

All the barbiturates in current use will produce anaesthesia if given in sufficiently large doses. In experimental

studies, there is a marked variation in rapidity of the onset of hypnotic or anaesthetic effect between the barbiturates. In contrast to long acting barbiturates, which take up to 30 minutes, pentobarbital will induce anaesthesia within 2 to 3 minutes after intravenous adminstration. Upon administration of barbiturates by any route, depression of the central nervous system of any degree from mild hypnosis to complete anaesthesia and finally death from respiratory arrest may be produced.

Pentobarbital is used extensively for preliminary sedation in spinal and ether anaesthesia. The heart rate of rabbit, cat and dog is usually increased by an anaesthetic dose of barbiturates. Pentobarbital increased the blood pressure of normal dogs, probably by increasing peripheral resistance (194). Intravenous injection in dogs produced increased blood flow in the femoral artery. Imig et al. (195) found a quickened pulse rate throughout anaesthesia.

The basal metabolism did not change significantly, even with administration of maximum anaesthetic doses of pentobarbital. Watts (196) reported no change in the blood sugar level of normal fasting animals. However, biosynthesis of ascorbic acid was increased (197). Ascorbic acid deficiency impaired the production enzymes connected with the destruction of pentobarbital and other barbiturates (198).

Pentobarbital is readily absorbed and is reversibly bound by serum albumin (199). It passes into all tissues and secretions, including milk. It is completely metabolized in man and in dog. In man its metabolism is slower than in

dogs (200). After oral administration of N^{15} pentobarbital to dogs, about 16% of N^{15} was excreted in the urine in 24 hours. Only 3% of this was unchanged pentobarbital. Less than 8% was ammonia and urea. The remaining 84% was not identified (201), but it was probably a product derived from the oxidation of the methyl-butyl side chain to a secondary alcohol (202).

(iii) Nitrous Oxide

Nitrous oxide was prepared by Priestly in 1776, and Sir Humphrey Davy in 1799 suggested it be used to obscure pain during surgical procedures. Colton, in 1842, introduced it as an anaesthetic for dental extractions. Organe and Broad in 1938 (203) introduced the concomitant use of nitrous oxide and oxygen during pentothal anaesthesia.

The inhalation of nitrous oxide has a definite anaesthetic action: 30 to 70% in oxygen produces analgesia (204), while 80 to 85% in oxygen produces light anaesthesia. The respiratory disturbances, convulsive features and cyanosis produced by undiluted nitrous oxide are purely asphyxial. The asphyxial phenomena are absent if the gas is mixed with 15 to 20% of oxygen and the anaesthesia can be prolonged indefinitely. Brown, Lucas and Henderson (205) found that, working with nitrous oxide under pressures up to two atmospheres, anaesthesia with muscle relaxation was not produced in the rabbit as long as the pressure of oxygen was equal to 156 mm Hg. These results were not supported by Seevers et al. (204).

Nitrous oxide, in the absence of anoxia, has few if any undesirable action on the subject. The blood pressure is

elevated during induction, but later returns to normal. Respiration is full and regular. The heart rate is increased due to anoxia (206).

Significant differences from typical narcotics are shown in failure of nitrous oxide to narcotize excised tissue (muscle or heart) and invertebrates. On these it merely acts as an indifferent gas. Wieland (207), therefore, ascribed its effects to interference with oxidative process in the central nervous system.

Nitrous oxide is soluble in blood to the extent of 45 volumes per cent. It probably does not combine with haemoglobin. The blood catalase is diminished by nitrous oxide as in other forms of anaesthesia (208).

In <u>in vitro</u> respiration of guinea pig brain preparations, oxidation of glucose and pyruvate, and oxidative phosphorylation are not affected by 20% of nitrous oxide, 78% of nitrogen with 2% of oxygen (209). However, a mixture of 80% nitrous oxide and 20% oxygen inhibited oxygen consumption of rat brain cortex slices (210).

Nitrous oxide is rapidly absorbed from the alveoli and is excreted equally as rapidly from this tissue.

(iv) <u>Ethyl Alcohol</u>

Ethyl alcohol depresses the central nervous system, especially the "higher centers". Nitzescu (211) described the intravenous alcohol anaesthesia for animals. Full anaesthesia

is obtained with 2 to 2.5 ml. of ethyl alcohol per Kg. of body weight, diluted twice its volume of 5% glucose solution. The anaesthetic stage sets in as consciousness, sensation and muscular tone are gradually lost. The paralytic stage is shown by medullary paralysis: the respiration is slow, pulse rate is scarcely discernible, the pupils are dilated and reflexes are abolished (212).

In man, intravenous injection of alcohol, in doses sufficient to produce mild inebriation, has practically no effect on the blood flow, vascular resistance and metabolism of the brain. In severe acute intoxication, the cerebral vascular resistance is decreased, the blood flow is markedly increased, but the oxygen consumption of the brain is markedly decreased, notwithstanding the greater blood supply (213). Cerebrospinal fluid formation is increased by alcohol (214). The pressure of the cerebrospinal fluid is not changed by alcohol unless it is lowered consequent on fall of systemic blood pressure (215). Coronary blood flow in anaesthetized dogs is increased (216). The effect of ethyl alcohol on the heart rate of animals is inconsistent, although in general there is quickening. Rapid intravenous injection of alcohol in unanaesthetized animals caused a sharp drop in blood pressure, followed by a rapid recovery.

Weak ethyl alcohol solutions cause gelatinization of cells of the frog epithelium.

Deep alcohol narcosis of rats and guinea pigs results in slow but marked decrease of adrenal ascorbic acid and

cholesterol, with very slow recovery. It is probably a stress reaction, mediated through the hypophysis. The response is not potentiated by pentobarbital (217). The liver ascorbic acid of guinea pigs is increased in 48 hours after alcohol intoxication (218). Ethyl alcohol increases the blood sugar at the expense of liver glycogen (219). In well-fed rats, anaesthetic doses produce a marked increase of blood concentration and decrease of liver glycogen; this effect is absent if the liver glycogen has been depleted by fasting.

Tissue slices oxidize ethyl alcohol in vitro. Liver is most active, using part of the alcohol for the synthesis of fatty acids and cholesterol, and oxidizing the remainder to carbon dioxide and water. Kidney does not use it for synthesis, but oxidizes it more rapidly. Brain and skeletal muscle metabolize only an insignificant part (220).

Over 90 to 98% of administered ethyl alcohol disappear in the body, being completely oxidized to carbon dioxide and water. The oxidation occurred chiefly in the liver, apparently first to acetaldehyde by alcohol dehydrogenases (221) and then by aldehyde oxidase to acetic acid, which is then oxidized through the tricarboxylic cycle.

Rats receiving a single dose of 1 gm. alcohol per Kg. of body weight of radio-labelled alcohol oxidized an average of 74% to carbon dioxide in 4 hours, 83.7% in 8 hours. They excreted 4% unchanged by the urine in 8 hours - 3.7% by the lungs, 0.2% by the faeces (222).

(v) Magnesium Sulphate

Magnesium administration depresses all nervous and muscular functions and this effect is observed even in very dilute solutions (1:25,000). It produces direct sensory paralysis, both central and peripheral. Magnesium sulphate is used as a local intraspinal and general anaesthetic and therapeutically against tetanus, but its effective dose is dangerously close to being fatal (paralysis of respiration), hence its usefulness is limited (223).

Meltzer and Auer (224) found that general anaesthesia, with abolition of reflexes, may be produced by the subcutaneous injection of magnesium salts (1.5 gm. of crystalline magnesium sulphate per kg. of body weight, used as a 25% solution). The anaesthesia was complete in half an hour or an hour and lasted about 2 hours. The blood pressure was but little lowered; there was some diuresis but no diarrhea. Recovery was perfect. Moore and Wingo (225) found that, in dogs and cats, analgesia was produced when the serum level of magnesium reaches 15 mg.%, complete anaesthesia with 23 mg.% and respiratory arrest with 33 mg.%. The rabbit is completely anaesthetized when the serum magnesium is 15 mg.%.

Intravenous injection of magnesium sulphate in human subjects produces immediate, severe and often fatal depression of blood pressure (226). The fall of blood pressure, presumably, is due to peripheral vasodilation and myocardial depression (227). The heart rate is quickened. The suppression of reflexes by magnesium is due to neuromuscular block, but the

anaesthetic action is central (228).

Parenteral injection of magnesium sulphate to animals results in a fall of the serum potassium by 34 to 53%, but sodium and calcium levels remain unchanged (229). In hibernation, the serum level of magnesium is increased in various species. It falls to normal about an hour after awakening (230).

Magnesium ions activate the enzyme system phosphomonoesterases at pH 9, and inhibit them at pH 5.3; in both cases they protect against inhibition by phosphate ions. They increase the affinity of the enzyme for the substrate at pH 9, and decrease it at pH 5.3 (231). Magnesium activation of bone phosphatase is decreased by cyanide and by amino acids (232). Magnesium ions also activate certain enzymes of intermediatory metabolism such as phosphorylase, enolase and phosphoglucomutase. They suppress the calcium ion activation of the adenosine triphosphatase of myosin (233).

After parenteral administration and absorption, the excretion of magnesium is mainly by the kidneys. When magnesium is taken by mouth, some of it is excreted by the bile, in proportion to the amount ingested (234).

K. Acetylcholine Content of Brain Tissue in the Narcotized State.

The first recorded data on the effect of narcotics in relation to variations of ACh content in brain were incidentally reported by Welsh (149) as part of a different type of investigation. He found an increase of free ACh in pentobarbital narcotized rats. In subsequent years, the increase in ACh content during narcosis has been by Tobias et al. (235), Richter

and Crossland (141), Elliott et al. (236) and Crossland and Merrick (237). Richter and Crossland (141) found that the ACh content of young rats killed by immersion in liquid air varied with the state of activity in the nervous system. These authors showed that (see Table II) during decreased cerebral activity induced by narcotic drugs, the amount of ACh extractable from the brain was increased, while during increased cerebral activity induced by convulsant drugs, the ACh content of the brain was temporarily decreased. Elliott et al.(236) also found that pentobarbital, ether or chloralose anaesthesia increased the ACh content of rat and cat brain. Picrotoxin and pentylenetetrazol lowered the ACh content of the anaesthetized cat but not the normal cat. At various times, explanations to account for the increase in ACh content have been put forward (141,236,237), but there is as yet no experimental evidence for the operative mechanism. Recently, Giarman and Pepeu (238) confirmed and extended this observation for narcotics and sedative-hypnotics which would tend to suggest that there is an accumulation of ACh in the depressed brain, independent of the type of depressant drug used. Hosein and Ara (239) have chromatographically demonstrated that the increase in brain ACh activity during pentobarbital and ether narcosis was due mainly to a mixture of betaine CoA esters. Kurokawa et al. (72) studied the influence of phenobarbitone on the subcellular distribution of ACh in rat brain. Hosein and Proulx (73) provided evidence that the betaine CoA esters during ether and pentobarbital narcosis accumulated mainly in the brain subcellular fraction P_2.

STATEMENT OF THE PROBLEMS STUDIED

The ACh theory was established on the burden of indirect evidence. The biologically active material present in tissue extracts was assumed to be ACh mainly on bioassay. Only in one instance (142) was the actual chemical identification done on material believed to be ACh in brain extract and this involved 99% loss of the desired derivative. Narcotics were known to cause an increase in biologically active material in the brain and this accumulated material was also assumed to be ACh. These unanswered questions prompted us to carry out the following investigation.

1. The systematic study of the influence of representatives of various classes of narcotics on the subcellular distribution of materials with ACh-like activity in brain has not been described in the literature, but these data could be important if we knew the relative distribution of this activity in brain homogenates. This was studied with ether, pentobarbital, ethyl alcohol, nitrous oxide and magnesium sulphate.

2. The narcotic influence on ACh content of cardiac tissue also has not been reported. This was studied with pentobarbital and ether because of the reported heart block during anaesthesia.

3. Chemical identification of active substances in normal brain and cardiac tissues was performed because the reliability of differential assay is uncertain.

4. In view of the complete lack of chemical identification

of active substances in narcotized brain and cardiac tissue extracts, this analysis was carried out.

5. An attempt was made to determine, by means of parallel quantitative assay on different test objects, whether or not ACh could be differentiated from other active substances which are present in brain extracts.

6. An attempt was also made to devise a method which would differentiate ACh from other active substances which are present in brain extracts by parallel colorimetric and bioassay determinations.

METHODS

Part 1. Acetylcholine-like Substances in Brain and Heart

A. Separation of Subcellular Particles from Brain Homogenates

Essentially, the methods used were those described by Hebb and Whittaker (20) and by Whittaker (21).

(i) Homogenization

Black-hooded rats weighing between 200-250 gm. from a colony at the Royal Victoria Hospital were used throughout this investigation. Each animal was decapitated with a guillotine and the brain was removed as quickly as possible. The weight (usually 1.5 gm. $\pm$ 0.1) was recorded and in most cases the brain was divided into half hemispheres by cutting along the central sulcus. The tissue was then thoroughly homogenized in sufficient ice-cold 0.32 M sucrose to make a 10% tissue homogenate. Usually, when one half of the brain was homogenized in 0.32 M sucrose medium with eserine, the corresponding half was homogenized in 0.32 M sucrose without eserine. When eserine was used, it was added before homogenization so that its final concentration in the homogenate was 10^{-5} M. The type of homogenizer used by Whittaker differed from that used by us in that Whittaker used a glass Perspex homogenizer with a clearance of 0.025 cm. between pestle and mortar, while we used an all-glass hand homogenizer (Ace Glass Co., Vineland, New Jersey) with a similar clearance.

(ii) Centrifugation

The brain homogenate was centrifuged at 2,500 g for

3 minutes with the Precision centrifuge. The material which sedimented at this centrifugal force was the nuclear fraction, P_1. The resulting supernatant S_1 was further centrifuged at 22,500 g. for 20 minutes in the International centrifuge model HR1, using the No. 1608 cups. The sedimented material was the crude mitochondrial fraction of P_2, and the supernatant S_2.

B. Extraction of Acetylcholine from the Subcellular Fractions

The method of TCA extraction of Chang and Gaddum (34), and Banister et al. (57) and of Hosein et al. was used.

The solid fractions P_1 and P_2 surrounded with crushed ice were suspended in 5 ml. of water and,in the case of the liquid fraction S_2, TCA was added directly to it to make a 10% TCA solution. The sample was then thoroughly homogenized. The TCA treated homogenate was allowed to stand for 30 minutes at 10°C, for protein precipitation. Periodic resuspension of the precipitate was made during this interval.

C. Removal of Trichloroacetic Acid from Extracts

The precipitate formed from each tissue fraction after TCA treatment was collected by centrifuging for 5 minutes at 2,000 g, and discarded. The clear supernatant was repeatedly shaken (5 times) with 5 times its volume of ether to extract the TCA. When most of the TCA had been removed, (indicated when the pH of the supernatant was between 4.0 and 4.5) this solution was neutralized with 0.1 N NaOH to between pH 6.5 and 7.0.

D. Preparation of Extracts for Chromatography

The neutral aqueous extracts were evaporated to dryness

in vacuo at 37°C. Ten ml. of methanol were added to the residue and the content mechanically shaken for 10 minutes. Impurities such as sucrose, nucleotides and protein remained undissolved. Especially with the S_2 fraction which contained the homogenizing medium, most of the sucrose could be precipitated out by more prolonged shaking with methanol. The methanol extracts were then centrifuged and the volume of the clear supernatant was reduced to 0.5 ml. under jets of air.

E. Chromatography

Paper chromatography was carried out as described by Banister et al. (57) and by Hosein et al. (108). Each sample was applied as a narrow band on a 6" by 20" sheet of Whatman No.1 paper. The paper, secured between two glass plates, was placed into a trough. The whole 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 equilibration period, solvent consisting of water-saturated butanol was poured into the trough as quickly as possible. The whole system was then allowed to develop for 17-18 hours at room temperature (22°C). Development was considered complete when the solvent front moved about 17-18 inches away from the starting line. The paper was then taken out, the solvent front noted and allowed to dry in air.

Individual bands occupying positions corresponding to the R_F values of the quaternary ammonium compounds can be made visible by spraying with 2% iodine solution in ethanol. Iodine development caused the appearance of dark coloured bands within

a few minutes. In most cases where the components were to be assayed on the frog rectus abdominis muscle, the material was eluted with methanol and assayed directly without use of iodine. For routine purposes, however, narrow 1-inch strips were cut from the chromatograms and sprayed with iodine. The remainder of the paper could then be eluted and assayed as usual.

F. Elution of the Various Bands on the Chromatograms

Two specific bands, delineated by their R_F values, were cut from the paper. These were the R_F 0.05-0.15 (ACh) and the R_F 0.50-0.70 (betaine CoA esters) bands. The remainder of the paper, which includes the starting line, the region between R_F 0.15-0.45 and the solvent front, was collected together as one single sample. Therefore, from each chromatogram, three samples were obtained for assay purposes. Each zone of the paper was cut into tiny pieces and eluted by shaking in 10-15 ml. of methanol for 2 hours. The methanol eluate was collected and fresh methanol was again added to the paper squares and eluted for another hour. The eluates were pooled and evaporated to dryness under air jets at 37°C or under vacuum at this temperature. The dry material was then ready for assay.

G. Preparation of Heart Extract

In this instance also, black-hooded rats from a colony at the Royal Victoria Hospital were used. The rats were killed by decapitation with a guillotine, the chest cavity was opened and the heart (which includes the auricle and ventricle) was excised. Blood on the heart was gently removed with tissue paper, and the organ was immediately weighed and placed directly

in a volume of ice cold 10% TCA, sufficient to make a 10% homogenate. Because of the toughness of cardiac tissue, homogenization was carried out in a Virtis blender (model 45) for 3 minutes at 8,000 rpm. After homogenization, the TCA tissue extract was treated in exactly the same manner as described above for preparation of the extract for chromatography and bioassay.

H. Bioassay on the Frog Rectus Abdominis Preparation

The saline solution for the perfusion of the frog rectus abdominis (Rana temporaria) was essentially Locke solution and consisted of the following:

NaCl	9.0 gm.
$NaHCO_3$	0.56
$CaCl_2$	0.24
KCl	0.42

made up in 1,400 ml. of 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 methods of isolating the frog rectus abdominis muscle was that of MacIntosh and Perry (240). The frog's skin on the ventral surface of the abdominal wall was cut open with fine scissors and the rectus abdominis muscle was identified. The muscle was split longitudinally into two equal strips. Threads were tied at the origin of the muscle at the sternum and at a point as near its insertion as possible. The attachments were cut and the muscle tied with one end on a silver hook mounted on a rubber cork through which a glass tubing was

perforated to permit oxygenation, and the other end was tied to a writing lever. Care was taken not to stretch the muscle and keep it moist with Locke solution during the dissection. The bath was a 2 inch length of glass tube with a capacity of 3 ml. Thus set up, the muscle was allowed to relax for one hour under a constant tension of about 1 gm. in ordinary oxygenated Locke solution. After this period of relaxation, the muscle was sensitized by changing the solution to neostigmine-containing Locke solution. Sensitization was complete in an hour. The amount of neostigmine needed for sensitization varied with each set of muscles, but was within the range of 10^{-6}M. Eserine was not used, since this substance tends to be less stable than neostigmine. The sensitivity of the muscles usually ranged between 30-75 µg. ACh-Cl and, after standardization, the muscles were ready to assay. One and a half minutes were allowed for the development of contracture and 8 minutes allowed for relaxation between successive contractions. The muscle was washed twice with Locke solution after each contraction.

Each dry sample of tissue extract was dissolved in 1 ml. of Locke solution and aliquots of it or of standard ACh solutions were administered into the muscle bath by means of a 1 ml. hypodermic syringe. Test solutions were given between alternate doses of standard ACh solution. In this manner of "bracketing", the amount of ACh activity in the extracts could be estimated with a reasonable degree of accuracy with an error of not more than 5%. Doses of the sample were given until reproducible results were obtained. In many instances, the assays were

checked by using different muscle preparations. In cases where the amount of ACh activity was small, a known amount of pure ACh was added to the sample and the difference in height of the contraction less the height of the contraction elicited by the amount of pure ACh was taken to be due to the tissue extract sample itself.

I. Varying the Physiological State of the Animal

(i) Narcosis

(a) Ether

Induction of ether narcosis in the rat was produced by enclosing the animal in a bell jar in which cotton wool soaked with ether had been placed. After inhaling the ether vapour for 3 to 5 minutes, the rat lost consciousness. Narcosis was prolonged for a period of half an hour during which time the bell jar was slightly lifted to permit adequate oxygenation. If this was not done, death ensued. To maintain deep narcosis, more cotton wool soaked with ether was intermittently introduced into the jar. The deep stage of narcosis was reached when the animal lost its righting reflex and also became insensitive to pain induced by pinching its toes. After half an hour of narcosis, the animal was decapitated with a guillotine and the brain quickly excised.

(b) Pentobarbital

Pentobarbital was administered to the rat by intraperitoneal injection; deep narcosis was obtained with 13 mg. pentobarbital in saline per 200 gm. of body weight. Narcosis could be maintained with this amount of pentobarbital for

30 minutes, after which time the rats were decapitated with a guillotine and the brains and hearts quickly excised.

(c) Nitrous Oxide

Narcosis was produced in the rat by inhalation of a mixture of nitrous oxide (85%) and oxygen (15%). At this concentration of oxygen, asphyxiation was avoided and narcosis was maintained for half an hour. The rat was placed in a jar and the amount of nitrous oxide and oxygen admitted was regulated by valves on the compressed gas tanks. The volume of each gas admitted was indicated by mercury manometers. After half an hour of narcosis, the rats were decapitated with a guillotine and the brains removed as quickly as possible and extracted in the manner already described.

(d) Ethyl Alcohol

The solution used for intraperitoneal injection was made with an equal volume of absolute ethyl alcohol and 5% glucose solution. Narcosis was produced in about 3 minutes after administration of 3 ml. of this solution. Since it was found that the susceptibility to the alcohol varied from rat to rat, further injection of alcohol was necessary to maintain the animal under the deep stage of narcosis. In all instances, narcosis was maintained for 30 minutes before the animals were decapitated and the brains and heart removed. The tissues were extracted as described above.

(e) Magnesium Sulphate

Magnesium sulphate in 25% aqueous solution was administered subcutaneously to the rabbit, since this animal was found

to be more susceptible to magnesium sulphate solution per kilogram of body weight. The period of induction was long, about half an hour but narcosis, once attained, could be maintained for half an hour. The rabbits were then killed by decapitation and the brains rapidly removed. The brains were extracted as described above.

Part 11. Chemical Identification

A. Preparation of Extracts

Black hooded rats from a colony kept in the Royal Victoria Hospital were used throughout. Rat brain and heart tissue contain only minute amounts of ACh-like activity. In order to obtain a sufficient yield of active substances to permit chemical identification, especially in cases where procedure manipulations caused unavoidable loss, 100 rats were used in each experiment. The rats were decapitated with a guillotine, the brains and hearts quickly excised, and immediately homogenized in TCA (6 ml. of 10% TCA per brain) using a Waring Blender. In view of the time lapse between successive dissections of the brains, the brains and hearts were placed in a solution of TCA first, in batches of five, and then homogenized. The TCA homogenate was allowed to stand for half an hour and then centrifuged. The supernatant was extracted five times with ether, neutralized with 1 N NaOH and brought to dryness *in vacuo* exactly as described in the first part of the "Methods".

B. Isolation by Complexing with Reinecke Salt

This method is essentially that of Bregoff *et al.* (128)

and of Banister et al. (57). The residue was dissolved in 5 ml. of distilled water and the pH adjusted to 8.0-8.5 with ammonia. Five ml. of 5% Reinecke Salt in methanol was immediately added and the mixture was stored at 0°C for 3 hours to allow complete precipitation. More Reinecke solution was added if, after some precipitation, the supernatant had lost its red colour. The pink precipitate formed was collected by centrifugation. It was then washed with 1 ml. of propan-1-ol, during which a small amount of the reineckate precipitate went into solution. The washed precipitate was again collected by centrifugation and dissolved in a minimum volume of acetone-water (I:IV/V) (128). Silver Nitrate solution (2M) was added drop by drop until precipitation of the silver reineckate ceased; this precipitate was removed by centrifugation and discarded. The supernatant now contained a slight excess of $AgNO_3$ and saturated NaCl solution was added drop by drop until precipitation of AgCl ceased. The AgCl precipitate was removed by centrifugation and discarded. The supernatant was brought to dryness at 37°C in vacuo and the residue was extracted twice with anhydrous methanol. The pooled extract was centrifuged and the methanol insoluble NaCl was discarded. The clear supernatant was brought to dryness under jets of air at 37°C. If chromatography was intended, the volume was brought down to 0.5 ml. and applied on the chromatogram, as described in the first part of the "Methods".

C. Removal of Contaminating Material with Water-Saturated Butanol

The tedious procedure of Reinecke Salt purification

unavoidably causes loss of material (57). With practice, consistent 60% recovery of ACh-like activity could be obtained. It was found that washing the extract with water-saturated butanol removed most of the contaminating material without any loss of ACh-like activity. Accordingly, the extracts were washed with water-saturated butanol in which contaminating material remained undissolved. The residue was discarded and the supernatant collected. The supernatant was then brought to a small volume at 37°C under jets of air and applied on chromatograms as described in the first part of the "Methods". In order to ensure proper development of the chromatograms by eliminating overloading, the extract was applied on as many chromatograms as were needed.

D. Elution

The following bands were cut from the chromatograms: starting line R_F 0.0-0.05, R_F 0.05-0.15, R_F 0.15-0.25, R_F 0.25-0.45, R_F 0.50-0.70 and the solvent front R_F 0.70-1.00. Elution was carried out as described in the first section of "Methods".

E. Preparation of the Tetrachloraurates and Chloroplatinates

The method employed was essentially that of Krimberg and Wittandt (116) and of Carter et al. (241).

The residue, after purification with Reinecke Salt or by water-saturated butanol, was dissolved in 2-3 drops of 1% HCl. To this solution, 0.2 ml. of 10% aqueous gold chloride solution (W/V) was added. The solution was boiled for 5 minutes and placed at 0°C for 72 hours. The crystals formed

were collected by centrifugation.

The preparation of the chloroplatinates was the same as that for the tetrachloroaurates, except that the 10% platinum chloride solution was made up in absolute alcohol. The crystals were also formed in the cold and were collected by centrifugation.

F. Conversion of Carnitine to Crotonbetaine

The method was described by Carter et al. (241). A sample suspected of containing carnitine or AC was heated in an oven at 120 - 130°C for one hour with 2 ml. of concentrated sulphuric acid (Sp. Gr. 1.84). The solution was poured to 5 gm. of ice and the sulphuric acid was neutralized with an excess of $BaCO_3$. The precipitate of $BaSO_4$ was removed by centrifugation and the supernatant brought to dryness in vacuo. The residue, which was now principally crotonbetaine was taken up in 2 ml. of water and transferred to a test tube. This step was necessary because the amount of material available from tissue extracts was small. After drying under jets of air, the material was converted to the tetrachloroaurate derivative as described above.

G. Determination of the Melting Points

Since the quantities of crystals were very small, all but the necessary transferring steps were avoided. The crystals were collected with a glass rod and transferred to cover slips

and crushed by pressing with another cover slip so as to obtain uniform size particles. The melting point was determined with an Olympus melting point apparatus, calibrated before use. By adjusting the polaroid filter attached to the eye piece of the microscope, the crystals were made to appear as yellow, shiny particles. The melting point was taken at that temperature when the colour and the crystal shape were extinguished (242). Tetrachloroaurate and chloroplatinate derivatives of authentic choline and betaine compounds were prepared as described above. The melting points were determined and used as reference standards. Tetrachloroaurate derivatives were also prepared from mixtures of several choline and betaine compounds. The characteristic melting points of individual compounds which made up these mixtures were observed under the microscope.

H. Ultraviolet Spectrophotometric Analysis of the Betaine CoA Ester Fraction

Acetylcholine and other acyl esters of choline have no distinctive absorption spectrum in the ultraviolet region and thus differ from the betaine CoA esters which have absorption peaks at 260 mμ. due to the adenine moiety and at 232 mμ. due to the thiol ester linkage (243). Other parts of the molecule would account for absorption beyond 260 mμ. The two absorption peaks characteristic of CoA esters could, therefore, be used for their detection. These thiol esters lose their absorption at 232 mμ. when treated with alkali, but that at 260 mμ. remains unchanged.

Accordingly, betaine CoA esters obtained by eluting

the band with R_F 0.50-0.70 of experimental chromatograms matched against a control obtained from the same region of blank chromatograms, were read using quartz cells in a Beckman DK spectrophotometer. For alkaline hydrolysis of the thiol ester linkage, the experimental sample was treated with 0.5 ml. of 0.2 M NaOH and allowed to react for 20 minutes at room temperature and neutralized with 0.5 ml. of 0.2M HCl. One ml. of 0.5M $CaCl_2$ was added to the control sample. The experimental and the control samples were again read in the Beckman DK spectrophotometer.

I. Phosphate Determination

This method was described by Fiske and Subbarow (244). The preparation of the tetrachloroaurate derivatives of material eluted from the R_F 0.50-0.70 band involved boiling in 1% HCl for 5 minutes. This procedure cleaved the CoA molecule into its moieties (245). Consequently, inorganic phosphate appeared in solution and could be determined by the Fiske and Subbarow method of phosphate identification. After crystallization of the tetrachloroaurate derivatives, the supernatant was bubbled with H_2S gas to get rid of the unreacted gold chloride. The black precipitate was discarded and the supernatant aerated to remove excess H_2S. This solution, concentrated to a smaller volume, could be used for phosphate determination.

(a) Reagents

1) 10 N H_2SO_4; prepared by adding 250 ml. concentrated H_2SO_4 to 650 ml. water.

2) 2.5% ammonium molybdate solution; prepared by dissolving 25 gm. ammonium molybdate in 200 ml. water, then adding

300 ml. 10 N H_2SO_4 and finally making up to one litre in a volumetric flask.

3) 15% sodium bisulphite solution; prepared by dissolving 75 gm. of sodium bisulphite in water and diluting to a volume of 500 ml.

4) 20% sodium sulphate; prepared by dissolving 40 gm. sodium sulphate in water and diluting to a volume of 200 ml.

5) 10% TCA; prepared by dissolving 10 gm. of TCA in water and diluting to a volume of 100 ml.

6) The Fiske-Subbarow Reagent was prepared by grinding 0.5 gm. 1-amino-2-naphthol-4-sulphonic acid in a mortar. This was added to 195 ml. of 15% sodium bisulphite in a glass stoppered cylinder. The cylinder was shaken until the solid was dissolved. Five ml. of 20% sodium sulphate was added. Reagent was found usable for 4 weeks if kept in a brown glass bottle in the refrigerator (8°C).

(b) Procedure

A sample suspected of containing inorganic phosphate was placed in a calibrated 20 ml. tube and the volume brought up to 2 ml. with water. To this was added 8.0 ml. of 10% TCA and the volume was then made up to 15 ml. with water.

One ml. of 2.5% ammonium molybdate was added and followed by 0.4 ml. of Fiske-Subbarow reagent. Qualitative rather than quantitative determination was attempted. A positive test was indicated by the formation of a blue colour.

J. Ribose Determination

This method was described by Tauber (246). The reagent

was made up of 1 gm. benzidine dissolved in 25 ml. glacial acetic acid. A 0.5 ml. solution suspected of containing ribose was boiled vigorously with 0.5 ml. of the reagent and cooled. A positive test was indicated by the development of a very stable cherry red colour.

Part 111. Bioassay of Acetylcholine-like Activity

A. The Frog Rectus Abdominis Method

(a) The Neostigmine Sensitized Preparation

This method for the quantitative estimation of material which ACh-like activity in tissue extracts has been described in the first part of the "Methods".

(b) The Normal Preparation

It was found that the frog rectus abdominis was in some cases sufficiently sensitive, without the use of neostigmine, to material with ACh-like activity in tissue extracts. These normal muscle preparations were used in conjunction with other test objects for the differential assay of material with ACh-like activity in tissue extracts or of synthetic AC samples.

B. The Leech Dorsal Muscle Method

This method was described by Fuehner (77) and by MacIntosh and Perry (240).

(a) Saline Medium

The aerated saline medium used was that described by Quastel *et al.* (247) and had the following composition.

NaCl	7.10 gm.
KCl	0.32
$CaCl_2$	0.18
$NaHCO_3$	0.12
Glucose	0.77

made up to 1 litre with distilled water.

The leech used in this study (obtained from the Dominion Herb Supplies Co., Point Claire) required 0.01 mg. of eserine sulphate per 7 ml. of saline for sensitization. The leeches were kept in a refrigerator (8°C) in the saline medium described above for muscle perfusion. They remained alive for 6 months or longer when kept in this manner (248).

(b) Apparatus

The apparatus used for the assay was the same one used by Harpur in 1949 (248). The inner vessel measured 7.0 ml. and the outer jacket was provided with an inlet and outlet through which cold tap water (18°C) continually coursed. In this way, constant temperature was maintained. The muscle was kept below 23°C, otherwise spontaneous contractions developed. Oxygen and saline were supplied by glass tubings. An outflow tube attached to the top of the muscle bath permitted overnight perfusing of the muscle.

(c) Dissection

The leech was pinned on its back (anterior sucker is smaller than the posterior and the dorsal surface is darker than the ventral) and its ventral wall was removed with fine scissors. All the tissue adherent to the inner surface of the dorsal wall were cut away as rapidly and cleanly as possible. The muscle

was kept moist with the perfusion fluid during its preparation. The dorsal muscle was cut longitudinally to provide a muscle strip 4 cm. long and about 0.25 cm.in width. Threads were ligatured, one at either end. One of the ligatures was tied to a silver hook at the bottom of the bath and the other attached to the writing lever of a kymograph. The lever was balanced by placing bits of plasticine on it. When first set up, the muscle strip was fully contracted. Relaxation, under a tension of about 1 gm. of plasticine placed on the writing lever, was achieved by perfusing the muscle strip overnight with perfusion fluid at a rate of about 1 ml. of saline per minute. When this was done, the preparation was found to be very suitable for the estimation of ACh (248). To sensitize the muscle for assay, 0.01 mg. eserine sulphate was added. After allowing half an hour for sensitization with eserine, the muscle was washed and assay begun, care being taken to keep the volume (7 ml.) of the perfusion fluid constant.

(d) Procedure for Assay

Solutions of ACh-Cl (Merck and Co.) were used as a standard and all the results are reported in terms of this substance. In the calibration of the leech muscle, 0.25 to 0.50 ml of a solution of ACh-Cl were added. Three minutes were allowed for the development of contracture and 8 minutes allowed for relaxation between successive contractions. The muscle was washed twice with saline after each contraction and, after each contraction, eserine sulphate, 0.01 mg. per 7 ml. of saline, was added. For assay of ACh in tissue extracts, the technique

of "bracketing" was used. Estimations were repeated until constant results were obtained so as to reduce experimental error as far as possible. Error of not more than 5% was obtained.

C. The Guinea Pig Ileum Method

The guinea pig ileum preparation used was essentially similar to that described by Guggenheim and Loeffler (249).

(a) Saline Medium

The Locke-Ringer solution was made up as follows:

NaCl	9.00 gm.
KCl	0.42
$CaCl_2$	0.24
$MgCl_2$	0.005
$NaHCO_3$	0.50
Glucose	1.00

made up with glass distilled water to 1,000 ml.

The solutions were made up a few hours before use. The $CaCl_2$ and $MgCl_2$ were added as stock solutions (20% and 5.0% respectively). The other components were weighed out at the time of preparation. The mixing procedure was found to be important; precipitation of carbonates occurred if the sodium bicarbonate was added to a solution of other salts. Therefore, a separate solution of the former was prepared and added. In the assay of ACh with this preparation, it was necessary to inhibit the action of histamine which might be present in tissue extracts. For this purpose, the highly specific antihistamine, neoantergan (mepyramine maleate, courtesy of Poulenc Ltd., Montreal) was added to the Locke-Ringer solution to make a final concentration of 10^{-8} M of the drug. In assaying the tissue

extracts, neoanterganised Locke-Ringer solution was always used.

(b) Apparatus

The guinea pig ileum apparatus was constructed by Mr. K. Holeczek of the Dept. of Physiology, McGill University. It consisted of a constant temperature bath maintained at 37 ± 1°C.

A glass tube made into an S shaped hook held in position by a clamp was inserted into a 5 ml. muscle bath. The hook served a dual purpose, in that the muscle was tied to it and oxygen, from a compressed oxygen tank at a rate sufficient to form a continuous stream of small bubbles, was supplied through it. Locke-Ringer solution from a reservoir was fed in and drained out from the muscle bath by suction via a two way stopcock. The writing lever was held in position by a clamp placed immediately on top of the bath. A vibrator was placed on the rotating arm of the kymograph drum to eliminate any sticking of the lever on the smoked paper. It was found that the writing lever capped with glass tips minimized the amount of friction.

(c) Dissection

Guinea pigs of either sex weighing 200-250 gm. were killed by a blow to the head, the throats cut and the animals bled. The abdominal wall was opened and the ileocaecal junction was identified. A piece of terminal ileum about 20-30 cm. long was removed by stripping from the attached mesentery. The ileum strip was divided into sections of about 10 cm. long and

placed in warm Locke-Ringer solution. By passing warm Locke-Ringer solution with a pipette from the cephalic to the caudal end, the ileum strip was washed five times to ensure thorough removal of intestinal content. Sections of the ileum touched by hand were discarded.

Sections of ileum 2-2.5 cm. long were cut and threads were passed through both ends with a sharp needle and tied so that the gut remained open at each end. One end of the gut was then attached to the hook on the oxygen tube and the other end to the writing lever. After about 15 minutes of equilibration and after several washings, the preparation was usually ready for use in assays.

Sections of the gut which were not immediately used were kept in saline in the refrigerator at 8°C. If used within a few hours, their sensitivity to ACh remained unchanged.

(d) Procedure for Assay

The muscle was first calibrated with aliquots of 0.15-0.50 ml. of 10^{-7} gm. per ml. of ACh-Cl (Merck & Co.) solution. Thirty seconds were allowed for the development of contracture and 3 minutes allowed for relaxation between successive contractions. The muscle was washed twice with Locke-Ringer solution after each contraction.

The technique of "bracketing" was strictly adhered to. In some cases, the gut had a tendency to undergo spontaneous contractions and consequently a good base line was hard to obtain. However, it was found that very small doses

of morphine sedated the gut and made possible the establishment of a good base line. In all instances, no "slow contracting" activity was seen so that the "true" contraction elicited within the 30 seconds allowed could not be due to substance P, bradykinin or serotonin. In some cases, abolishment of contractions was checked with atropine.

D. The Cat's Blood Pressure Method

The bioassay of tissue extracts on the cat's blood pressure was performed by Miss M. Luttman of the Dept. of Physiology, McGill University.

Part IV A. Concerning l-Carnitine

A. Isolation of l-Carnitine from Meat Extract

The method was described by Gulewitsch and Krimberg (88).

Difco beef extract was dissolved in a small amount of water. Solid TCA was added until its concentration was 10%. The mixture was homogenized with an all-glass homogenizer (Ace Glass Co., Vineland, New Jersey) and allowed to stand at 8°C for 30 minutes. The TCA precipitate so formed was discarded. The supernatant was extracted five times with ether until it reached the pH range between 4.0 and 4.5. It was then neutralized with 10% NaOH to pH 6.5 to 7.0 and evaporated to a syrup in vacuo 37°C.

The syrup was dissolved in absolute ethanol and centrifuged. The precipitate which was obtained after centrifugation

was re-extracted with absolute alcohol. The combined ethanol extract was evaporated to dryness and the residue again extracted with absolute alcohol. The new alcoholic extract was precipitated with a hot concentrated alcoholic solution of $HgCl_2$. The precipitate which became crystalline on standing was collected by centrifugation, washed with absolute ethanol and, after drying, extracted three times with boiling water, in which a small part remained undissolved.

The hot aqueous solution was filtered from a hot water funnel maintained at 61°C and the precipitate formed on cooling to room temperature (22°C) was discarded. The concentrated mother liquor was left overnight in the refrigerator (8°C). The precipitate formed was collected by centrifugation. Itwas then decomposed with hot water and H_2S was passed through the solution to remove the $HgCl_2$. The HgS precipitate was collected by centrifugation and discarded. The supernatant was neutralized with Na_2CO_3 to pH 7.0 and then brought to dryness.

The residue obtained (l-carnitine) was yellowish and was deliquescent.

B. Acetylation of l-Carnitine

This method was described by Krimberg and Wittandt (116). The l-carnitine obtained was mixed with approximately 10 times its volume of acetylchloride in a round bottom flask which was then connected to a reflux condenser and sealed with a $CaCl_2$ tube. The whole assembly stood for 2 days at room

temperature (22°C) with occasional shaking. During this interval, heavy colourless crystals precipitated out and the supernatant fluid turned slightly yellow.

The contents were then transferred on to a big watch glass and placed over Na_2CO_3 in a vacuum dessicator. On evacuation of the dessicator, the excess acetylchloride evaporated and reacted with the Na_2CO_3. The white precipitate (AC) was weighed and collected by dissolving in hot absolute ethanol. Material which remained undissolved in the absolute alcohol was discarded. The alcoholic solution was brought to dryness and the residue (AC) was obtained.

Part IV B. Colorimetric Analysis

A. The Bromophenol Blue Method

This method was a modification of the Mitchell and Clark procedure (85) and was described by Nowell and Wilson (87), except that half quantities were used. It was deemed desirable to measure the active components of tissue extracts by means of bioassay and the bromophenol blue method. Eluates from Reinecke Salt extracts were measured by this bromophenol blue method. Crude tissue extracts could not be measured by this method because of interfering substances.

The bromophenol blue reagent was prepared by shaking 30 mg. 3-3:5-5 tetrabromophenol sulphonephthalein (British Drug Houses Ltd.) in 100 ml. 30% K_2HPO_4 (W/V) for 30 minutes. The solution was then filtered through filter paper.

To each of a series of 15 ml. stoppered centrifuge tubes containing 1.5 gm. sodium carbonate and 5 ml. chloroform, 2.5 ml. bromophenol blue reagent were added by pipette, taking care to avoid running through the chloroform layer. A volume of 2.5 ml. of the standard or test solution dissolved in 0.2 M K_2HPO_4 were then pipetted carefully into the upper aqueous layer. Each tube was shaken vigorously by hand for 30 seconds and centrifuged for approximately 1 minute. The upper purple aqueous layer was removed by suction and discarded. The lower blue chloroform layer was transferred to a 5 ml. colorimetric tube and the intensity of the blue colour was measured in a Coleman spectrophotometer at 600 mμ.

The concentration of the substance forming the blue colour complex was expressed in terms of ACh by reference to a calibration curve obtained with different concentrations of ACh-Cl.

EXPERIMENTAL

Preliminary Experiments

A. Homogenization

In the isolation of subcellular particles from brain homogenates in preparation for extraction of material with ACh activity, it was important to pay particular attention to the homogenization. If the brain tissue was too severely homogenized, the major part of the ACh-like activity was recovered in the S_2 fraction. Conversely, if it was not well homogenized, the activity sedimented with the P_1 fraction. It was found that adequate homogenization with subsequent "good" distribution of activity in the various subcellular fractions, with the recovered values similar to those found by Hosein and Proulx (73), could be obtained consistently if the brain tissue was macerated with 40 up and down excursions of the homogenizer. Since the clearance of the glass homogenizers became wider with use, it was necessary to replace them once every two months.

Such precaution need not be taken with homogenization of heart tissue, since in this case it was of interest only to extract ACh from the whole tissue and not from intact subcellular fractions. Mechanical homogenizers were adequate and the "Virtis" type was always used.

B. Chromatography

It has been the experience of Hosein's group that proper preparation and method of application of the extracts

on the chromatogram ensured proper development of the chromatogram by the solvent with a subsequent reasonably good degree of separation of the active components. Overloading of the chromatogram was avoided by applying only small amounts of the extracts on each chromatogram sheet and in such a manner that the applied extract did not exceed 0.25 cm. Such precautions were always taken. The S_2 fraction contained sucrose which, when applied directly, formed a crusty layer on the chromatogram which prevented its development. However, sucrose was precipitated out by prolonged shaking of the S_2 fraction in methanol with intermittent scratching of the flask surface to provide nuclei for crystallization. After such treatment, the non-viscous extract proved suitable for the application and development of the chromatograms. Reinecke Salt purified extracts were never a problem, as the extract was never viscous.

C. The Influence of Trichloroacetic Acid on Acetylcholine Mobility with the Butanol-water Chromatographic System

The R_F value (butanol-water solvent system) of pure synthetic ACh-Cl is 0.13. The possibility that TCA accelerated the mobility of this substance (125) 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 and, in particular, it was of importance to make sure that tissue ACh itself did not contaminate the band with R_F 0.5-0.7 normally occupied by the betaine CoA esters.

Four chromatograms with 2,3,4 and 5 ug. of ACh-Cl were run in the butanol-water system. The percentage recoveries in these experiments were 79, 76, 83 and 79 respectively, with bands located at R_F 0.17, 0.15, 0.17 and 0.16.

In order to ascertain the effect of TCA on the mobility of ACh on the chromatogram, experiments were performed with 2 ug. of ACh in 10 ml. of water to which solid TCA was added to make a 10% TCA solution. After an hour, the solution was extracted with ether, dried and chromatographed. The different bands, thus developed, were assayed. Almost all the ACh-Cl activity was recovered in a band spreading from R_F 0.05-0.15. No activity was found beyond R_F 0.40. When compared to the chromatograms run with ACh alone, the results indicated that TCA had no apparent effect on ACh mobility.

D. Chromatographic Separation by Augustinsson System

In order to cross-check the reliability of chromatographic separation of extracts on the butanol-water system, extracts were also chromatographed on the Whatman No.4 paper and resolved on the Augustinsson system (58). Acetylcholine in this solvent system has an R_F of 0.40 (56,58,59) and the betaine CoA esters an R_F of 0.80 (Hosein et al. unpublished data). Four experiments were done, 2 samples prepared from the P_2 fraction in eserine and 2 samples prepared from P_2 without eserine were chromatographically resolved on the Augustinsson system, specific bands were eluted and assayed on the frog rectus preparation.

The results are presented in Table V and, when compared

with those to be described in Table VI, show that the pattern of distribution of ACh-like substances on the butanol-water and Augustinsson chromatographic solvent systems are similar.

TABLE V

Chromatographic Distribution (Augustinsson System) of Substances with ACh-like Activity in the P_2 Fraction Prepared from Half Rat Brain Homogenate

µg. ACh-Cl (Frog Rectus Assay)

Probable Substances	P_2 with Eserine		P_2 without Eserine	
	I	II	I	II
R_F 0.42-0.52 (ACh)	0.02	0.03	0.03	0.02
R_F 0.70-0.90 (Betaine CoA esters)	1.02	0.88	0.62	0.54
R_F 0.00-0.42 0.52-0.70 0.90-1.00 (Pooled Substances)	0.58	0.42	0.24	0.15

E. Rechromatography of Material in the Eluate of the Band with R_F 0.50-0.70

Enzymically synthesized gBBCoA has an R_F of 0.65 in the butanol-water system (130). In order to ascertain that the betaine CoA esters were situated in the region delineated by R_F 0.50-0.70, eluates from this band were rechromatographed in the butanol-water system. The activity was recovered in the band with R_F 0.50-0.70 without appreciable loss. The recovery was 80%.

McLennan et al. (250) have claimed that the material in the band with R_F 0.50-0.70 is mainly an ACh-TCA complex.

They have cited evidence from Levy and Pierron (125) that such a complex has faster mobility than pure ACh, but they have neglected to report that Levy and Pierron also found a fast moving component in cadmium hydroxide and acidified alcohol extracts of brain and that these reagents do not form a complex with ACh. Moreover, McLennan's conclusions are not borne out by his experiments since, to regard the band with R_F 0.50-0.70 as comprising of ACh-TCA, he should have shown that any other added substance did not run to the band with R_F 0.50-0.70. Since the TCA added is much in excess of ACh, complexing should have been complete, although McLennan *et al*. always found two distinct (R_F 0.1 and R_F 0.6) bands in their chromatograms.

McLennan *et al*. (250) have said that treatment of the eluate by acidification to pH 3 at room temperature (22°C) for 3 hours cleaves the hypothetical ACh-TCA complex; on rechromatography after neutralization, ACh activity should be found only in the band with R_F 0.1.

Accordingly, 2 samples of eluates containing 1.7 µg. and 5.5 µg. ACh-Cl equivalent from the band with R_F 0.50-0.70 were acidified with 0.1N HCl to pH 3 and left at room temperature (22°C) for 3 hours, neutralized to pH 7, rechromatographed and then assayed on the frog rectus preparation. The results are shown in Table VI.

The recoveries of the 2 experiments shown in Table VI represented 50 and 60% respectively. These results, showing that most of the activity was recovered in the band with R_F 0.50-

0.70 on rechromatography after acid treatment indicated that the betaine CoA esters rather than ACh-TCA complex contributed to the ACh activity. The loss of activity was attributed to cleavage of the acetyl group from acetyl-l-carnityl CoA.

TABLE VI

The Distribution of Material in the Eluate of the Band with R_F 0.50-0.70 after Acid Treatment and Rechromatography on the Butanol-Water System.

µg. ACh-Cl (Frog Rectus Assay)

Probable Substances	Experiment 1	Experiment 11
R_F 0.05-0.15 (ACh)	0.37	0.24
R_F 0.50-0.70 (Betaine CoA Esters)	0.66	2.47
Pooled Substances	0.02	0.01

Pooled Substances: R_F 0.15-0.45 (PCh and BuCh), R_F 0.00-0.05 (SL) and R_F 0.70-1.00 (SF). SL: Starting line. SF: Solvent front. This terminology is used in all subsequent tables.

F. Chromatography after Reinecke Precipitation

In order to check the chromatographic behaviour of ACh after TCA and Reinecke treatment, a sample of authentic ACh was mixed with TCA and the Reinecke precipitable material was chromatographed in the butanol-water system. Iodine spraying revealed that the ACh had an R_F of 0.13.

When ACh was added to brain homogenates and the reineckate residue chromatographed, the added ACh was found in the band with R_F 0.1. The results of this type of experiment

completely disagree with those of Pepeu, Schmidt and Giarman (251) who found added ACh in the R_F 0.50-0.70 band. In our experiments, the position of the added ACh on the chromatogram was ascertained both by iodine development and bioassay on the frog rectus.

G. Recovery after Water-Saturated Butanol and Reinecke Salt Purification

When a tissue extract, which had been treated with TCA, was washed with water-saturated butanol, there was no loss of activity on subsequent bioassay. A TCA tissue extract, when treated with Reinecke Salt, showed some loss of activity but a consistent recovery of 50-60% could be obtained with practice. In an experiment, the TCA extract of 2 rat brains was purified with Reinecke Salt and the residue was chromatographed. The material in specific bands was eluted and assayed on the frog rectus preparation.

TABLE VII

Chromatographic Distribution (Butanol-water System) of Material with ACh-like Activity in Brain Extracts before and after Reinecke Purification.

µg. ACh-Cl (Frog Rectus Assay)

Probable Substances	TCA Extract of 2 Rat Brains	Reinecke Purified TCA Extract of 2 Rat Brains	Reinecke Purified TCA Extract of 2 Rat Brains
R_F 0.05-0.15 (ACh)	Trace	0.92	0.40
R_F 0.50-0.70 (Betaine CoA Esters)	7.20	2.92	3.66
Pooled Substances	0.17	0.20	0.40

From the data shown in Table VII, it was calculated that the total activity in terms of ACh-Cl of the Reinecke Salt purified brain extract, when compared with the control (no Reinecke purification), represented recoveries of 54 and 61% respectively. On the average, 75% of the total activity was found in the band with R_F 0.50-0.70.

H. Ultraviolet Spectrophotometric Analysis of the Material in the Band with R_F 0.50-0.70.

Adenine-containing compounds have absorption at 260 mμ. and thus betaine CoA esters should also possess this property. Thiol esters have absorption at 232 mμ. which is decreased when the linkage is destroyed by alkali. Betaine CoA esters, which possess the thiol ester linkage, should also exhibit absorption at 232 mμ. Absorption at 232 mμ. by the betaine CoA esters should decrease on alkali treatment and, since the adenine moiety is not affected by alkali treatment, absorption at 260 mμ. should remain unchanged.

The presence of the betaine CoA esters in the eluate of the band with R_F 0.50-0.70 was, therefore, checked by ultraviolet spectrophotometric analysis. Their presence was indicated by absorption at 232 and 260 mμ. It was found that alkaline hydrolysis decreased absorption at 232 mμ. but that at 260 mμ. remained unaltered.

I. Reliability of Bioassay

The frog rectus abdominis, leech dorsal muscle and guinea pig ileum preparations are accepted standard test objects

for bioassay of ACh and ACh-like substances. Using the technique of "bracketing", errors of not greater than 5% were obtained. The estimation by the frog rectus method in part depended on the sensitivity of each particular preparation to ACh. Consequently, only muscles which responded to 30-70 mμg. ACh-Cl were used.

J. Chemical Identification

The melting points of the tetrachloroaurate and the chloroplatinate derivatives of quaternary ammonium compounds are sharp and characteristic for each individual compound and are suitable for identification purposes. These compounds crystallized out of solution with homogeneous composition from a mother liquor containing a mixture of these substances (105). The melting points of some of these derivatives were determined.

Tetrachloroaurates of	MP (°C)	Chloroplatinates of	MP (°C)
ACh	169	Carnitine	210-212
PCh	250-255	Acetyl-dl-carnitine	185-187
BuCh	85-90	ACh	256
Choline	230		
Carnitine	148-152		
Acetyl-dl-carnitine	120-124		
GBB	180-182		
Crotonbetaine	208-211		

It is well known that a substance will depress the melting point of another. This is usually determined with the naked eye with relatively large amounts of material collected in a capillary tube. In the experiments to be described, the well separated and characteristic melting points of quaternary ammonium compounds together with the scattered and discrete crystals of homogeneous composition (105) seen on the microscopic

field enabled the identification of individual compounds determined under the microscope.

For these reasons, it was found possible to observe the melting points of each individual compound which made up these mixtures.

Mixture of Tetrachloroaurates of	MP (°C) Observed	Identity
ACh and GBB	164-167	ACh
	175-177	GBB
ACh, GBB, Carnitine and AC	114-116	
	122-126	AC
	140-142	
	148-152	Carnitine
	164-167	ACh
	173-177	GBB

Of great significance was the finding that when 200 µg. of pure ACh-Cl were added to a rat brain and then homogenized in 10% TCA, the substance was recovered as its tetrachloroaurate derivative, with a melting point of 162-166°C.

K. The Bromophenol Blue Reaction

The bromophenol blue reaction is for quaternary ammonium compounds; tertiary nitrogen compounds do not react (85). It was important to ascertain whether brain tissue extracts contained materials which interfered with the reaction. Although it was found that TCA extracts interfered with colour development, control samples obtained from Reinecke purified tissue extracts, which were boiled in alkali to destroy esters, neutralized and then filtered, did not form a colour with the bromophenol blue dye. The bromophenol blue reaction was,

therefore, suitable for the determination of betaine or choline esters in Reinecke purified tissue extracts. Since betaine and choline esters exhibited different absorbance sensitivity to bromophenol blue, this method was used in conjunction with bioassay to determine the chemical nature of the quaternary ammonium compounds in brain extracts.

SECTION 1

Normal Brain Subcellular Acetylcholine-like Activity

A. Subcellular Distribution of Substances with Acetylcholine-like Activity from Rat Brain Homogenates Isolated in Eserinized and Eserine-free 0.32 M Sucrose

This study was conducted in part to confirm the subcellular distribution of ACh described by Hebb and Whittaker (20), Whittaker (21), Gray and Whittaker (19) and Kurokawa et al. (72) and in part to reaffirm the pattern of subcellular distribution of ACh-like substances described by Hosein and Proulx (73). Particularly, these results served as controls for the subsequent study of the influence of various narcotic agents on the distribution of materials with ACh-like activity in the various subcellular fractions

Rat brains, weighing approximately 1.5 gm. each, were divided in halves by section across the central sulcus. The corresponding halves were homogenized in eserinized or eserine-free 0.32 M sucrose as required and separated by differential centrifugation into subcellular fractions P_1, P_2 and S_2. The

subcellular fractions were extracted with 10% TCA and their methanol extracts were chromatographed in the butanol-water system. After development, specific bands as described in "Methods" were cut and eluted with methanol. The eluates were assayed for ACh-like activity on the frog rectus abdominis preparation. Because of the physiological significance of ACh and of our prime concern with the betaine CoA esters, the bands containing these substances were assayed as individual samples. Other substances, which were present on the chromatogram, were "pooled" and assayed as a mixed sample. The values obtained are shown in Table VIII.

TABLE VIII

Subcellular Distribution of Substances with ACh-like Activity from Half Rat Brain Prepared in Eserinized 0.32 Sucrose.

Equivalent Activity in µg. ACh-Cl. Mean and S.D.
(Frog Rectus Assay)

Probable Substances	P_1	P_2	S_2
R_F 0.05-0.15 (ACh)	0.02	0.02	0.01
R_F 0.50-0.70 (Betaine CoA esters)	0.26 ± 0.13	0.95 ± 0.14	0.34 ± 0.04
Pooled substances	0.24 ± 0.14	0.26 ± 0.10	0.09 ± 0.01
Total	0.52 ± 0.23	1.23 ± 0.16	0.44 ± 0.04
No. of Experiments	9	9	4

S.D. : Standard Deviation. This abbreviation is used in all subsequent tables.

The values of the activity for the ACh band are not presented in statistical terms, since the small amount of activity obtained made accurate assay impossible. All values represent equivalent ACh-Cl activity in µg. and not the amount of actual substances.

It is seen that the subcellular fraction P_2 accounts for 57%, P_1 24% and S_2 19% of the total activity recovered on the chromatogram. This pattern of subcellular distribution of total ACh resembles the results of Whittaker (21) and Kurokawa et al. (72). It also suggests that the ACh-like activity found in P_1 and S_2 may be due to particle contamination from P_2 (21).

In every subcellular fraction, the betaine CoA esters account for the major part of the ACh-like activity. In the P_2 fraction, this amounts to 75%, in agreement with the data of Hosein, Proulx and Ara (108) and of Hosein and Proulx (73).

If the equivalent ACh activity of P_1, P_2 and S_2 are added together and then divided by the average brain weight of 1.5 gm., the value of 2.9 µg. per gm. is obtained. This is within the range of approximately 2.5-3.0 µg. ACh-Cl per gm. fresh rat brain, reported by Hosein's group and is comparable with the data of Whittaker (21) and Kurokawa et al. (72).

Since particulate ACh is stable to cholinesterase, it was of interest to determine its subcellular distribution in eserine-free 0.32 M sucrose. The results are presented in Table IX.

Residual activity was recovered in all subcellular fractions isolated from brain homogenates without the protection of eserine. The small amount of activity obtained in the ACh band from subcellular fractions prepared in eserinized and eserine-free 0.32 M sucrose makes quantitative comparison difficult. It appears, however, that the quantity of extractable ACh was unaltered and that eserine did not increase the yield of ACh. Since particulate ACh is equated with cholinesterase stable "bound ACh" and since free ACh is largely an artifact (40), the conclusion drawn is not without some justification, particularly when Hosein and Proulx (73) showed pure ACh-Cl added to non-eserinized brain homogenates resulted in its total destruction. If the total activity of P_2, isolated under eserinized conditions, is compared with that isolated without eserine (Tables VIII and IX), there is a difference of 0.47 µg. ACh-Cl. Again, if the ACh-Cl equivalent activity of the betaine CoA ester band (Tables VIII and XI) is compared, the difference is 0.40 µg. Since the loss is identical, it appears that the betaine CoA esters were not labile to cholinesterase under these conditions and that their partial destruction (cleavage of the acetyl moiety of acetyl-l-carnityl CoA) accounted for the loss of biological activity.

In the main, the subcellular distribution of materials with ACh-like activity from brain homogenates prepared in an eserine-free medium resembles the pattern described for materials with ACh-like activity in the subcellular fractions isolated in an eserinized medium.

TABLE IX

Subcellular Distribution of Substances with ACh-like Activity from Half Rat Brain Prepared in Eserine-free 0.32 M Sucrose.

Equivalent Activity in µg. ACh-Cl. Mean and S.D.
(Frog Rectus Assay)

Probable Substances	P_1	P_2	S_2
R_F 0.05-0.15 (ACh)	0.01	0.02	0.01
R_F 0.50-0.70 (Betaine CoA esters)	0.22 ± 0.11	0.55 ± 0.12	0.12 ± 0.02
Pooled Substances	0.20 ± 0.16	0.19 ± 0.07	0.07 ± 0.02
Total	0.43 ± 0.21	0.76 ± 0.17	0.20 ± 0.04
No. of Experiments	6	6	4

B. Subcellular Distribution of Substances with Acetylcholine-like Activity of Rabbit Brain Homogenates Isolated in Eserinized and Eserine-free 0.32 M Sucrose.

The subcellular distribution of ACh-like substances was also investigated in another species, namely the rabbit. This study was conducted to provide control data for the subsequent study of the narcotic influence of magnesium. Since the rat is not susceptible to magnesium narcosis, this was done on the rabbit.

The isolation of the subcellular fractions of rabbit brain, chromatography and subsequent bioassay were carried out in exactly the same manner as described in the "Methods" for the rat.

TABLE X

Subcellular Distribution of Substances with ACh-like Activity in Rabbit Brain Isolated in Eserinized 0.32 M Sucrose.

Equivalent Activity in µg. ACh-Cl per gm., Mean and S.D. (Frog Rectus Assay)

Probable Substances	P_1	P_2	S_2
R_F 0.05-0.15 (ACh)	0.02	0.02	0.02
R_F 0.50-0.70 (Betaine CoA Esters)	0.13 ± 0.04	0.71 ± 0.07	0.27 ± 0.02
Pooled Substances	0.06 ± 0.04	0.08 ± 0.02	0.10 ± 0.03
Total	0.21 ± 0.05	0.81 ± 0.07	0.39 ± 0.02
No. of Experiments	3	3	3

TABLE XI

Subcellular Distribution of Substances with ACh-like Activity in Rabbit Brain Isolated in Eserine-free 0.32 M Sucrose.

Equivalent Activity in µg. ACh-Cl per gm., Mean and S.D. (Frog Rectus Assay)

Probable Substances	P_1	P_2	S_2
R_F 0.05-0.15 (ACh)	0.02	0.02	0.02
R_F 0.50-0.70 (Betaine CoA Esters)	0.11 ± 0.04	0.47 ± 0.10	0.17 ± 0.01
Pooled Substances	0.06 ± 0.02	0.12 ± 0.04	0.14 ± 0.03
Total	0.19 ± 0.01	0.61 ± 0.07	0.33 ± 0.03
No. of Experiments	3	3	3

Tables X and XI illustrate the pattern of subcellular distribution of equivalent ACh activity of choline and betaine esters. The data suggest that the activity in the P_1 and S_2 fractions was due to particle contamination by the P_2 fraction. The equivalent ACh-Cl activity of the betaine CoA esters again accounted for the major part of the activity. When isolated in the absence of eserine, there was a loss in the P_2 fraction of 40% of the equivalent ACh-Cl activity of the betaine CoA esters. This loss is comparable to that of 50% obtained with the rat.

C. Chemical Identification of Acetylcholine-like Substances in Normal Rat Brain

The subcellular distribution of ACh-Cl equivalent activity in rat brain was established through tentative chromatographic identification of substances which contributed to the overall equivalent ACh-Cl activity. In order to define clearly the nature of these substances, it was necessary to attempt chemical analysis of the material(s) in the extract.

There was no necessity to separate the brain into subcellular fractions, because activity in other subcellular fractions could be due to particle contamination from P_2. Since the quantity of active substances in brain tissue is small, it was necessary to use a large number of rats for each experiment, so that a sufficient yield of material was available for chemical identification. Accordingly, 100 rats were used for this experiment. The brains from these rats were excised and, as described under "Methods", extracted with TCA and the

methanol residue was precipitated with Reinecke at pH 8.5 (128). The reineckate was collected, washed and the precipitate made Reinecke-free by heavy metal precipitation. The residue was then chromatographed in the butanol-water system. After chromatographic development, substances in the eluates of the bands with R_F 0.05-0.15, 0.15-0.45 and 0.50-0.70 were converted to their tetrachloroaurate derivative by boiling with 10% gold chloride solution in 1% HCl. On cooling, the tetrachloroaurates crystallized out of solution. As described in "Methods", when the mother liquor was left in the refrigerator for 3-4 days, more tetrachloroaurates crystallized out of solution. The melting points were determined and are shown in Table XII.

TABLE XII

Chemical Identification of Material Present in the Various Bands of the Chromatograms Made from Reinecke Precipitable Material from Brain Extracts.

Tetrachloroaurates from	M.P. (°C)	Identity
R_F 0.05 - 0.15	169 - 173 228 - 230	ACh Choline
R_F 0.15 - 0.45	89 - 92	BuCh
R_F 0.50 - 0.70	122 - 126 148 - 150 182 - 185 205 - 206	AC l-Carnitine GBB Crotonbetaine

The melting points of the tetrachloroaurate derivatives of these compounds have been reported and affirmed the identity of these substances.

Tetrachloroaurate derivatives of	M.P. (°C)	Author
ACh	169	Stedman and Stedman (142).
	165	Hosein et al. (108).
Choline	230	Hosein et al. (108).
BuCh	85 - 90	Hosein et al. (108).
AC	128	Krimberg and Wittandt (116).
	129 - 130	Strack et al. (252).
l-Carnitine	152 - 153	Linneweh (105).
	153 - 154	Krimberg (90).
	157	Strack et al. (252).
	155	Tomita and Sendju (92).
GBB	182 - 184	Linneweh (105).
Crotonbetaine	212 - 214	Linneweh (105).

From the data presented in Table XII, the material in the band with R_F 0.50-0.70 consisted of a mixture of AC, l-carnitine, GBB and crotonbetaine. The amounts obtained were too small to be weighed but, from experimental observation, these substances were present in relatively decreasing amounts in the order listed.

The CoA molecule is composed of adenine, ribose, phosphate, pantothenic acid and β-mercaptoethylamine. Acid or alkaline hydrolysis of CoA is known to break the molecule into these components (245). The acid hydrolysate of the eluate from the band with R_F 0.50-0.70 after reacting with the gold chloride and precipitation of the tetrachloroaurate derivative, yielded a supernatant which contained excess gold chloride. The excess gold chloride was removed with hydrogen sulphide and the solution was evaporated to dryness. The dry methanol extract of the residue was tested for ribose (246) and for

phosphate (244). Both were found to be present.

The eluate from the band with R_F 0.50-0.70 showed the presence of the CoA ester linkage with ultraviolet absorption at 260 mμ. Alkaline hydrolysis decreased absorption at 232 mμ. showing cleavage of the CoA ester linkage but it did not alter absorption at 260 mμ. as expected, since this reaction does not affect the absorption of adenine.

The conclusion was, therefore, drawn that the material in the band with R_F 0.50-0.70 consisted of the CoA esters of AC, l-carnitine, GBB and crotonbetaine. This is in accord with the results of Hosein, Proulx and Ara (108) and Hosein (127) and reaffirmed the presence of acetyl-l-carnityl CoA.

It is known that Reinecke precipitation of tissue extracts causes a loss of ACh-like activity. To avoid this, TCA extracts from 50 rat brains were chromatographed, taking the precaution to apply the extract on as many strips of 6" x 20" Whatman No. 1 chromatographic paper as were required to ensure proper development. This procedure caused no loss of active material. The substances in the bands with R_F 0.05-0.15 and 0.50-0.70, as before, were converted to their tetrachloroaurates. The melting points were determined and they are presented in Table XIII.

Crotonbetaine could not be found in the band with R_F 0.50-0.70 and this may be due to its relatively slight occurrence. These results did, however, show that in this band all the active substances can be accounted for by the CoA esters of betaines and in the band with R_F 0.05-0.15 by both ACh and AC.

TABLE XIII

Chemical Identification of Material Present in the Various Bands of the Chromatograms Made from TCA Extraction of Brain Tissue

Tetrachloroaurates from	M.P. (°C)	Identity
R_F 0.05 - 0.15	172 - 174 122 - 124	ACh AC
R_F 0.50 - 0.70	125 - 126 148 - 150 182 - 184	AC l-Carnitine GBB

SECTION 11

Brain Subcellular Acetylcholine-like Activity during Narcosis

This study was based on the results of experiments reported by Richter and Crossland (141), Hosein, Proulx and Ara (108), Hosein and Ara (239), Hosein and Proulx (73) and Kurokawa et al. (72) on the effect of narcosis on the bound ACh and ACh-like substances of brain tissue. These results have previously been summarized in the "Introduction". In the present investigation, the effects of a whole spectrum of narcotics such as ether, pentobarbital, ethyl alcohol and nitrous oxide were studied in vivo in the rat and that of magnesium in the rabbit.

A. The Effect of Ether, Pentobarbital, Ethanol and Nitrous Oxide Narcosis on the Acetylcholine-like Activity of Substances in the P_2 Fraction of Rat Brain Homogenates Prepared in Eserinized 0.32 M Sucrose

Narcosis was induced for half an hour in the

experimental animal by intraperitoneal injection for pentobarbital and ethanol and by inhalation for ether and nitrous oxide. The subcellular fractions were prepared as previously described and the active substances were assayed on the frog rectus preparation.

The effect of narcosis was reflected in an increase in total equivalent ACh-Cl activity. From Table XIV, column 2, the statistically significant increase in equivalent ACh-Cl activity was accounted for mainly by the betaine CoA ester fraction. A significant increase was also noted in the sample of "pooled substances", except in the case of pentobarbital. The small amount of activity in the ACh fraction did not permit accurate assay and hence statistical comparison with the control values.

TABLE XIV

Distribution of ACh-like Activity of Substances in the P_2 Fraction Isolated from Rat Brain Homogenates in the Presence of Eserine.

Treatment	No. of Animals	Equivalent Activity in µg. ACh-Cl in P_2 per half Rat Brain. Mean and S.D. (Frog Rectus Assay)		
		ACh R_F 0.05-0.15 (1)	Betaine CoA Esters R_F 0.50-0.70 (2)	Pooled Substances (3)
None (controls)	9	0.02	0.95 ± 0.14	0.26 ± 0.10
Ether	5	0.02	1.71 ± 0.16	0.82 ± 0.35
Pentobarbital	5	0.04	1.61 ± 0.11	0.39 ± 0.22*
Ethanol	3	0.04	1.71 ± 0.22	0.65 ± 0.20
Nitrous Oxide	3	0.06	1.41 ± 0.12	0.55 ± 0.20

All changes in columns (2) and (3) are significant when subjected to "Student's" t test, except that marked *.

B. The Effect of Ether, Pentobarbital, Ethanol and Nitrous Oxide Narcosis on the Acetylcholine-like Activity of Substances in the P_2 Fraction of Rat Brain Homogenates Prepared in Eserine-free 0.32 M Sucrose

As previously discussed, residual equivalent ACh-Cl activity can be recovered from subcellular fractions isolated in absence of eserine. The amount recovered is about 60% of the extractable ACh-Cl activity from subcellular fractions isolated in eserinized medium.

TABLE XV

Distribution of ACh-like Activity of Substances in the P_2 Fraction Isolated from Rat Brain Homogenates in the Absence of Eserine.

Treatment	No. of Animals	Equivalent Activity in µg. ACh-Cl in P_2 per half Rat Brain. Mean and S.D. (Frog Rectus Assay)		
		ACh R_F 0.05-0.15 (1)	Betaine CoA Esters R_F 0.50-0.70 (2)	Pooled Substances (3)
None (Controls)	6	0.02	0.55 ± 0.12	0.19 ± 0.07
Ether	5	0.01	0.84 ± 0.14	0.56 ± 0.23
Pentobarbital	5	0.03	1.13 ± 0.13	0.50 ± 0.21
Ethanol	3	0.03	1.12 ± 0.28	0.67 ± 0.10
Nitrous Oxide	3	0.05	0.89 ± 0.10	0.46 ± 0.16

All changes in columns (2) and (3) and significant when subjected to "Student's" t test.

The effect of narcosis was reflected in an increase of the equivalent ACh-Cl activity. From Table XV, column (2) it is seen that the statistically significant increase in equivalent

ACh-Cl activity was again accounted for by the betaine CoA ester fraction. This increase raised the level of ACh-Cl like activity to that found in a normal P_2 subcellular fraction isolated in eserinized 0.32 M sucrose, suggesting the effect of prevention of release by narcosis is equatable with prevention of hydrolysis by eserine of the active substances. The increase in the "pooled" sample was also statistically significant. Again, the level of ACh itself appeared unaltered.

From Tables XIV and XV, the increase of equivalent ACh-Cl activity in the betaine CoA ester fraction P_2 isolated in the eserinized and in eserine-free medium was about 0.7 µg. and 0.5 ug. respectively. As there was no great significant difference between these two values, it would appear that the increased activity was due to a material which was not entirely sensitive to cholinesterase.

C. <u>The Effect of Ether, Pentobarbital, Ethanol and Nitrous Oxide Narcosis on the Acetylcholine-like Activity of Substances from S_2 Fraction of Rat Brain Homogenates Prepared in Eserinized 0.32 M Sucrose</u>

The total equivalent ACh-Cl activity found in this subcellular fraction was relatively low compared with that in the P_2 fraction.

The effect of narcosis was reflected in an increase in the equivalent ACh-Cl activity. From Table XVI, it is seen that the slight but significant increase in equivalent ACh-Cl activity was mainly accounted for by the betaine CoA esters.

The equivalent ACh-Cl activity in the "pooled" sample varied with each narcotic. S_2 is the supernatant fraction and, as such, contained the sucrose homogenizing medium. Although most of the sucrose could be removed by precipitating out of methanol, some of it may have remained behind and, on chromatography, stayed on the starting line. The "pooled" sample included the starting line and therein the sucrose most likely interfered with the bioassay. The assayed value may, therefore, not represent true ACh-like activity and hence no reliable conclusion can be derived from the statistical analysis of the results. The level of ACh itself remained unaltered.

TABLE XVI

Distribution of ACh-like Activity of Substances in the S_2 Fraction Isolated from Rat Brain Homogenates in the Presence of Eserine.

Treatment	No. of Animals	Equivalent Activity in µg. ACh-Cl in S_2 per half Rat Brain. Mean and S.D. (Frog Rectus Assay)		
		ACh R_F 0.05-0.15 (1)	Betaine CoA Esters R_F 0.50-0.70 (2)	Pooled Substances (3)
None (Controls)	4	0.01	0.34 ± 0.04	0.09 ± 0.01
Ether	4	0.01	0.50 ± 0.04	0.09 ± 0.03
Pentobarbital	5	0.01	0.48 ± 0.07	0.07 ± 0.01
Ethanol	3	0.04	0.41 ± 0.02	0.35 ± 0.03
Nitrous Oxide	3	0.04	0.61 ± 0.11	0.27 ± 0.11

All changes in column (2) are significant when subjected to "Student's" t test.

D. The Effect of Ether, Pentobarbital, Ethanol and Nitrous Oxide Narcosis on the Acetylcholine-like Activity of Substances in the S_2 Fraction of Rat Brain Homogenates Prepared in Eserine-free 0.32 M Sucrose.

TABLE XVII

Distribution of ACh-like Activity of Substances in the S_2 Fraction Isolated from Rat Brain Homogenates in the Absence of Eserine.

Treatment	No. of Animals	Equivalent Activity in µg. ACh-Cl in S_2 per half Rat Brain. Mean and S.D. (Frog Rectus Assay)		
		ACh R_F 0.05-0.15 (1)	Betaine CoA Esters R_F 0.50-0.70 (2)	Pooled Substances (3)
None (Controls)	4	0.01	0.12 ± 0.02	0.07 ± 0.02
Ether	4	0.01	0.25 ± 0.01	0.06 ± 0.01
Pentobarbital	5	0.01	0.21 ± 0.05	0.07 ± 0.02
Ethanol	3	0.04	0.30 ± 0.04	0.24 ± 0.05
Nitrous Oxide	3	0.03	0.29 ± 0.07	0.16 ± 0.03

All changes in column (2) are significant when subjected to "Student's" t test.

The effect of narcosis was reflected in an increase of total equivalent ACh-Cl activity as shown in Table XVII. This increase was accounted for by the betaine CoA esters. The significantly high activity in the "pooled" sample in the case of ethanol and nitrous oxide was probably due to sucrose interference of the bioassay. The activity in the "pooled" sample in the case of ether and pentobarbital did not differ significantly from the control. The level of ACh itself appeared unaltered.

E. The Effect of Ether, Pentobarbital, Ethanol and Nitrous Oxide Narcosis on the Acetylcholine-like Activity of Substances in the P_1 Fraction of Rat Brain Homogenates Prepared in Eserinized 0.32 M Sucrose

TABLE XVIII

Distribution of ACh-like Activity of Substances in the P_1 Fraction Isolated from Rat Brain Homogenates in the Presence of Eserine.

Treatment	No. of Animals	Equivalent Activity in µg. ACh-Cl in P_1 per half Rat Brain Mean and S.D. (Frog Rectus Assay)		
		ACh R_F 0.05-0.15 (1)	Betaine CoA Esters R_F 0.50-0.70 (2)	Pooled Substances (3)
None (Controls)	9	0.02	0.26 ± 0.13	0.24 ± 0.14
Ether	5	0.01	0.22 ± 0.12	0.48 ± 0.10*
Pentobarbital	5	0.03	0.17 ± 0.12	0.39 ± 0.18
Ethanol	3	0.02	0.11 ± 0.03	0.22 ± 0.03
Nitrous Oxide	3	0.02	0.15 ± 0.04	0.21 ± 0.08

All changes in columns (2) and (3) are not significant when subjected to "Student's" t test, except that marked *.

From Table XVIII, it is seen that narcosis did not alter significantly the level of total equivalent ACh-Cl activity. The equivalent ACh-Cl activity of 0.48 µg. in the "pooled" sample in the case of ether differed significantly from the control. No explanation can be given for this.

F. The Effect of Ether, Pentobarbital, Ethanol and Nitrous Oxide Narcosis on the Acetylcholine-like Activity of Substances in the P_1 Fraction of Rat Brain Homogenates Prepared in Eserine-free 0.32 M Sucrose

TABLE XIX

Distribution of ACh-like Activity of Substances in the P_1 Fraction Isolated from Rat Brain Homogenates in Absence of Eserine.

Treatment	No. of Animals	Equivalent Activity in µg. ACh-Cl in P_1 per half Rat Brain Mean and S.D. (Frog Rectus Assay)		
		ACh R_F 0.05-0.15 (1)	Betaine CoA Esters R_F 0.50-0.70 (2)	Pooled Substances (3)
None (Controls)	6	0.01	0.22 ± 0.11	0.20 ± 0.16
Ether	5	0.01	0.16 ± 0.02	0.51 ± 0.13
Pentobarbital	5	0.03	0.24 ± 0.12	0.29 ± 0.12
Ethanol	3	0.04	0.07 ± 0.05	0.17 ± 0.04
Nitrous Oxide	3	0.01	0.11 ± 0.04	0.23 ± 0.12

All changes in columns (2) and (3) are not significant when subjected to "Student's" t test.

From Table XIX, it is concluded that narcosis did not alter significantly the level of equivalent ACh-Cl activity attributable to the betaine CoA esters and to ACh itself.

G. The Effect of Magnesium Narcosis on the Acetylcholine-like Activity of Substances in Subcellular Fractions of Rabbit Brain Homogenates Prepared in Eserinized and Eserine-free 0.32 M Sucrose

The narcotic effect of magnesium ions was studied in the rabbit rather than in the rat, since the latter was found to be resistant to magnesium narcosis. Narcosis was induced for half an hour by subcutaneous injection of magnesium sulphate. The excised brain was extracted as previously described and assayed for ACh-like activity with the frog rectus abdominis preparation.

TABLE XX

The Effect of Magnesium Narcosis on the ACh-like Activity of Substances in Rabbit Brain Subcellular Fractions Prepared in Eserinized 0.32 M Sucrose.

µg. ACh-Cl per gm. (Frog Rectus Assay)

Probable Substances	P_1	P_2	S_2
R_F 0.05-0.15 (ACh)	0.03	0.05	0.05
R_F 0.50-0.70 (Betaine CoA Esters)	0.20 ± 0.01	0.98 ± 0.12	0.45 ± 0.13
Pooled Substances	0.07 ± 0.05	0.15 ± 0.03	0.03 ± 0.01
Total	0.30 ± 0.03	1.18 ± 0.05	0.53 ± 0.18
No. of Experiments	3	3	3

TABLE XXI

The Effect of Magnesium Narcosis on the ACh-like Activity of Substances in Rabbit Brain Subcellular Fractions Prepared in Eserine-free 0.32 M Sucrose.

µg. ACh-Cl per gm. (Frog Rectus Assay)

Probable Substances	P_1	P_2	S_2
R_F 0.05-0.15 (ACh)	0.04	0.03	0.04
R_F 0.50-0.70 (Betaine CoA Esters)	0.15 ± 0.04	0.84 ± 0.08	0.23 ± 0.05
Pooled Substances	0.08 ± 0.04	0.18 ± 0.04	0.13 ± 0.09
Total	0.27 ± 0.09	1.05 ± 0.08	0.44 ± 0.07
No. of Experiments	3	3	3

The data presented in Tables XX and XXI, when compared with those in Tables X and XI respectively, show an increase of total equivalent ACh-Cl activity during magnesium narcosis. This increase, which was statistically significant ($p < 0.05$), was accounted for by the betaine CoA esters in the P_2 fraction. There were no significant changes in the P_1 and S_2 fractions ($p > 0.05$).

H. Chemical Identification of Substances with Acetylcholine-like Activity in Narcotized Rat Brain

In the foregoing experiments, it was established that the effect of narcosis manifested itself in an accumulation of ACh-like substances in the brain. The chemical nature of these substances was tentatively and chromatographically identified as the CoA esters of the betaines. Since chromatography does not

by itself provide proof of identification, it was thought necessary to analyse chemically the substances which contributed to the increase in equivalent ACh-Cl activity in brain during narcosis.

One hundred rats were narcotized with pentobarbital and their excised brains were extracted with 10% TCA. After Reinecke precipitation at pH 8.5, the residue was resolved chromatographically in the butanol-water system. The substances in the eluates of the bands with R_F 0.05-0.15, R_F 0.15-0.25, R_F 0.25-0.45 and R_F 0.50-0.70 were converted to their tetrachloroaurate derivatives. After 3 days in the refrigerator, the tetrachloroaurates crystallized out of solution and their melting points were determined.

TABLE XXII

Chemical Identification of Material Present in the Various Bands of the Chromatograms Made from Reinecke Precipitable Material from Pentobarbital Narcotized Brain Extracts

Tetrachloroaurates from	M.P. (°C)	Identity
R_F 0.05 - 0.15	122 - 126 170	AC ACh
R_F 0.50 - 0.70	124 - 125	AC

No crystals were obtained from the eluates of other bands. From the results presented in Table XXII it was striking that the tetrachloroaurate derivatives of the R_F 0.50-0.70 region consisted only of AC. The sharp melting point testified to its homogeneous composition. The yield was in an amount

comparable with the combined quantity of GBB, crotonbetaine, l-carnitine and AC isolated from the brains of 100 normal rats.

Acetyl-l-carnitine was also found in the eluates from the band with R_F 0.05-0.15 in considerably higher amounts than ACh. This relative amount was estimated in terms of the number of crystals per microscope field. It was, however, not possible to weigh the crystals since the amount obtained was very small. In this experiment only ACh was found and this was probably due to the fact that the animals were not deeply narcotized. In subsequent experiments to be described, ACh could not be found. The isolated AC tetrachloroaurate was found to be difficultly soluble in water, insoluble in ether and soluble in alcohol, in accord with the observations of Krimberg and Wittandt (116).

The results were found to be reproducible as evidenced in a repeat experiment when AC was isolated and identified as the tetrachloroaurate.

In order to prove the presence of the betaine moiety of AC, another experiment was carried out in which the reineckate precipitate from the extracts of 100 pentobarbital narcotized rat brains was heated in concentrated sulphuric acid to hydrolyse the ester linkage and to dehydrate the resultant carnitine molecule. This was again converted to the tetrachloroaurate derivative which melted at 193-195°C. This substance was identified as crotonbetaine tetrachloroaurate. Carter *et al.* (241), using similar experimental conditions, reported a melting point of

196-200°C for crotonbetaine tetrachloroaurate, which lent support to the identification of crotonbetaine in the above experiment. These melting points are lower than that of crystalline crotonbetaine and it may be due to the fact that the crotonbetaine found from the reaction was not isolated, but was used directly in the reaction mixture. The probability that crotonbetaine was obtained was, therefore, likely.

Ribose determination by the Tauber method (246) and phosphate by the Fiske-Subbarow method (244) were found to be positive in the supernatant fluid remaining behind after the crystallization and precipitation of the tetrachloroaurate derivative. As previously mentioned, adenine and CoA ester linkage were found by means of ultraviolet absorption at 260 and 232 mμ. Alkali hydrolysis decreased the absorption only at 232 mμ. The conclusion was, therefore, drawn that the substance which accumulated in brain during narcosis was acetyl-l-carnityl CoA.

It is known that Reinecke precipitation causes quantitative loss of biological activity. It has been the experience of Hosein's group that with careful chromatography, TCA extract of brain tissue first washed with water-saturated butanol can be resolved into reasonably clean fractions from which tetrachloroaurates can be prepared. Experiments of this kind involve no loss of biological activity. With this view in mind, tetrachloroaurates were prepared from the material in the bands with R_F 0.05-0.15 and R_F 0.50-0.70 of butanol-water chromatographically resolved TCA extracts of 50 pentobarbital narcotized brains. The melting points of the tetrachloroaurate derivatives were

determined and these are presented in Table XXIII.

This experiment reaffirms the identification of AC from both the R_F 0.05-0.15 and R_F 0.50-0.70 bands. In the latter band, l-carnitine was also found but in a relatively small quantity compared with AC. It should be pointed out that, in this experiment, no ACh could be found in the R_F 0.05-0.15 band.

TABLE XXIII

Chemical Identification of Material Present in the Various Bands of the Chromatograms Made from Pentobarbital Narcotized Brain Extracts Washed with Water-Saturated Butanol.

Tetrachloroaurates from	M.P. (°C).	Identity
R_F 0.05 - 0.15	120 - 124	AC
R_F 0.50 - 0.70	124 - 128 145 - 148	AC l-Carnitine

Hitherto, pentobarbital was used in the experiments on chemical identification. Chemical analysis of the active substances which accumulated in brain during ethanol narcosis was also performed. As in the previous experiment, the procedure involved only washing of the TCA extract from 50 ethanol narcotized rat brains with water-saturated butanol before chromatography. No Reinecke precipitation was performed.

The results presented in Table XXIV show that l-carnitine was identified in the bands with R_F 0.05-0.15 and R_F 0.50-0.70, but in the latter band AC predominated. The presence of l-carnitine was due, most likely, to hydrolysis of its conjugated

TABLE XXIV

Chemical Identification of Material Present in the Various Bands of the Chromatograms Made from Ethanol Narcotized Brain Extracts Washed with Water-Saturated Butanol

Tetrachloroaurates from	M.P. (°C).	Identity
R_F 0.05 - 0.15	150 - 154	l-Carnitine
R_F 0.50 - 0.70	120 - 124 150 - 154	AC l-Carnitine

parent compound during the lengthy experimental procedure.

In order to prove more conclusively the identity of AC, another 100 rats were narcotized with pentobarbital, and the brains extracted as described, and precipitated with Reinecke at pH 8.5. However, the chloroplatinate in addition to the tetrachloroaurate derivative was prepared and its melting point was determined. In this experiment with the chloroplatinate, no chromatography was performed, since in previous experiments, only AC was found. Acetyl-l-carnitine was identified by the melting point of 185°C of its chloroplatinate derivative. Krimberg and Wittandt (116) reported a melting point of 187°C for AC chloroplatinate, which was confirmed in preliminary experiments. The AC chloroplatinate isolated was difficultly soluble in water, in accord with the observation of Krimberg and Wittandt (116).

From this series of experiments where the isolation procedure caused loss of active material as in Reinecke pre-precipitation and in the modified procedure which involved

negligible or no loss of active substances, the conclusion was drawn that, during narcosis induced either by pentobarbital or ethanol, the active substance which accumulated in brain was acetyl-l-carnityl CoA.

I. The Physiological Activity of Acetyl-l-carnitine

The isolation and identification of AC in brain tissue made necessary a study of its physiological activity. l-Carnitine in meat extract was precipitated with a hot alcoholic mercuric chloride solution, and the complex was decomposed with hydrogen sulphide. l-Carnitine was obtained by extraction of the residue with absolute ethanol. It was acetylated with acetylchloride according to the method of Krimberg and Wittandt (116).

When assayed on the normal frog rectus, AC had 1/80 the activity of ACh-Cl; on the neostigmine sensitized frog rectus it had 1/100 the potency relative to ACh-Cl. This value is much lower than that reported by Dallemagne et al. (119) and was no doubt due to contamination by impurities which were not removed during the extraction procedure.

Hosein and Kato (unpublished) have chemically synthesized acetyl-d-carnitine. Acetyl-d-carnitine was found by this writer in a few preliminary experiments to block the stimulatory action of ACh on the neostigmine sensitized frog rectus abdominis preparation. Hosein (unpublished) has shown that acetyl-dl-carnitine is without ACh-like activity. This inhibitory action of acetyl-d-carnitine on ACh may be responsible for this lack of ACh-like activity of acetyl-dl-carnitine.

SECTION III

Differential Assay

Chang and Gaddum (34) stated that tissue extracts contained ACh alone, if the biological activity of the extracts, when estimated quantitatively in terms of ACh using several different test objects, the same result should be obtained in each case. As previously discussed, this conclusion is uncertain. Where differential assay results differ, several biologically active substances are present in tissue extracts. Conversely, no reliable conclusion can be drawn from experiments in which tissue extracts assay identically on several test objects.

A. A Comparison of the Acetylcholine-like Activity of Brain Extracts from Normal and Narcotized Rats Determined by Different Assay Preparations

In order to support further the chemical identification of active substances in normal and narcotized brain, differential assay employing the neostigmine sensitized frog rectus abdominis, the eserinized dorsal muscle of the leech and the guinea pig ileum as test objects was performed. Brain extracts from normal and pentobarbital narcotized rats were, therefore, tested with these preparations.

The data presented in Table XXV show that normal and pentobarbital narcotized brain extracts did not assay identically on the different test objects. This fact, by itself, justifies the conclusion that the equivalent activity of the brain extracts was not contributed by ACh alone.

TABLE XXV

The Activity in µg. ACh-Cl of Brain Extracts from Normal and Narcotized Rats as Assayed on Three Different Test Preparations

Condition	Neostigmine Sensitized Frog Rectus Abdominis	Eserine Sensitized Leech Dorsal Muscle	Guinea Pig Ileum
Normal	1.21 0.96 1.04	1.67 1.21 1.42	0.87 0.68 0.67
Narcosis	2.01 1.86 1.65	2.85 2.68 2.25	1.35 1.15 0.94

Statistical analysis by the method of two-way classification of variance (kindly computed by Dr. A.F. Naylor of the Dept. of Genetics, McGill University) shows that the ACh-Cl equivalent of normal as well as narcotized brain extracts as assayed on the different test objects differed significantly (in both types of samples, $p < 0.005$) from one test object to another. Furthermore, the deviation in the narcotized samples was significantly different ($p < 0.005$) from that in the normals, taking into account the higher values in the narcotized samples. This indicates that the nature of the active substances in the normal brain extracts differed from that in the narcotized brain extracts, a conclusion which is in accord with the results of chemical identification.

Brain extracts from normal and narcotized rats were also assayed on the frog rectus and the cat's blood pressure preparations. The assay on the cat's blood pressure was

performed by Miss M. Luttman of the Dept. of Physiology, McGill University. The results are shown in Table XXVI.

TABLE XXVI

The Activity in µg. ACh-Cl of Brain Extracts from Normal and Narcotized Rats as Assayed on Two Different Test Preparations

Condition	Neostigmine Sensitized Frog Rectus Abdominis (a)	Cat's Blood Pressure (p)	Ratio a/p
Normal	1.45 1.59 0.96 1.04	1.13 1.27 1.01 1.02	1.3 1.3 1.0 1.0
Narcosis	3.17 2.52 1.65 1.55	2.86 2.19 1.67 1.35	1.1 1.2 1.0 1.1

Statistical analysis of the data presented in Table XXVI shows there was no significant difference between the equivalent ACh-Cl values as assayed on the frog rectus abdominis and the cat's blood pressure preparations. In pursuance with previous discussion, differential assay results are of value only if they differ. Since chemical analysis has shown acetyl-l-carnityl CoA alone in brain taken from rats during narcosis, it would appear that the frog rectus abdominis and the cat's blood pressure are not sufficiently sensitive distinguishing test objects.

SECTION IV

Colorimetric Analysis

A. A Parallel Colorimetric and Bioassay Determination of Substances with Acetylcholine-like Activity in Brain Extracts Prepared from Normal and Narcotized Rats

A second method used to substantiate the chemical of the brain extracts was the bromophenol blue reaction of Nowell and Wilson (87). This reaction depends on the formation of a chloroform soluble complex, when micro-quantities of quaternary ammonium compounds are allowed to react in alkaline media with bromophenol blue. The colour intensity is read at 600 mu. The reaction is specific for quaternary ammonium compounds, and is especially sensitive to esters of quaternary ammonium compounds (87). These compounds exhibit different sensitivities to bromophenol blue. As shown in the "Introduction" of this thesis, AC is 10 times and BuCh 20 times more sensitive than ACh. It was possible, therefore, to distinguish ACh from other quaternary ammonium compounds in tissue extracts, if the bromophenol blue reaction was used in conjunction with biological assay.

A standard curve for ACh-Cl-bromophenol blue complex was constructed. For the purpose of parallel colorimetric and bioassay determinations, TCA extracts of brain tissue normal and 30 pentobarbital narcotized rats were purified with Reinecke and chromatographed on the butanol-water system. Specific bands were eluted with methanol. Each eluate was divided into 3 equal portions. One portion was boiled in 0.1 N NaOH to destroy the

esters and then neutralized and used as the tissue blank, the second portion was used as the experimental and the third assayed for ACh-like activity on the frog rectus preparation. The results are presented in Tables XXVII and XXVIII.

TABLE XXVII

The Equivalent ACh-Cl Activity of Brain Extracts Prepared from Normal Rats as Determined by the Colorimetric and Bioassay Methods

Material from	Optical Density	Calculated ACh µg. for Bromophenol Blue Complex	Rectus Assay ACh µg. in Duplicate Sample
R_F 0.05-0.15	0.002	0	0.4
" " " boiled blank	0.004	0	-
R_F 0.50-0.70	0.125	480	10.2
" " " boiled blank	0.002	0	-
"Pooled" band	0.018	0	3.8
" " boiled blank	0.004	0	-
Reagent blank	0.000		

In the extract prepared from normal rats, the bromophenol blue colour was obtained only from the material in the band with R_F 0.50-0.70; no colour was obtained with the material in either the R_F 0.05-0.15 band or from the "pooled" sample. Calculated from the standard curve, the colour absorbance from the material from the band with R_F 0.50-0.70 was equivalent to 480 µg. ACh-Cl, yet the rectus assay showed it had only 10.2 µg. Obviously the material responsible for the production of colour intensity and for the biological activity could not have been ACh.

TABLE XXVIII

The Equivalent ACh-Cl Activity of Brain Extracts Prepared from Pentobarbital Narcotized Rats as Determined by the Colorimetric and Bioassay Methods.

Material from	Optical Density	Calculated ACh µg. for Bromophenol Blue Complex	Rectus Assay ACh µg. in Duplicate Sample
R_F 0.05-0.15	0.002	0	0.6
" " " boiled blank	0.000	0	-
R_F 0.50-0.70	0.086	320	5.8
" " " boiled blank	0.002	0	-
"Pooled" band	0.004	0	0.5
" " boiled blank	0.002	0	-
Reagent blank	0.000		

In the extracts obtained from pentobarbital narcotized rats, the colour absorbance from the material in the band with R_F 0.50-0.70 was equivalent to 320 µg. ACh-Cl but the rectus assayed only 5.8 µg. ACh-Cl. The same conclusion was also drawn that the material could not have been ACh. The results from these experiments also indicate that the substance responsible for the bromophenol blue colour complex was yet more sensitive to bromophenol blue than that found in extracts from normal animals. They, therefore, support the chemical analysis data, that the material present in the extract is most likely acetyl-l-carnityl CoA.

SECTION V

Acetylcholine-like Activity in Heart

The heart is an abundantly innervated organ and its ACh-like material does not behave like ACh in respect to its chromotographic mobility (56). Thiamine deficient heart tissue extract does not assay for ACh-like activity identically on the frog rectus and the guinea pig ileum (56). Again, dog coronary vessels constrict on vagus stimulation, but dilate on administration of ACh (253). These factors prompted a study on the distribution of substances with ACh-like activity in normal and in narcotized hearts.

A. The Effect of Pentobarbital and Ethanol Narcosis on the Distribution of Substances with Acetylcholine-like Activity in the Rat Heart

Hearts excised from normal and pentobarbital or ethanol narcotized rats were macerated in TCA in the "Virtis" homogenizer. The extracts were chromatographically resolved on the butanol-water system and eluates from specific bands were assayed on the frog rectus preparation, all as previously described for brain tissue.

From the data presented in Table XXIX, the chromatographic distribution of ACh-like substances suggests that betaine CoA esters accounted for about 75% of the total activity of normal heart extract. The contribution made by ACh itself was small and accurate biological assay was not possible.

TABLE XXIX

The Distribution of ACh-like Activity of Substances in Heart Extracts Prepared from Normal and Narcotized Rats

Treatment	No. of Animals	Equivalent Activity in µg. ACh-Cl per gm. of Tissue Mean and S.D. (Frog Rectus Assay)		
		ACh R_F 0.05-0.15 (1)	Betaine CoA Esters R_F 0.50-0.70 (2)	Pooled Substances (3)
None (Controls)	5	0.01	0.68 ± 0.06	0.11 ± 0.02
Pentobarbital	6	0.02	0.85 ± 0.04	0.08 ± 0.02*
Ethanol	5	0.01	1.26 ± 0.22	0.22 ± 0.04

All changes in columns (2) and (3) are significant when subjected to "Student's" t test, except that marked *.

The effect of pentobarbital and ethanol narcosis was manifested in an accumulation of betaine CoA esters. The increase in equivalent ACh-Cl activity was statistically significant. While changes of ACh level were not statistically analysed, it would appear that its concentration remained unaltered during narcosis.

B. Chemical Identification of Substances with Acetylcholine-like Activity in Normal and Pentobarbital Narcotized Rat Heart

In order to identify chemically and to compare qualitatively the ACh-like substances in normal and narcotized rat, 50 hearts taken from the narcotized rats were homogenized in 10% TCA. The residue, after its deproteinization by TCA, was

washed with water-saturated butanol and converted to the tetrachloroaurate derivatives as previously described for brain. The melting points were determined and these are shown in Table XXX.

TABLE XXX

Chemical Identification of Material Present in Heart Extracts Prepared from Normal and Narcotized Rats

Normal		Pentobarbital	
M.P. (°C) of Tetrachloroaurate	Identity	M.P. (°C) of Tetrachloroaurate	Identity
124 - 126	AC	120 - 124	AC
170 - 171	ACh	144 - 148	l-Carnitine
178 - 182	GBB		
228 - 232	Choline		
254 - 256	PCh		

The results presented in Table XXX show that normal rat hearts contained choline and its acetyl and propionyl esters, GBB and AC. In contrast, narcotized hearts contained predominantly AC and a slight amount of l-carnitine. Because the amounts obtained were too small to be weighed, quantitative estimation was made in terms of number of crystals per microscopic field. Ribose and phosphate were detected in the supernatant after crystallization of the tetrachloroaurate derivatives, by the Tauber (246) and the Fiske Subbarow (244) tests respectively. The betaines, therefore, were probably derived from their CoA esters, as shown in greater detail with extracts from brain. From the results

obtained in this study, it would appear that heart metabolism, like that in brain is altered by narcotics so that in both tissues there is the accumulation of acetyl-l-carnityl CoA during narcosis.

DISCUSSION

Since the classical work of Loewi, Dale, Gaddum, Feldberg, Nachmansohn and others, little attention has been devoted to the problem of whether choline esters other than ACh, such as PCh, BuCh and/or betaine esters are present in animal tissue; all of which may play a role in nerve function. Biological methods for the assay of ACh are not specific, insofar as the chemical nature of the assayed compound is concerned. These biological preparations are highly sensitive test objects and measure only minute amounts of active substances in tissue extracts. It is emphasized that the substance(s) remain unknown without chemical identification (117). For example, Dale and Dudley (78) demonstrated ACh-like activity in spleen extracts. From identical differential assay results, these authors logically concluded that the active material was ACh. Twenty five years later, Gardiner and Whittaker (144) found that the active material in spleen extracts was a mixture of ACh and PCh. This emphasizes the value of chemical analysis of materials in tissue extracts. Florey (254) also has stated that the nature of active materials can only be revealed by means of chemical analysis. With respect to this, the most serious flaw in the ACh theory is the lack of chemical identification. Where ACh was recognized in mammalian brain (142), only 1% of the tetrachloroaurate derivative was identified as such. The possibility that other active substances were present in the discarded material cannot be excluded. The introduction of microanalytical methods has made possible advances in the

separation and identification of biologically active substances present in tissues in small amounts. The technique of paper chromatography has been shown to be suitable for the separation of various active components (59). Using this method in combination with classical biological methods, Banister, Whittaker and Wijesundera (57) demonstrated that ox spleen contained ACh, PCh and an unidentified component F of higher R_F in butanol-water. Component F was later identified as a mixture of PCh and ACh, in combination with an unknown Zwitterion X^{+-} which conferred on these esters a high R_F value (255). According to Gerard (256), ACh as a neurotransmitter is now being supplemented by PCh. Koelle (257) contends that other substances besides ACh and PCh are involved in nerve function. Another successful identification of an ester of choline other than ACh was demonstrated by Erspamer and Benati (285). The highly active murexine, which is present in the hypobranchial gland of Murex trunculus and has been known since 1938 to be closely related to ACh, was identified as urocanylcholine. Holtz and Schumann (150), using the combined technique of paper chromatography and differential assay, successfully identified BuCh in ox brain. Thus there are various reports in the literature, where a combination of more modern techniques and old classical methods have produced results which indicate other esters besides ACh are present in tissues and which may participate in neural function. It was with this view in mind that this project was undertaken. The fundamental background of this project was based on the work of Richter and Crossland (141) and of Hosein, Proulx and Ara (108).

Using a parallel technique of paper chromatography and chemical identification, Hosein et al. (108,129), produced evidence that the transmitter substances involved in the central nervous system are a group of substances, namely ACh, PCh, BuCh and the CoA esters of gBB, crotonbetaine, l-carnitine and AC. This finding may require a reevaluation of the theory of cholinergic transmission, because earlier workers considered ACh as the only transmitter substance in the cholinergic nervous system, whereas Hosein et al. (108) found that ACh and other choline esters contributed only about 20% of the total ACh-Cl activity.

The subcellular distribution in rat brain of these ACh-like substances has been described in the "Experimental". The identity of the betaine CoA esters has been reaffirmed through chemical analysis and is supported by differential bioassay and colorimetric determination.

The phenomenon of narcosis and brain ACh has been investigated from two directions. In 1949, Richter and Crossland (141) showed that the accumulation of brain ACh content was one of the end effects of narcosis. These results were confirmed by Elliott et al. (236) in the following year. From another direction, MacIntosh and Oborin (12) provided evidence that the rate of transudation of ACh from the cerebral cortex was decreased during pentobarbital and ether narcosis. It seems reasonable to interpret the results of these workers in terms of prevention of release of free ACh from nerve cells during decreased cerebral activity induced by narcotics. In

this respect, the active substance in the extract and perfusate was assumed to be ACh on the basis of bioassay procedure. In effect, Richter and Crossland (141) used methods which most closely approximated the technique of differential assay. The test objects employed, frog rectus, leech dorsal muscle and cat's blood pressure, did not identically assay the brain extracts. In accordance with the discussion of pharmacological identification by Chang and Gaddum (34), Richter and Crossland's data provide the first hint that biological activity in tissue extracts may not have been due to ACh alone.

It is known that the action of narcotics is to depress the intact nervous system, the deepest stages being marked by an almost complete quiet cerebral cortex (258). Perhaps it is reasonable to assume that the utilization of neurohormones is reduced in the depressed brain, although there is as yet no rigorous proof of this. Another possibility is that narcotics prevent or reduce the release of ACh from its "bound" form. MacIntosh (259) has emphasized that the amount of ACh in the nerve ending depot is maintained at a fixed value, in spite of the substantial turnover of ACh during rest. During increased or decreased cerebral activity and at rest, the storage of ACh is determined by its synthesis, its rate of release from the depot and its destruction. Whatever mechanism is operative in the narcotic-induced increase in the level of ACh activity in the brain, the most striking effect of this phenomenon is the relative efficiency of ACh metabolism in maintaining the level of the neurohormone within relatively narrow limits. This

imposition of a ceiling on the level of ACh activity in the brain may be related to a depression of the synthetic process of ACh itself. This observation agrees with the data of MacIntosh and Oborin (12), which may mean that the synthetic process of ACh itself is reduced during narcosis. Direct evidence for the narcotic inhibition of ACh synthesis by brain in vitro is available. Ether in quantities that suppress respiration, suppress ACh synthesis in brain (31). McLennan and Elliott (185) and Johnson and Quastel (186) have obtained evidence that various narcotic drugs depress the synthesis of ACh by brain slices.

Section II of the "Experimental" described the distribution of bioactive substances in subcellular particles of brain taken during narcosis. On the basis of bioassay of the substances occupying characteristic chromatographic positions, the narcotic-induced increase in ACh-Cl activity is due to the betaine CoA esters. Insofar as this does not determine the nature of the active substance, chemical analysis did show that the identifiable active substance was acetyl-l-carnityl CoA.

In section II of the "Experimental", the conclusion was drawn that the accumulation of equivalent ACh-Cl activity in brain during narcosis was due to acetyl-l-carnityl CoA, which was thought to be only partially labile to cholinesterase 1. The acetyl group may be cleaved but the CoA part remains stable. Experiments by Parmar et al. (260) have shown that cholinesterase preparations are not very active against butyryl-thiocholine. The accumulation of acetyl-l-carnityl CoA was

equated with the prevention of its release from the vesicular organelles. The possibility is considered that the prevention of release is not the factor but rather inhibition of brain cholinesterase (cholinesterase 1) by narcotics themselves causes accumulation of acetyl-l-carnityl CoA. Narcotic inhibition of cholinesterase 1, however, can be excluded in view of the finding of Schultz (261) that prolonged administration of barbiturates in animals does indeed decrease cholinesterase activity of the spinal cord but a single dose of drugs sufficient to cause deep sleep does not have this effect. In in vitro experiments, phenobarbitone does not inhibit brain cholinesterase (262) and the more common anaesthetics inhibit brain cholinesterase only in much higher concentrations.

The results reported in sections I, II and V of the "Experimental" indicate that acetyl-l-carnityl CoA is present in the normal and is the principal substance in narcotized brain and cardiac tissue. Only in one instance was ACh found in narcotized tissue. This stressed that ACh synthesis is inhibited during narcosis, while the metabolism of betaine CoA esters is altered, causing a shift of the equilibrium to acetyl-l-carnityl CoA.

Although the pharmacology of pure synthetic acetyl-l-carnityl CoA has not been studied, that of AC is well authenticated. The discovery of l-carnitine in living tissue (88) has provided another quaternary ammonium base structurally similar to choline. The demonstration of the pharmacological behaviour of AC (117,119) and its occurrence in brain suggests

it as another transmitter substance. The chemical and pharmacological behaviour of AC, like ACh, resembles the activity of tissue extracts. Acetyl-l-carnitine has muscarinic action and is active on neuromuscular and synaptic transmission (119).

Both ACh and AC are products of acetylation of weakly active tissue quaternary ammonium bases. They exhibit a high degree of similarity in their physiological mode of action in that both exert a rapidly appearing, easily reversible negative inotropic effect on the frog heart, which is influenced antagonistically by atropine and synergistically by eserine and as a consequence are to serve as stimulants of parasympathetic nervous elements. Apart from its identification in brain and heart, the physiological mode of action of AC is abolished by enzymic hydrolytic activity. These factors indicate that this substance, like ACh, is of physiological significance. The knowledge that AC is relatively less active than ACh on isolated test organs does not in any way contradict the physiological significance of this substance. The real significance is that by acetylation, l-carnitine, which is physiologically inert, is made active. The high similarity in physiological and chemical behaviour of ACh and AC is important. The battery of properties employed for the identification of ACh such as selective parasympathetic activity, restoration of activity after reacetylation, slight alkali resistance and hydrolysis by an enzyme which is inactivated at 56°C and by eserine (30) cannot be regarded as specific properties of ACh.

Cholinesterase and its substrate ACh hold an important position in the physiology of the nervous system. They are essential to the metabolism of the nerve cells. Cholinesterase hydrolyses ACh and this is considered the role that it plays in the organism. Thus cholinesterase is said to destroy ACh immediately after transmission has occurred and within the refractory period of the muscle or ganglion cells.

As early as 1939, Gilman et al. (263) described two types of hydrolytic enzymes in rat tissue, specific and non specific cholinesterase; the former was inhibited by minute amounts of eserine and, unlike the specific cholinesterase, the pseudo type was little affected by high concentration of eserine. The question arose as to whether the ACh hydrolysing factor could be ascribed to a single enzyme. The results of Hawkins and Gunter (264) have given evidence that pseudocholinesterase plays no essential part in the hydrolysis of ACh in vivo. The classes of cholinesterase were fully explored by Mendel et al. (265,266) who found that specific or cholinesterase 1 was chiefly found in the nervous system and in the red blood cells of many animal species. On the basis of present knowledge, cholinesterase resides chiefly in the brain cortex and cholinesterase 11 is limited to cell nuclei and glial tissue (267,268). In the vascular system, cholinesterase 1 is in the erythrocytes and cholinesterase 11 in the serum (269).

Substrate specificity of cholinesterase 1 and 11 has been studied. Alles and Hawes (269,270) observed that excess ACh depressed activity of cell cholinesterase, but did not

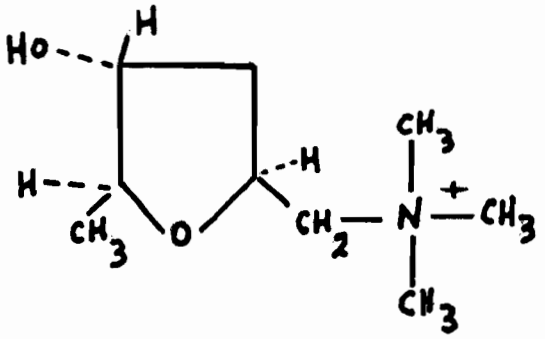
affect serum cholinesterase. Acetyl-β-methylcholine was split by the cells at the same rate as ACh, but the serum cholinesterase on the other hand hydrolysed the β-methyl derivative only slightly. While Alles and Hawes deserve the priority of initial discovery, the main substrate characteristics of cholinesterase 1 and 11 were put forward by Mendel and coworkers (265,266,271). Thus cholinesterase 11 hydrolyses ACh, benzoylcholine, tributyrin and methylbutyrate but not acetyl-β-methylcholine and that the activity toward any ester should be inhibited by low concentration of eserine. Cholinesterase 1, on the other hand, hydrolyses ACh and acetyl-β-methylcholine but not benzoylcholine, tributyrin or methylbutyrate. Tributyrin and methylbutyrate are hydrolysed by the ali esterase, but these enzymes do not hydrolyse choline esters and are not inhibited by low concentrations of eserine. In contrast to mammalian cholinesterase 1, the avian type apparently hydrolyses acetyl-β-methylcholine (272,273,274). That mammalian cholinesterase 1 alone most specifically cleaves acetyl-β-methylcholine and mammalian cholinesterase 11 has no effect on this substrate enabled Pfeiffer (132) to state that the true natural substrate for mammalian cholinesterase 1 should occur with a β carbon substitution. The same conclusion was also derived from a study of the analgesic drugs, isomethadone and methadone (157). We have observed from bioassay that the equivalent ACh-Cl activity of subcellular particles isolated in eserine free medium is reduced by about 40%. Chemical analysis has shown the presence of bioactive acetyl-l-carnityl CoA and it is not unreasonable to assume, on the basis of

eserine protection, that the acetyl moiety is cholinesterase 1 labile. Furthermore, it is also not unreasonable to assume, on the basis of Pfeiffer's (132) structure activity studies, that acetyl-l-carnityl CoA, possessing a substituted β carbon atom, is a more natural substrate than ACh for rat cholinesterase 1.

Cholinesterase 1 seems to have stereochemical specificity. Thus Beckett et al. (275) have shown that bovine cholinesterase 1 hydrolyses L(+)-acetyl-B-methylcholine, but the enzyme activity is inhibited by the D(-)-enantiomorph. While Hosein et al. are investigating the phenomenon of stereochemical factors in cholinesterase 1 activity, it is not a prioi to state that the stereochemical specificity of cholinesterase 1 is likely to be complemented by specific stereoisomers of acetylcarnitine and of acetylcarnityl CoA. On the basis of absolute structural and stereochemical characteristics of specific substrates for mammalian cholinesterase 1, the terminology of this enzyme system may need revision.

l-Carnityl CoA was discovered by Hosein et al. (108) and its presence in brain tissue was reaffirmed in experiments reported in sections 1 and 11 of the "Experimental". This compound possesses a β hydroxy group. In cerebral tissue, epinephrine, which has an isomeric ratio of 20 (132), and ACh, maintain homeostatic activity. Structure activity relationship requires the β hydroxy group of epinephrine be mirrored by an isosteric β hydroxy group in ACh, which, however, possesses no such group but which is fulfilled by l-carnityl CoA.

The observation that the true natural substrate for mammalian cholinesterase 1 should have its chemical structure β carbon substitution and higher muscarinic effect (132) has been discussed. Besides considering susceptibility to cholinesterase attack, optimum structural requirements for fit at cholinergic receptor must be taken into account. The activity of a drug or a neurohormone is in part determined by its goodness of fit to its receptor; primarily and most probably the goodness of fit depends on stereochemical configuration. Knowledge of the absolute configuration of these drugs or neurohormones may allow conclusions about the parasympathetic receptor. In a recent review, Waser (276) has emphasized the importance of stereochemical factors in muscarinic activity. Muscarine possesses three asymmetric carbon atoms which make possible 8 enantiomorphs or stereoisomers, of these only the natural form L(+)-muscarine is significantly active and is about 3 times as active as ACh.



L(+)-muscarine

Stereo specificity between L(+)- and D(-)- muscarine is high, by a factor of 1000 (276). The absolute configuration of L(+)-muscarine resembles that of L(+)-acetyl-B-methylcholine; the latter compound is 250 times as active as its D(-)-enantiomorph.(277,278). These active molecules seem to be complementary

with the cholinergic receptor since diastereoisomers differ largely in potency. L(+)-acetyl-β-methylcholine is equally as active as ACh (278). Steric localization of a substituent on the α carbon is unimportant, since both stereoisomers of acetyl-α-methylcholine are only weakly active (278); this is due to the fact that α-methyl substitution exerts a steric effect on the cationic head, thereby preventing a close drug receptor association (275).

The stereochemical configuration of the cholinergic receptor cannot be rigidly complemented by ACh, PCh or BuCh, since they do not offer possibilities of stereoisomerism. Certain stereoisomers of optically active acetylcarnitine, carnityl CoA and acetylcarnityl CoA, however, can complement stereochemical configuration of the cholinergic receptor. In a few preliminary experiments, acetyl-(+)-carnitine (acetyl-d-carnitine) was found to inhibit the action of ACh on the frog rectus. Acetyl-(-)-carnitine (AC) is active (117,119) and the racemic acetyl-(±)-carnitine (acetyl-dl-carnitine) is inert. Thus it would appear that the stereospecificity between acetyl-(-)-carnitine and acetyl-(+)-carnitine is indeed very high. Furthermore, it would appear that acetyl-(-)-carnitine is highly complementary to the cholinergic receptor.

The biochemical evidence implicating ACh in the chemical transmission of nerve impulses rests not only on its hydrolytic enzyme, but also on its synthetic enzyme. The choline acetylase system is not specific for the synthesis of ACh. This was first incidentally reported by Nachmansohn, Hestrin and

Voripaieff (145), who used this enzyme from a brain source to synthesize, in the absence of choline, an acetate ester which was biologically active. Subsequently, the extraordinary unspecificity of this enzyme system in respect to the alcohol part of the molecule was demonstrated by Burgen et al. (279). The role of acetyl-l-carnityl CoA in nerve function must ultimately rely on experimental demonstration of the existence of its biological synthetic mechanism. Hosein and Smoly (unpublished), using the fatty acid activating enzyme, have synthesized this compound. In relation to the non-specificity of the choline acetylase system, these authors (147) have also used this enzyme system to synthesize AC from dl-carnitine and acetate at a rate comparable to that of ACh. Only the l-isomer is acetylated, which suggests that the choline acetylase system also possesses stereochemical specificity. The synthetic mechanism for the production of AC is apparently not limited to the choline acetylase system. Friedman and Fraenkel (280) found an enzyme in both pigeon and sheep liver, which catalyses the acetylation of carnitine in the presence of CoA, ATP and acetate:

$$\text{Acetyl CoA} + \text{carnitine} \rightleftharpoons \text{acetylcarnitine} + \text{CoA}$$

In the work of Bremer (281), no requirement for CoA has been demonstrated, either in AC formation or in the deacetylation reaction. CoA-independent mechanism of these reactions in mitochondria cannot, therefore, be excluded. Fritz et al. (282) have isolated an enzyme system termed carnitine acetyltransferase from heart tissue which specifically

acetylates l-carnitine and of significance, choline does not react. Norum (283) has traced the enzyme system called acetyl-carnitine-CoA acetyltransferase which mediates the mitochondrial acetylation of carnitine from an incubation mixture of pyruvate and carnitine to the kidney, liver, muscle, heart and brain. In the brain, this enzyme system exists totally in the mitochondria. Because of the similar source, the brain acetylating enzyme described by Norum may be related to the choline acetylase system.

We have observed with bioassay that the total ACh-Cl equivalent activity of brain and heart increases during deep narcosis. Through chemical identification we have shown that only one substance (acetyl-l-carnityl CoA) was present in the extract from narcotized brain. It would appear, therefore, since this substance possesses ACh-like activity, one of the pharmacological effects of narcosis in brain is to alter the metabolism of the betaine esters, resulting in the accumulation of acetyl-l-carnityl CoA.

In normal animals, we have found a mixture of CoA ester derivatives of GBB, crotonbetaine, l-carnitine and AC. Chemically these substances are trimethylammonium derivatives of butyric acid and its derivatives. The metabolism of butyric acid as a CoA ester is known to go through the crotonic and the B-hydroxy derivatives. It is, therefore, likely that the metabolism of GBBCoA could be identical to butyryl CoA because the same types of intermediates are common to both pathways. With respect to the metabolism of GBB, this may not be unreasonable,

since in 1929 Linneweh (105) postulated just such a metabolic pathway for GBB:

GBB ⟶ crotonbetaine ⟶ l-carnitine

The relation of GBB with the protein molecule linked through GABA was established in 1909 by Engeland and Kutscher (99), who stated that the precursor of GBB was GABA which, in turn, could arise from glutamic acid.

Protein ⟶ glutamic acid ⟶ GABA ⟶ GBB

Because of the recognition of the betaines in brain tissue as their CoA derivatives by Hosein et al. (108), it is possible to interrelate this finding with the postulates of Linneweh (105) and of Engeland and Kutscher (99) as follows:

Glutamic acid ⟶ GABA ⟶ GBBCoA ⟶ crotonbetaine CoA
⟶ l-carnityl CoA ⟶ acetyl-l-carnityl CoA

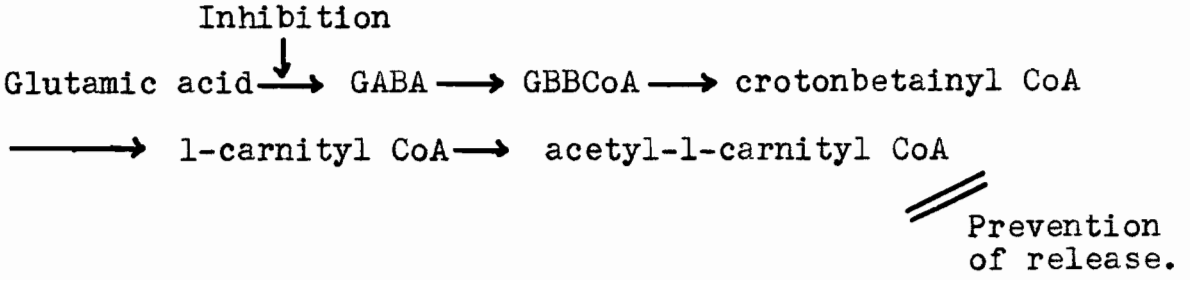
Assuming that such a pathway does exist, GBB CoA can be converted to crotonbetaine and then to l-carnityl CoA, which can then be acetylated. Since we found in the narcotized animal that acetyl-l-carnityl CoA was the only substance present with ACh-like activity, perhaps it is a product of GBB metabolism.

Narcosis is characterized by the inability of neurones of the central nervous system to metabolize glucose, lactic acid, pyruvic acid and glutamic acid (174). In narcosis, because the ability to metabolize carbohydrates is deficient, the oxidation

of fatty acids is incomplete and the products of this incomplete combustion, namely β-hydroxy butyric acid and aceto-acetic acid, accumulate (284). By analogy, narcosis results in an accumulation of acetyl-l-carnityl CoA, which is the acetylated derivative of l-carnityl CoA, γ-trimethylammonium-β-hydroxy butyric acid CoA ester. The β-keto derivative of GBB, analogous to aceto-acetic acid, has never been found in tissue extracts.

Studies by Whittaker (21) and de Robertis et al. (71) have shown that most of the ACh-like activity is present in subcellular particles. Hosein and Proulx (73) have shown that, when these particles are incubated at 37°C, material with ACh-like activity is released from within the particles into the incubation medium. Recent studies by Hosein and Levy (unpublished) have shown that, on incubating these particles from brain with 20mM concentration of various narcotics, there is inhibition of release of these materials with ACh-like activity from within subcellular particles.

It is possible to understand the neuronal mode of action of narcosis by (a) suppression of glutamic acid conversion to GABA, (b) accumulation of acetyl-l-carnityl CoA by prevention of its release from the subcellular particles.



SUMMARY

1) The subcellular distribution of the choline esters ACh, PCh and BuCh and the CoA esters of the betaines, namely gamma-butyrobetaine (GBB), crotonbetaine, l-carnitine and acetyl-l-carnitine (AC) in rat brain is described. In brain homogenates, the nuclear fraction, P_1, accounts for 24, the crude mitochondrial fraction, P_2, 57 and the supernatant fraction, S_2, 19% of the total ACh-like activity. The betaine CoA esters account for 75% and the choline esters 20% of the equivalent ACh-Cl activity. When isolated in eserine-free medium, the total equivalent ACh-Cl activity of subcellular fractions is reduced by about 40%. This decrease occurs mainly in the betaine CoA component of the crude mitochondrial fraction, P_2.

2) Chemical analysis shows the presence of ACh, PCh, BuCh and the CoA esters of GBB, crotonbetaine, l-carnitine and AC in normal rat brain.

3) The subcellular distribution of choline esters and the betaine CoA esters in narcotized rat brain is also described. The effect of ether, pentobarbital, ethanol and nitrous oxide narcosis is manifested in an increase in general of 50% in equivalent ACh-Cl activity in homogenates prepared in eserinized medium. This increase is accounted for by the betaine CoA component of the crude mitochondrial fraction, P_2. There is also on the average an increase of 50% in equivalent ACh-Cl activity in homogenates prepared in eserine-free medium. This

increase also occurs in the betaine CoA component of the crude mitochondrial fraction, P_2.

The effect of magnesium narcosis is manifested in an increase of equivalent ACh-Cl activity in rabbit brain homogenates prepared either in eserinized or eserine-free medium. This increase in ACh-like activity is accounted for by the betaine CoA component of the crude mitochondrial fraction, P_2.

4) Chemical analysis shows the presence of only one substance in narcotized rat brain, namely acetyl-l-carnityl CoA.

5) The structural complementarity of acetyl-l-carnityl CoA to the choline acetylase system, the cholinergic receptor and the cholinesterase system is discussed.

6) Parallel quantitative assay employing the frog rectus abdominis, eserinized leech dorsal muscle and the guinea pig ileum supports the chemical analysis that brain contains, besides ACh, other bioactive substances.

7) The bromophenol blue reaction determines in conjunction with the eserinized frog rectus assay that bioactive substances other than ACh contribute importantly to the ACh-like activity of normal and narcotized rat brain.

8) The distribution of choline esters and betaine CoA esters in normal and narcotized rat heart is described. The betaine CoA esters account for 85% of the total equivalent ACh-Cl activity. There is an increase of 20% during pentobarbital narcosis and 85% during ethanol narcosis; this activity increase

is accounted for by the betaine CoA ester component.

9) Chemical analysis shows the presence of ACh, PCh and the CoA esters of GBB, l-carnitine and AC in normal rat heart, but the presence of AC only in narcotized rat heart.

CLAIMS TO ORIGINAL RESEARCH

1) Ethanol, nitrous oxide and magnesium sulphate, in common with pentobarbital and ether narcosis, increase the extractable ACh-like activity of rat brain, mainly in the crude mitochondrial fraction, P_2.

2) The increase in ACh-like activity is due to the accumulation of acetyl-l-carnityl CoA in brain during narcosis.

3) Chemical analysis of normal rat heart extract shows the presence of ACh, PCh and the CoA esters of GBB, l-carnitine and AC.

4) Ethanol and pentobarbital narcosis increase the extractable ACh-like activity of rat heart.

5) The increase in extractable ACh-like activity is due to acetyl-l-carnityl CoA in rat heart.

6) It is suggested that one of the effects of narcotics is to prevent the release of acetyl-l-carnityl CoA from the subcellular particles.

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