

1980

A STUDY ON THE ABILITY OF PRECURSOR AND ACETYLATED PRODUCT TO REFILL CHOLINERGIC VESICLES INDEPENDENTLY OF THE CYTOPLASM

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A STUDY ON THE ABILITY OF PRECURSOR AND ACETYLATED
PRODUCT TO REFILL CHOLINERGIC VESICLES INDEPENDENTLY
OF THE CYTOPLASM

BY

STEPHEN HAROLD NELSON

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

IN

PHARMACOLOGY AND TOXICOLOGY

UNIVERSITY OF RHODE ISLAND

1980

MASTER OF SCIENCE THESIS
OF
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1980

ABSTRACT

Mouse forebrain minces were incubated in a Krebs or a lithium high K⁺ (L.K.) Krebs medium, cytoplasmic (S₃) and crude vesicular (P₃) fractions prepared and the levels of choline and acetylcholine (ACh) in each, determined. ACh levels were depleted by 70% in the P₃ fraction of L.K. Krebs as compared to Krebs, incubated minces; while, S₃ ACh levels were not significantly altered by the lithium incubation. Lithium treatment lowered the S₃ choline content by 29%, with no significant effect upon P₃ choline levels, when compared to S₃ and P₃ fractions of contralateral minces incubated in Krebs media. Mince, depleted of P₃ ACh, were subsequently incubated in Krebs containing paraoxon (0.1μM) and 0.1mN concentrations of either ¹⁴C choline or ¹⁴C homocholine. Depletion of P₃ ACh resulted in an enhanced transport of extracellular ¹⁴C choline (84%) and ¹⁴C homocholine (76%), into this fraction but not into the S₃ fraction. Furthermore, the depleted P₃ fraction was repleted with newly synthesized ¹⁴C ACh or ¹⁴C acetylhomocholine (¹⁴C AHc). The ratio of ¹⁴C ACh to the total ACh in the repleted P₃ fraction (0.63), exceeded that attained in the S₃ fraction, (0.35). Additionally, the ratio of ¹⁴C AHc to ACh in the repleted P₃ fraction (7.26), was greater than that attained in the S₃ fraction (0.44). Similar results were obtained when mouse brain hippocampal minces were depleted of P₃ ACh and subsequently

incubated in Krebs containing ^{14}C homocholine (0.1mM). The ratio of ^{14}C AHC to ACh in the repleted P_3 fraction (1.50), exceeded that ratio attained in the S_3 fraction. Incubation of depleted forebrain minces in Krebs containing the preformed products ^{14}C ACh or ^{14}C AHC, in 0.1mM concentrations, did not result in an increased transport, or the repletion of the P_3 fraction with labelled product. However, depletion of P_3 ACh in hippocampal minces, did result in a greater accumulation of preformed ^{14}C AHC into the repleted fraction, resulting in a higher ratio of ^{14}C AHC to ACh in the repleted P_3 fraction (0.17), as compared to the P_3 fraction of nondepleted minces, (0.11). The amount of preformed ^{14}C AHC transported into the repleted P_3 fraction was insufficient, however, to replace lost transmitter. These results indicate that depletion of P_3 transmitter stores, occurs independently of the cytoplasm with newly synthesized product formed from extracellular precursor.

TO MY PARENTS
FOR THEIR CONSTANT SUPPORT AND ENCOURAGEMENT

ACKNOWLEDGEMENTS

The author wishes to express his appreciation to Dr. Paul T. Carroll for the guidance and encouragement throughout this study. Additionally, I would like to thank Dr. Raymond Panzica and other members of the Department of Medicinal Chemistry for help and advice on the synthesis of the labelled compound acetylhomocholine.

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INTRODUCTION

The majority of acetylcholine, in brain tissue, is located within the nerve terminals of cholinergic neurons (Hebb and Whittaker, 1958; Gray and Whittaker, 1962). Acetylcholine (ACh) is synthesized by the enzyme choline-O-acetyltransferase (ChAT; E.C. 2.3.1.6.), from the precursor choline and acetyl-coenzyme A (Hebb, 1972) and, similar to ACh, the enzyme is primarily found within cholinergic nerve endings (Hebb and Smallman, 1956). The subcellular localization of nerve ending ACh and ChAT has been studied extensively, and there is general agreement that the former is bimodally distributed between the cytoplasm and synaptic vesicles (De Robertis et al., 1963; Whittaker et al., 1964; Whittaker and Sheridan, 1965). In vitro, ChAT also appears bimodally distributed, existing in a soluble and a membrane associated form (Whittaker et al., 1964). The significance of the latter form, in vivo, has been the subject of much controversy.

The development of a unified model, mechanistically depicting ACh metabolism, requires the characterization of precursor uptake and neurotransmitter synthesis, storage and release. According to the current model, all ACh is synthesized by cytoplasmic ChAT and subsequently transported into synaptic vesicles (Fonnum, 1968, 1970). Fonnum (1967, 1968) has reported that in vitro, as much as 85% of the total nerve terminal ChAT can be solubilized by high ionic strength media,

the remainder being associated nonionically with membrane components of the terminal. Thus, given similar in vivo conditions, the majority of ChAT may be cytoplasmic, suggesting that the bulk of ACh synthesis occurs here and that little physiologically significant synthesis occurs elsewhere.

ACh, formed in the cytoplasm, is believed to be translocated into synaptic vesicles (Whittaker, 1959) where upon depolarization all release is said to originate in a Ca^{2+} dependent manner (del Castillo and Katz, 1954, 1955, 1957; Hebb, 1972; Fonnum, 1973). The current hypothesis places little importance, in terms of maintaining releasable stores of ACh, upon the membrane associated enzyme. Essentially, according to this model, the vesicles are entirely dependent upon the cytoplasm as a source of releasable transmitter. In turn, the enzyme activity of cytoplasmic ChAT is dependent upon the existing vesicular transmitter levels. Thus the two compartments are envisioned as operating in unison and as being interdependent.

Recent investigations have provided evidence that certain aspects of the present model require some modification. That all ACh is synthesized by cytoplasmic ChAT has been questioned on the basis of the observations of Hattori et al. (1976) and Feiganson and Barnett (1977), that ChAT also appears adsorbed to the outer vesicle membrane and possibly contained within the vesicles (Hattori et al., 1976). Others (Haubrich and Chippendale, 1977) have reported that ChAT may be associated with the neuronal membrane, possibly near the

vesicular attachment sites. Smith and Carroll (1980) have demonstrated enzyme activity in a crude vesicular fraction capable of acetylating choline and certain choline analogs. The authors have suggested that membrane associated ChAT is capable of maintaining vesicular transmitter levels.

Furthermore, investigations characterizing the release mechanism of ACh, have indicated that both compartments may function independently of each other. Release of ACh, both centrally and peripherally, is believed to occur in two ways--spontaneously and by depolarization (Somogyi and Szerb, 1972; Grewaal and Quastel, 1973; Carroll and Goldberg, 1975). Spontaneous release of ACh originates from the cytoplasm of nerve terminals (Carroll and Goldberg, 1976), occurs during and in the absence of depolarization and does not require extracellular choline or Ca^{2+} . In contrast the depolarized release of transmitter is dependent upon both extracellular choline and Ca^{2+} , exceeds the amount released spontaneously, and appears to originate from the vesicles (Carroll and Goldberg, 1976).

Several investigators have attempted to ascertain the origin of vesicular ACh by measuring the specific radioactivities (sp. act.) of ACh in the two subcellular compartments after incubating tissue in media containing labelled choline. Essentially, if transmitter, stored in synaptic vesicles, is ultimately derived from the cytoplasm, the sp. act. of ACh in the latter compartment should be equal to or exceed that measured in the vesicles.

Marchbanks (1969), Marchbanks and Israel (1972) and Richter and Marchbanks (1971), using in vitro tissue preparations, have reported that most newly synthesized ACh formed from extracellular choline, appeared in the cytoplasm relative to the vesicles resulting in a greater sp. act. in the former compartment. Similar qualitative results were reported by Chakrin and Whittaker (1969) after injecting labelled choline into the cerebral cortex of anesthetized animals.

Systemic administration of labelled choline results in the synthesis of labelled ACh in brain tissue (Schuberth et al., 1969, 1970; Jenden et al., 1974). However, different from the results reported above, the sp. act. found in the vesicular fraction was higher than that attained in the cytoplasm (Aquilonius et al., 1973; Schuberth et al., 1969, 1970), indicating that newly synthesized and not preformed ACh can refill vesicular stores of transmitter.

Katz et al. (1973), found that accumulation of preformed labelled ACh did not differ between stimulated and nonstimulated tissue and that stimulation of tissue after preloading with labelled ACh released only unlabelled transmitter. Furthermore, prolonged stimulation in media containing no added choline resulted in lowering total ACh levels. These results suggest that preformed ACh does not replenish reduced stores of transmitter, despite the fact that labelled ACh was accumulated by the nerve endings. Lastly, prepara-

tions of isolated vesicles, containing endogenous ACh (Marchbanks, 1968) and depleted of vesicular contents (Suskiw, 1976), have failed to accumulate preformed labelled ACh above diffusional levels, suggesting that the active transport of cytoplasmic ACh into vesicles does not occur.

The purpose of this study was to examine the ability of vesicles to refill independently of the cytoplasm and to determine whether only newly synthesized ACh, formed from extracellular choline can refill a depleted vesicular pool of transmitter. The influence of ACh levels, presynaptically, upon stimulation of precursor or preformed product uptake as suggested by Jenden et al. (1976), was also determined. The present investigation differed in an important way from previous studies in that vesicular levels of ACh, within intact nerve terminals of forebrain minces, were depleted, thus placing this compartment in a state of required transmitter synthesis prior to exposure to labelled precursor or acetylated product. Depletion of vesicular ACh was attained by incubating brain minces in a lithium containing incubation medium essentially as described by Carroll and Nelson (1978).

The nature of vesicular refilling was further examined using the choline and ACh analogs - homocholine and acetylhomocholine, respectively. According to Barker and Mittag (1975) and Collier et al. (1977a, b), homocholine is not acetylated by cytoplasmic ChAT in vitro even though in the latter studies homocholine was taken up by rat brain

synaptosomes and the cat superior cervical ganglion, a percentage of which was acetylated. Release of the acetylated analog was also demonstrated from the ganglionic tissue by a Ca^{2+} -dependent process. Thus if vesicles, previously depleted of ACh, could be refilled with newly synthesized acetylhomocholine, it would further suggest that a vesicular refilling process operates independently of the cytoplasm.

The significance of the present study is that if vesicular stores of transmitter can be refilled, as well as depleted, independently of the cytoplasm, the vesicles and the cytoplasm may represent two physiologically, as well as morphologically, separate compartments.

LITERATURE REVIEW

Differential centrifugation of brain tissue which has been homogenized in iso-osmotic media, led to the observation that acetylcholine (ACh) is nonhomogeneously distributed within a cholinergic neuron (Hebb and Whittaker, 1958; Whittaker, 1959). Whittaker et al. (1964), using sucrose density gradient centrifugation, have defined several distinct pools of ACh in central neuronal tissue. The physiological significance of sequestering this transmitter into subcellular compartments has yet to be established, but suggests a separate role for each pool. Two of the most common questions asked regarding neurotransmitter compartmentation are - to what extent, and in what way are these pools interdependent?

The degree of interdependence is of pharmacological import. Several neural disorders, such as Huntington's chorea (Eckernas et al., 1977) and tardive dyskinesia (Davis et al., 1975), have been associated with central cholinergic pathways. If the processes of ACh synthesis, storage and release occur separately within each compartment, then each transmitter pool may be differentially effected by disease processes.

Neuronal Pools of ACh

Neuronal ACh can be separated into three pools by

differential centrifugation of brain homogenates in iso-osmotic media (Hebb and Whittaker, 1958; Whittaker, 1959; Whittaker et al., 1964; see Fig. 1).

Free ACh

Homogenization of brain tissue in 0.32M sucrose, liberates a soluble ACh containing fraction (S_1), not bound by membranes and referred to as "free" ACh. This fraction, derived primarily from that ACh originally contained within axons, represents 20-30% of the total tissue ACh store and requires a cholinesterase inhibitor present during homogenization to protect it from hydrolysis by acetylcholinesterase (AChE; E.C. 3.1.1.7). Free ACh is thought to be formed by ChAT being transported from the cell body to the nerve terminals. At present no physiological function has been attached to this transmitter pool.

Bound ACh

Separation of the S_1 fraction at 17,000g gives rise to a pellet (P_2 in Fig. 1), referred to as the crude mitochondrial fraction. Distribution studies by Hebb and Whittaker (1958), Whittaker (1959), and later by Gray and Whittaker (1962), demonstrated that 70-80% of tissue ACh is localized within this fraction. Microscopic analysis of this pellet has revealed that it consists of mitochondria and pinched off nerve endings formed during the initial homogenization of tissue in iso-osmotic (0.32M) sucrose. In such media,

the nerve endings become resealed and form membranous sacks which Whittaker et al. (1964) have termed synaptosomes. Synaptosomes retain the original component organelles of the intact nerve terminal and thus are metabolically active (Bradford, 1969). Purification of the P_2 fraction on discontinuous sucrose density gradients showed that essentially all of the P_2 ACh was recovered in the synaptosomal fraction (Marchbanks, 1966) and that little ACh was associated with mitochondria of this fraction.

When the synaptosomal pellet is resuspended in hypoosmotic media and homogenized, the synaptosomal cytoplasm is liberated along with other nerve ending organelles. High speed centrifugation of the synaptosomal homogenate produces a soluble fraction (S_3) corresponding to the nerve terminal cytoplasm. Whittaker (1959) and Whittaker et al. (1964) found that approximately 50-60% of the nerve ending ACh was liberated upon rupturing the synaptosomes. The ability to measure brain ACh in the S_3 fraction depends upon the use of a cholinesterase inhibitor during hypo-osmotic rupture of fraction P_2 and therefore S_3 ACh is referred to as "labile-bound" ACh. The remaining 40-50% of synaptosomal ACh is found associated with the high speed pellet (P_3) which consists of small mitochondria, partially disrupted synaptosomes and vesicles. Whittaker et al. (1964) and Whittaker and Sheridan (1965) have demonstrated that P_3 ACh is contained within the vesicles.

Much of what is presently understood regarding the sequence of events prior to, during and after release of ACh, has been derived from electrophysiological studies at peripheral synapses. Fatt and Katz (1950, 1952), observed minute spontaneous fluctuations in membrane potentials (miniature endplate potentials; m.e.p.p.¹⁵) that occurred with a mean frequency of approximately one per second, with a potential of less than 1mV. These m.e.p.p.'s are believed to be the result of ACh release in the form of discrete packets or quanta at the neuromuscular junction (Fatt and Katz, 1952) and probably at central synapses (Katz and Miledi, 1963). Each quantum, consisting of several thousand ACh molecules, has been equated with a m.e.p.p.; however, this relationship is not well established. Inorganic calcium, Ca^{2+} , is required for the quantal release of ACh and its role in neurotransmission has been examined by del Castillo and Katz (1954), Katz and Miledi (1967, 1971) and Miledi (1973). When tissue is depolarized, Ca^{2+} influx is increased and the quanta are released more frequently, producing an endplate potential (e.p.p.) postsynaptically.

At about the same time that the quantal nature of ACh release was demonstrated, electronmicroscopic analysis of brain tissue (DeRobertis and Bennett, 1954, 1955), and of the neuromuscular junction (Robertson, 1956), demonstrated the existence of synaptic vesicles (400-500 \AA in dia.), concentrated at the presynaptic nerve terminal membrane. These

vesicles became the structural counterpart of the release mechanism, providing an understanding as to how ACh is released as quanta. Each vesicle, depending upon the animal species used, may consist of as many as 1,500 to 2,000 molecules of ACh (Krnjevic and Phillis, 1963), although a much more conservative estimate of 306 molecules ACh/vesicle was derived by Whittaker and Sheridan (1965); however, the low distribution of cholinergic terminals in the tissue used was not considered by these investigators.

The observations that ACh is released as quanta and that the vesicles are the likely means by which quantal release takes place, gave rise to the vesicle hypothesis of neurotransmission. Essentially, it states that ACh is packaged within nerve terminal vesicles, and upon depolarization and the simultaneous influx of extracellular CA^{2+} , the contents of the vesicles, each of which consists of a quantum of ACh, are released into the synapse.

Although the vesicle hypothesis readily offers a plausible explanation as to how the quantal release of transmitter is achieved, there are reports suggesting that quantal release occurs from the cytoplasm. Those proponents of cytoplasmic transmitter release, base much of their argument on the fact that a high proportion of the nerve terminal ACh, along with ChAT, is present in the cytoplasm. Israel and Dunant (1975), observed that cytoplasmic ACh levels oscillated when tissue of the electric organ of Torpedo sp. were electrically stimulated. That only cytoplasmic, and not

vesicular, ACh was shown to oscillate in content, led the authors to conclude that the source of releasable ACh was from the cytoplasmic compartment. However, Israel and Dunant were unable to measure fluctuations in the postsynaptic membrane potential, which is necessary for supporting their hypothesis. Tauc et al. (1974), noted that hydrolysis of cytoplasmic ACh, through microinjection of AChE into the neuron, reduced the synaptic transmission. However, it was not shown whether any AChE was also incorporated into synaptic vesicle and whether this source of ACh was also significantly depleted.

Israel and Dunant (1975), reported that after stimulation of the electric organ of Torpedo sp., at 10 Hz for 5-10 minutes, cytoplasmic ACh, but not vesicular transmitter, was lowered. However, several others, including Zimmerman and Whittaker (1974), have indicated that both vesicular content of ACh, and vesicle numbers, were diminished upon continuous electrical stimulation of the electric organ, indicating that the vesicles are the source of transmitter release. A problem associated with this conclusion is that the correlation between the recovery of vesicle number and the return of normal synaptic transmission is unclear. Often, neurotransmission recovers from fatigue before the vesicle population returns to pre-stimulatory levels. However, Heuser and Reese (1978), have suggested that not all of the vesicles observed in electron micrographs contain releasable transmitter and thus it would depend upon the percent of the original vesicle

population that was present at any given time after stimulation (Heuser, 1978). These investigators have further reported evidence, obtained through freeze fracturing techniques supporting an exocytotic-endocytotic release cycle. Actual membrane alterations (indentation) have been observed where synaptic vesicles are believed to have fused with the neuronal membrane and released their contents. Excitation of such tissue in the absence of Ca^{2+} , which is necessary for quantal release (del Castillo and Katz, 1954), results in a lack of these membrane indentations.

Fractionation of brain (Barker et al., 1972) and electric organ (Zimmerman and Denston, 1977) by differential and sucrose gradient centrifugation, has led to the separation of two metabolically distinct vesicle populations; a less dense monodispersed fraction (D or VP_1) and a fraction in close association with the neuronal membrane (H). Incubation of tissue in the presence of labelled choline (Barker et al., 1972) or the analog-homocholine (von Schwartztenfeld, 1979) results in newly synthesized transmitter of high specific activity (sp. act.), found primarily within the vesicles in closest association to the neuronal membrane. Labelling of presynaptic stores of transmitter with subsequent stimulation of the tissue, release preferentially, newly synthesized transmitter (or false transmitter), with a sp. act. similar to the sp. act. associated with fraction H vesicles, and higher than that measured in the cytoplasm or monodispersed vesicles (Suskiw et al., 1978; von Schwarzenfeld,

1979). The preferential release of newly synthesized transmitter has also been shown by Collier (1969), using the cat superior cervical ganglion.

A question that remains unresolved is that if ACh is synthesized in the cytoplasm and released from vesicles as quanta, by what mechanism is the transmitter concentrated within the latter structures. Presently, no firm biochemical evidence exists that supports the concept of ACh mobilization into depleted vesicle stores, above diffusional levels. It was the purpose of this study therefore, to examine whether vesicles, depleted of ACh, are refilled with preformed transmitter, derived from the cytoplasm, or are repleted with newly formed transmitter derived from extracellular precursors.

MATERIALS AND METHODS

Male (CD-1) albino mice were killed by cervical dislocation in the cold (4°). The brains (minus the pons, medulla and cerebellum) were sectioned through the median sagittal fissure and each hemisphere washed in ice cold normal Krebs or lithium Krebs media (media described below), to remove extracellular choline and AChE. A characteristic postmortem elevation of brain choline levels, which can alter the rate of ACh metabolism has been reported by several investigators (Schuberth, 1966; Schuberth et al., 1970; Dross and Kewitz, 1972; Nordberg, 1977). Washing the brains in ice cold media minimizes this effect. The forebrains were then blotted dry, weighed, minced and placed on a chilled petri dish until the onset of incubation.

Incubation of Brain Tissue

Objectives

Tissue incubations were routinely conducted at 37-38° under 95%O₂-5% CO₂ in a Dubnoff Metabolic Shaker set at 90 cycles/min. The overall design of the tissue incubations, as described below, was to: 1) selectively reduce the ACh content of the vesicle-bound pool without altering cytoplasmic ACh levels; 2) determine whether the reduced vesicle-bound pool could be refilled with newly synthesized product formed from extracellular precursors and/or with extra.

cellular preformed (i.e., acetylated) products; and 3) determine if the refilling process occurs independently of the cytoplasm.

Incubation Media

Several investigators (Whittaker et al., 1975; Jenden et al., 1976) have suggested that the amount of extracellular choline taken up presynaptically, is determined by the existing nerve-ending levels of ACh. To examine the ability of the vesicle-bound pool to be refilled with newly synthesized or preformed product, minces were preincubated in 10 mls of a nondepolarizing Krebs phosphate buffer medium (composition in mM; NaCl 117; KCl 3.5; KH_2PO_4 1.2; CaCl_2 2.5; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1.2; NaHCO_3 28.0 and glucose 11.1) while the contralateral minces were similarly preincubated in a lithium Krebs medium, in which LiCl (117 mM) replaced NaCl and KHCO_3 (25.0 mM) replaced NaHCO_3 (see below).

Several investigators have shown that lithium ions depolarize nerve terminals (Schou, 1957; Keynes and Swan, 1959) and by an unestablished mechanism blocks choline uptake presynaptically (Diamond and Kennedy, 1969; Simon and Kuhar, 1976; Jope, 1979). In an initial set of experiments (performed by Dr. P.J. Carroll), incubation of brain minces in a lithium Krebs medium, containing NaHCO_3 , resulted in a 70% reduction of vesicle-bound ACh relative to contralateral minces incubated in a Krebs medium. No significant alteration of cytoplasmic ACh levels occurred as a result of the incubations.

According to Schou (1957), lithium ions (Li^+), appear to depress cellular respiration in several tissues (e.g. renal and cardiac tissues). Accordingly, a modified lithium Krebs medium was utilized in which KHCO_3 replaced NaHCO_3 (maintaining ion-osmolarity). Potassium, in concentrations up to 40 mM, is known to stimulate tissue respiration (Canzanelli, 1942) and when added to a lithium medium reverses the lithium-induced depression of respiration. However, it should be pointed out that in brain tissue, Li^+ concentrations up to 100mM also stimulate cellular respiration (Canzanelli, 1942).

Incubation of brain minces in lithium Krebs containing NaHCO_3 or KHCO_3 has the same effect on lowering vesicle-bound ACh levels by 70% independently of the cytoplasm (Carroll and Nelson, 1978) and releases equivalent amounts of ACh into the incubation media (Nelson et al., 1980), thereby indicating that the inclusion of potassium is not disrupting central ACh metabolism. Therefore, KHCO_3 replaced NaHCO_3 in all lithium Krebs media used, unless otherwise stated. It should also be noted that Li^+ are unique in that they can replace Na^+ in several processes (Schou, 1957) and of particular importance Li^+ can support ChAT activity whereas the absence of Na^+ cannot (Potter et al., 1968).

Tissue Incubation

Minces were preincubated for a total of 30 minutes in 10 mls of normal Krebs (K.) or lithium K^+ Krebs (L.K.) media. The tissue was centrifuged (1000 g; 4°) and placed in fresh

media after the initial 15 minutes to reduce the accumulation of extracellular choline in the media generated primarily from phospholipid turnover (Bhatnagar and MacIntosh, 1967; Browning and Schulman, 1968; Browning, 1971; Collier et al., 1972). If the choline levels in the media become sufficiently elevated the lithium-induced inhibition of choline uptake may be partially reversed (although this effect was not examined in this investigation).

Tissue samples were placed on ice at the end of the pre-incubation, centrifuged (1000 g, 4°) and the pellets washed (2 x 5 mls) with ice cold K. media (the second wash contained 0.1 uM paraoxon).

To ascertain whether the vesicle-bound fraction, previously depleted of its ACh content, could be refilled with newly synthesized ACh or acetylhomocholine (AHc) formed from the extracellular precursors [^{14}C]choline or [^{14}C]homocholine, minces were incubated an additional 30 minutes in 5 mls of K. containing labelled choline (0.1 mM) or homocholine (0.1 mM) and paraoxon (0.1 uM), (incubation of minces in labelled choline was performed by Dr. P.T. Carroll).

In a similar set of experiments designed to test the ability of preformed product to refill a depleted vesicle-bound pool, minces, previously incubated in K. or L. K. media, were subsequently incubated for 30 minutes in 5 mls of K. containing 0.1 mM concentrations of [^{14}C]ACh or [^{14}C]AHc and 0.1 uM paraoxon.

Subsequent to the 30 minute K. incubation in labelled precursor or preformed product, tissue samples were centrifuged (1000 g; 4°), the media discarded and the tissue washed (2 x 5 mls) with ice cold 0.32 M sucrose.

Hippocampus

Several investigators have determined that ACh uptake into brain tissue is not specific for cholinergic neurons (Katz et al., 1973; Kuhar and Simon, 1974), suggesting that ACh uptake into central tissues is of little physiological import.

To determine whether an acetylated product could enter the nerve terminals of cholinergic neurons, 10-20 mice were killed, the brains removed and half placed in a K. ice cold washing medium and half placed in ice cold L. K. media. The hippocampi of the K. washed brains were removed, pooled and minced on a chilled petri dish while the hippocampi of the L. K. washed brains were similarly prepared. Hippocampal tissue consists of approximately 85-90% cholinergic nerve terminals (Kuhar et al., 1973). There are two hippocampi per brain, each lying lateral to the median sagittal fissure at the level of the lateral ventricle floor. The hippocampal tissue was incubated in either K. or L. K., as previously described and then washed in ice cold K. (second wash containing paraoxon 3 uM). The tissue was then incubated for 30 minutes in K. containing 0.1 mM [¹⁴C]ACh and 3 uM paraoxon.

In another set of experiments, hippocampal tissue was similarly prepared and incubated in K. containing 0.1 mM [^{14}C]homocholine plus paraoxon (3 μM) to compare the uptake and acetylation characteristics between hippocampal (primarily cholinergic) and forebrain (approximately 15% cholinergic nerve terminals) tissue.

The tissue minces were then washed (2 x 5 mls) in ice cold 0.32M sucrose and prepared for subcellular fractionation.

Subcellular Fractionation Protocol

Subcellular fractions of minces were prepared according to the method of Gray and Whittaker (1962) as modified by Collier et al. (1972) and Salehmoghaddam and Collier (1976). The washed minces were resuspended in 5 mls of ice cold 0.32M sucrose vortexed and transferred to a teflon to glass homogenizer (0.025 cm clearance) set at 840 rpm and homogenized using 8 up and down strokes. The homogenates were transferred to chilled tubes and centrifuged at 1000 g for 10 minutes. The pellet (P_1), containing nuclear debris, myelin and unbroken cells, was discarded. The supernatant (S_1) consisting primarily of mitochondria and synaptosomes, was centrifuged for 15 minutes at 17,000 g. The S_2 fraction (microsomal fraction) consists of nearly 20% of the total tissue ACh. The P_2 fraction (synaptosomal fraction), was resuspended in 2 mls of glass distilled water (pH 4.0) containing paraoxon (0.1 μM) to hypo-osmotically rupture the

synaptosomes. Paraoxon was included to preserve the labile or cytoplasmic ACh. Subsequent to homogenization in a teflon to glass homogenizer (400 rpm; 8 up and down strokes) the homogenate was centrifuged at 100,000 g for 90 minutes in chilled tubes.

The resulting crude vesicular pellet (P_3) consists of monodisperse and neuronally associated vesicles. Evidence that the vesicles are not artifacts of the tissue fractionation scheme was derived from experiments in which the cholinesterase inhibitor-paraoxon, was omitted just prior to the initial tissue homogenization. The levels of P_3 ACh did not differ from tissue homogenates with or without inhibitor, whereas, omission of paraoxon resulted in nondetectable levels of S_3 ACh.

The S_3 supernates were collected and stored at -20° . After surface washing the P_3 pellets (2 x 8 mls) with glass distilled water (pH 4.0), the pellets were transferred to ground glass homogenizers (Duell-20; Thomas Co.) and homogenized in 500 ul ice cold 1N Formic Acid/Acetone (15/85; v/v) and allowed to set in ice, for 20 minutes. This procedure extracts nearly all tissue ACh and choline (Toru and Aprison, 1966). Subsequent to a 10,000 g 20 minute centrifugation in the cold, an aliquot (300 ul) of clear supernate was removed and stored at -20° .

Total Radiolabel Uptake

The total radiolabel uptake of labelled precursor or product into the S_3 or P_3 fractions was determined by

removing 40 ul aliquots of either fraction, adding it to Hydromix (15 mls) and counting in a liquid scintillation spectrometer (Packard Model 3330). Counting efficiency was determined by adding [^{14}C]toluene standard (20 ul) to samples. Quenching did not appear to reduce the counting efficiency.

Determination of Tissue ACh and AHC

Total ACh and AHC levels were determined using the assay method of Goldberg and McCaman (1973). In some cases, a modification of this method was employed where the extraction of tissue samples was omitted.

Extraction of Tissue

According to Goldberg and McCaman (1973) and McCaman and Stetzler (1977), consistent and reliable results derived from the assay described below are attainable only when tissue samples are initially extracted with a liquid cation exchange substance - sodium tetraphenylboron dissolved in 3-heptanone (TPB/3H). Using this procedure, Na-TPB exchanges Na^+ for quaternary species present in the aqueous solution. The resulting amine-TPB complex is insoluble in aqueous, but soluble in organic (e.g., 3-heptanone) solvents.

Cytoplasmic (S_3) samples were extracted by mixing 300 ul of TPB/3H (5 mg/ml) with 100 ul of sample. After separating the phases by centrifugation, 200 ul of the organic layer were re-extracted with 100 ul of 1NHC1 and again centrifuged. The organic layer was discarded and 20-25 ul of the aqueous

phase dried in vacuo for assay. Standards of ACh (200 pmoles; bromide salt) were similarly extracted from 100 ul of water or from one of the incubation media and assayed along with the samples. It should be noted that standards of AChBr, added to 100 ul of H₂O, 4.7 or 35mMK⁺ Krebs or L. K. media are extracted equally indicating that the ions present in the media do not inhibit the extraction of ACh.

The vesicle-bound (P₃) fraction was extracted by initially drying (in vacuo) 50 ul of the formic acid/acetone tissue extract. Subsequently, 50 ul of 10mM sodium phosphate buffer (pH 6.6) was added to the dried samples and then mixed with 200 ul TPB/3H (5 mg/ml). One hundred fifty microliters of the organic phase was removed and re-extracted with 75 ul of 1N HCl. Again, 20-25 ul of the acid phase was dried in vacuo for assay. Acetylcholine standards (200 pmoles) were similarly extracted and assayed.

For the determination of ACh levels in the S₃ and P₃ fractions of tissue incubated in [¹⁴C]choline, [¹⁴C] ACh and one preparation (i.e., 4 brains) using [¹⁴C] AHc, samples were not extracted. Instead, 10 ul of P₃ or 10-20 ul of S₃ sample were dried in vacuo and assayed directly. For one set of experiments, four brains were incubated in [¹⁴C]-homocholine and subcellular fractions prepared as described previously. Comparison of S₃ values for total acetylated product (ACh and AHc), obtained from extracted samples were similar to those of nonextracted samples (12.4±1.3 nmoles/gm tissue and 12.6±0.9 nmoles/gm tissue, respectively).

Additionally, similar total ACh values were obtained for extracted and nonextracted P_3 samples derived from six brains incubated in [^{14}C]choline (9.3 ± 1.4 nmoles/gm wet wt. tissue and 8.8 ± 1.4 nmoles/gm wet wgt. tissue, respectively). Thus whether the S_3 or P_3 samples, of a given tissue preparation, were extracted initially, or assayed directly, comparison of the values for total acetylated product obtained using either procedure were not significantly different. Detection of ACh (or AHc) in nonextracted tissue was linear through 20 μl of S_3 and 10 μl of P_3 sample. Also, measurement of ACh is linear when samples equivalent to 2.5 mg tissue or less are used.

Determination of Acetylated Product

Initially, 10 μl of a buffered substrate was added at 0-2° to each dried sample. The buffer is comprised of, in final concentration within each sample tube, 64mM sodium phosphate (pH 8.0); 0.8 mM ATP; 3.1 MgCl_2 and 1.2×10^{-3} units of choline phospholinase (CPK), E.C. 2.7.1.32. During the subsequent incubation of samples at 37-38°, all free choline (or homocholine), is phosphorylated to phosphorylcholine (or phosphorylhomochole). After placing the samples on ice, 2 μl of a second buffered substrate consisting of 35mM sodium phosphate (pH 8.0), 0.23 unit AChE (E.C. 3.1.1.7) and [$\text{V-}^{32}\text{P}$]ATP, 15-35 Ci/mmole, in final concentrations in assay samples, were added. The samples were incubated an additional 15 minutes during which time ACh or AHc is

hydrolyzed to choline or homocholine and subsequently phosphorylated to [^{32}P]phosphoryl-choline or -homocholine. The reaction was terminated by placing samples in ice water and adding 20 μl of 0.3M barium acetate to precipitate the unreacted ATP. According to Goldberg and McCaman (1973) this step removes nearly 98% of the unreacted ATP. After a one minute centrifugation (2500 rpm), an aliquot of supernate was removed and added to anion exchange microcolumns made up of ammonium formate resin (200-400 mesh; medium pore size). This procedure removes the remaining 2% of unreacted ATP while the phosphorylcholine or phosphorylhomocholeline is eluted with 1200 μl of 75mM ammonium acetate (pH 10.0). Samples were collected and counted in Hydromix (15 mls). Nonextracted external standards (200 pmoles ACh; bromide salt) were similarly assayed along with samples. Additionally, internal standards (100 pmoles AChBr) were added to some samples to determine if any tissue inhibition of the assay existed.

Conversion of Precursor to Product

When minces were incubated in K. containing the precursors [^{14}C]choline or [^{14}C]homocholine, the percent conversion (acetylation) of the total radiolabel uptake was determined by a method as described by Carroll and Goldberg (1975). Using this procedure, 10 μl of S_3 or P_3 (FA/A) samples were dried in vacuo and 10 μl of a buffered substrate, at 0-2°, added. This medium consisted of, in final

sample concentrations, 70.3mM sodium phosphate buffer (pH 8.0); 1.2mM ATP; γ - ^{32}P ATP, 15-35 Ci/mmmole 2.3mM MgCl_2 and 7.03×10^{-4} unit of CPK. Samples were incubated at 37-38° for 15 minutes during which time all nonmetabolized (free) choline or homocholine is converted to the phosphorylated species. The samples were set at 0-2° in an ice bath and extracted by initially adding 25 ul of water followed by 150 ul of TPB/3H (75 mg/ml). This process extracts the acetylated positively charged species (ACh and AHc) while the negatively charged phosphorylated species (phosphorylcholine and phosphorylhomocholeline) remain in the aqueous phase. The conversion of the precursors choline and homocholine to the phosphorylated forms is essentially 100% over a concentration range of 5-1000 pmoles. Additionally, extraction of each precursor from an aqueous medium with TPB/3H (75 mg/ml) is complete and linear throughout this same concentration range. After separation of the phases by centrifugation, 100 ul of the organic layer and 10 ul of the aqueous phase are counted in 10-15 mls Hydromix. The activity in the organic phase represents newly synthesized product, [^{14}C] ACh or [^{14}C]AHc, while the activity in the aqueous phase represents the [^{14}C]choline or [^{14}C]homocholine that was not metabolized (acetylated) during the tissue incubation. Additionally, the activity expressed within the aqueous phase of the assay sample was considered as that labelled choline or homocholine not phosphorylated endogenously by neuronal

CPK and ATP during tissue incubation with precursor. To determine whether the nonacetylated precursor present within the tissue was in fact free (i.e., nonphosphorylated) base, minces were preincubated in K. or L. K. with subsequent incubation in K. containing [^{14}C]choline (0.1mM), or [^{14}C]homocholine (0.1mM) and paraoxon (0.1uM). Subcellular fractions were prepared and the S_3 and P_3 samples assayed for newly synthesized product and precursor as described above with the exception that CPK and ATP were not incorporated into the assay. The amount of radiolabel remaining in the aqueous phase indicates the amount of endogenous phosphorylation of precursor occurring during the incubation with the labelled precursors.

Synthesis of Homocholine Iodide

Unlabelled methy iodide (1.5 ml; 24.1 mmoles) was added to 3-dimethylamino-1-propanol (3.5 ml; 29. mmoles) immediately producing a white precipitate. The solid was washed (2 x 10 ml) with anhydrous ether and centrifuged in the cold (4°) at 3,500 rpm. The supernatants were decanted and the product-homocholine iodide, was dried in vacuo. The reaction afforded 5.61 g (95%) homocholine iodide with the following physical characteristics: m.p. 196-198.5: pmr (DMSO- d_6) 4.75 τ , 1, J=5 Hz, OH), 3.49 (m, 4- $\text{CH}_2\text{-CH}_2\text{-CH}_2\text{-}$), 3.14 (s, 9, $\overset{+}{\text{N}}(\text{CH}_3)_3$), 1.92 (p, 2, $\text{-CH}_2\text{-CH}_2\text{-CH}_2\text{-}$). N-[Me ^{14}C] homocholine iodide was synthesized as described above with the exception that 2 u1 of [^{14}C]CH $_3$ I were diluted with 20 u1

of unlabelled CH_3I to a final specific activity of 4.85mCi/mmole. The washed product was dissolved in 10 ml of distilled water (pH 6.5) to give a final concentration of 8.04mM and stored at -20° .

Preparation of Acetylhomocholine Iodide

Homocholine iodide (2.0 gm; 8.2 mmoles), produced as described above, was refluxed with 20 ml of acetic anhydride while stirring under an atmosphere of $\text{N}_2(\text{g})$ according to the method of Waltz et al. (1951). The excess anhydride was removed under reduced pressure and heat, producing a colored precipitate. The solid was washed (2 x 10 ml) with anhydrous ether and recrystallized by dissolving it in a minimum amount of absolute ethanol and bringing the solution to the cloud point with anhydrous ether. After washing the precipitate with anhydrous ether, the product was dried in vacuo over P_2O_5 for 24 hrs. providing 2.15 g (91.5%) of acetylhomocholine iodide with the following physical characteristics: m.p. $96-97.5^\circ$; pmr (DMSO-d_6) \odot 4.12 (t, 2, $J=6\text{Hz}-\text{CH}_2-\text{CH}_2\text{O}-$), 3.51 (m, 2, $(\text{CH}_3)\text{N}-\text{CH}_2-\text{CH}_2-$) 3.17 (s, 9, $\overset{+}{\text{N}}(\text{CH}_3)_3$), 2.07 (s, 3, $\text{O}-\text{CH}_2-\text{CH}_2$), 2.07 (p, 2, $-\text{CH}_2-\text{CH}_2-\text{CH}_2-$).

Anal. Calcd. for $\text{C}_8\text{H}_{18}\text{NO}_2\text{I}$: C, 33.46; H, 6.32; N, 4.88.

Found: C, 33.29; H, 6.16; N, 5.01.

$\text{N}-[\text{Me}-^{14}\text{C}]$ acetylhomocholine iodide was similarly synthesized with the exception that the specific activity of the undiluted methyl iodide was 49.0 MCI/mmole. Additionally, the final recrystallized product was dried by a stream of

dried $(\text{H}_2\text{SO}_4)\text{N}_2(\text{g})$. The product was dissolved in distilled water (pH 6.5) and stored at -20° . The final specific activity of the product was 5.42 mCi/mmole.

Melting points were determined with a Thomas-Hoover apparatus and are uncorrected. The proton magnetic resonance spectra were obtained on a Varian A-60 spectrometer using TMS as an internal standard. Descending paper chromatography with a solvent system of *n*-butanol-absolute ethanol-1N acetic acid-water (8:2:1:3) was used to determine the homogeneity of the synthesized compounds.

Determination of Overlap Between Subcellular Fractions

LDH. The extent of overlap between the S_3 and P_3 samples was determined by measuring lactate dehydrogenase (LDH) activity in the P_2 (synaptosomal), S_3 and P_3 fractions using a modification of the method of Barker *et al.* (1972). This enzyme exists in a soluble form (i.e., nonmembrane-bound) and thus any activity observed in the P_3 sample represents cytoplasmic contamination.

Brain minces were incubated and subcellular fractions prepared as previously described. An aliquot of the hypo-osmotically ruptured P_2 fraction was removed prior to ultracentrifugation and separation of the S_3 and P_3 fractions. The P_3 fraction was subsequently homogenized in a total of 1.0 ml of water. Bovine serum albumen was added to the P_2 aliquot and the S_3 fraction to stabilize the enzyme. The final protein concentrations were equal for the three

fractions. Triton X-100 was added to each sample assayed to a final concentration of 1% (v/v) and allowed to stand in ice for 3-4 hrs. An aliquot of each sample was added to a buffered medium consisting of 0.05M TRIS-HCl (pH 7.4), NADH₂ (87.6uM) and sodium pyruvate (346.0uM). The rate of oxidation of NADH₂ was determined by measuring the change in absorbance at 340nm over 3-4 minutes at 25°C in a Gilford single beam spectrophotometer. Blanks consisted of sample, TRIS-HCl buffer and NADH₂ and demonstrated little auto-oxidation during this time interval. The results indicated that 65% of the P₂ LDH activity was associated with the S₃ fraction and approximately 35% with the P₃ fraction.

Distribution of Choline-O-Acetyltransferase

The extent of overlap between the S₃ and P₃ fractions was further demonstrated by determining the amount of ChAT activity present in each fraction. Subcellular fractions were prepared as described previously. The P₃ pellet was washed (2x1 ml) with a 100mM sodium phosphate buffer (pH 7.4) to remove the ChAT that became membrane-bound subsequent to the hypo-osmotic rupture of the P₂ fraction. ChAT activity was determined by the method of McCaman and Hunt (1965) as modified by Fonnum (1969) and described by Spyker et al. (1972). The majority of enzyme activity (84%) was found in the S₃ and combined washes, whereas, 15.6% of the recovered P₂ activity was associated with the P₃ pellet.

MATERIALS

Choline chloride 1,2 ^{14}C - spec. act. 6.34 mCi/mmol, acetylcholine iodide (acetyl-1- ^{14}C) - spec. act. 1.2 mCi/mmol, ATP ^{32}P spec. act. 17-35 Ci/mmol, toluene standard ^{14}C , acetyl Co A (acetyl-1- ^{14}C) - spec. act. adjusted to 2mCi/mmol, methyl ^{14}C spec. act. 20.0 and 49.0 mCi/mmol, were obtained from New England Nuclear Corporation, (Boston, MA). Unlabelled methyl iodide and 3-dimethylamino-1-propanol and 3-heptanone were purchased from Aldrich Chemicals (Milwaukee, WI). Acetic anhydride was obtained from Eastman Kodak Co. (Rochester, NY). Acetyl-CoA was obtained from Calbiochem (San Diego, CA). Choline kinase (E.C.2.7.1.32), ACHE (E.C. 3.1.1.7) and paraoxon were obtained from Sigma Corporation (St. Louis, MO). Sodium tetraphenylboron was obtained from Fisher (Medford, MA).

RESULTS

Alteration of Nerve Ending Choline and ACh Levels in Mouse Forebrain Minces.

Brain minces were incubated for 30 minutes in a Krebs or a lithium high K^+ (L.K.) Krebs medium and the ACh and choline content of the cytoplasmic (S_3) and crude vesicular (P_3) fractions determined. The results shown in Table 1 indicate that although S_3 ACh content did not differ between treatments, lithium incubation did result in a 70% reduction of P_3 ACh levels, when compared to minces incubated in Krebs media. Conversely, S_3 choline levels were significantly reduced (29%) as a result of L. K. Krebs treatment, while the amount of choline in the P_3 fraction was only slightly (11%) lowered, (Table 3).

The transport of extracellular choline into the presynaptic nerve terminal for ACh synthesis has been established as a sodium dependent process (Dowdall and Simon, 1973; Kuhar et al., 1973; Simon et al., 1976). Thus the observed lowering of P_3 ACh levels of minces incubated in the lithium containing solutions with either low Na^+ ($NaHCO_3$, 28mM) or no added Na^+ ($KHCO_3$ substituted for $NaHCO_3$, 28mM), may reflect the lack of an adequate sodium ion concentration rather than the presence of lithium. To test this possibility, one set of forebrain minces were incubated in a normal Krebs medium

(total Na⁺ = 145mM), while contralateral minces were similarly incubated in a low Na⁺ Krebs medium (LiCl replaced by equiosmolar sucrose, total Na⁺ = 28mM). Incubation of tissue in the low Na⁺ Krebs solution resulted in a 50% reduction of P₃ ACh levels relative to minces incubated in the normal Krebs medium. However, unlike that observed after L. K. Krebs treatment, the levels of S₃ ACh were also reduced by nearly 30% relative to control tissue. The effect of lowered Na⁺ on S₃ and P₃ choline levels was not determined in this study.

Ability of Extracellular Choline to Refill A Vesicle-Bound Transmitter Pool With Newly Synthesized ACh.

Incubation of forebrain minces in a Krebs solution containing lithium results in the depletion of P₃, but not S₃, ACh (Table 1). To determine whether a depleted vesicular transmitter pool can be refilled with newly synthesized ACh formed from extracellular choline, minces, preincubated in Krebs or L. K. Krebs were incubated an additional 30 minutes in Krebs containing [¹⁴C]choline (0.1mM). The results shown in Table 4 indicate that the total P₃ ACh content of minces pretreated in Krebs or L. K. Krebs did not differ significantly. However, more newly synthesized [¹⁴C]ACh was measured in the repleted than in the nondepleted P₃ fractions, (5.1 nmoles/gm and 2.6 nmoles/gm, respectively) resulting in a ratio of [¹⁴C]ACh to total ACh in the repleted P₃ fraction

(0.63) that exceeded that achieved in the same fraction of Krebs preincubated minces (0.35), (Fig. 2). Preincubation of tissue in the lithium containing medium did not result in any greater amount of [^{14}C]ACh appearing in the S_3 fraction, when compared to the same fraction of nondepleted minces pretreated in Krebs media.

Additionally, when compared to the P_3 fraction of Krebs preincubated minces, substantially more unacetylated [^{14}C] choline was found associated with the repleted P_3 fraction of minces previously exposed to the Krebs solution containing lithium (Table 5). Conversely, similar amounts of [^{14}C] choline were measured in the S_3 fraction of Krebs and L. K. Krebs preincubated minces.

Repletion of the P_3 Fraction with Newly Formed Acetylhomocholine Derived from the Extracellular Precursor-Homocholine

As previously stated, homocholine differs from the natural precursor choline in that it appears not to be a suitable substrate for solubilized (i.e., cytoplasmic) ChAT (Currier and Mautner, 1974; Barker and Mittag, 1975; Collier *et al.*, 1977). Thus if a depleted P_3 fraction was refilled with newly synthesized acetylhomocholine (AHc) formed from extracellular homocholine, an alternative site of synthesis would be proposed to exist which serves the purpose of vesicular refilling.

To examine this possibility, forebrain minces were preincubated in Krebs or L. K. Krebs and incubated an additional

30 minutes in Krebs containing [^{14}C]homocholine (0.1mM). As seen in Table 4, the total amount of acetylated product (ACh and AHc) in the P_3 fraction of minces after Krebs or L. K. Krebs pretreatment, was similar (9.3 nmoles/gm and 7.3 nmoles/gm, respectively). However, the ratio of newly synthesized [^{14}C] AHc to ACh in the P_3 fraction of minces exposed to the lithium solution (7.26) exceeded that attained in the same fraction of minces pretreated in Krebs media (0.46) (Fig. 3). As with [^{14}C]choline incubated minces described above, the repleted P_3 fraction contained greater amounts of [^{14}C]AHc (4.7 nmoles/gm) than the nondepleted P_3 fraction of Krebs preincubated minces (2.1 nmoles/gm) (Table 4). Additionally, the ratio of [^{14}C]AHc to ACh in the repleted P_3 fraction (7.26) exceeded that attained in the S_3 fraction (0.44) of the same minces.

A net accumulation of unacetylated $\frac{1}{4}$ [^{14}C]homocholine occurred in the P_3 fraction of L. K. Krebs preincubated minces (7.2 nmoles/gm) relative to the nondepleted P_3 fractions (4.1 nmoles/gm). Lithium high K^+ Krebs preincubation did not augment the amount of unacetylated precursor existing in the S_3 fraction when compared to Krebs preincubated minces. However, a greater amount of newly synthesized [^{14}C]AHc existed in the S_3 fraction of depleted minces (5.3 nmoles/gm) when compared to those minces pretreated in Krebs media (3.4 nmoles/gm), thus resulting in a higher ratio of [^{14}C]AHc to ACh in this fraction of L. K. Krebs (0.44) relative to Krebs (0.26) preincubated minces (Fig. 3). It

should be noted that the total amount of acetylated product (ACh and AHc) did not differ between pretreatments (Table 4).

Similar results were obtained when mouse brain hippocampal minces were exposed to [^{14}C]homocholine (0.1mM). As seen in Table 8, the total amount of acetylated product in the P_3 fraction did not differ between Krebs (4.25 nmole/gm) and L. K. Krebs (4.0 nmoles/gm) pretreated minces. However, the amount of newly synthesized [^{14}C]AHc in the repleted P_3 fraction (2.03 nmoles/gm) was greater than that measured in the P_3 fraction of Krebs preincubated minces (0.61 nmoles/gm). Thus the ratio of labelled AHc to ACh in the P_3 fraction of L. K. Krebs preincubated minces exceed that in the same fraction after Krebs pretreatment (1.05 and 0.29, respectively; $n=2$). Additionally, the ratio of labelled AHc to ACh in the repleted P_3 fraction (1.05) exceeded that of the S_3 fraction (0.16) of minces preincubated in L. K. Krebs.

Greater amounts of unacetylated [^{14}C]homocholine existed in the repleted P_3 fraction (3.0 nmoles/gm) relative to the nondepleted P_3 compartment (1.6 nmoles/gm); a result similar to that observed in the repleted P_3 fraction of fore-brain minces. Additionally, more [^{14}C]homocholine was transported into the S_3 fraction of repleted hippocampal minces with greater amounts of [^{14}C]AHc being synthesized when compared to the same fraction of Krebs preincubated minces. As seen in Table 8, the ratio of [^{14}C]AHc to ACh in the S_3 fraction of repleted minces (0.16) exceeded that attained

in the same fraction of Krebs preincubated minces (0.04; n=2).

Accumulation of the Extracellular Products - $[^{14}\text{C}]\text{ACh}$ and $[^{14}\text{C}]\text{AHc}$ by the S_3 and P_3 Compartments

The results reported above indicate that the lithium induced depletion of P_3 ACh results in the refilling of this fraction with newly synthesized product formed from extracellular precursors. It was of interest to ascertain the disposition of the depleted P_3 fraction towards the utilization of the extracellular preformed products - ACh or AHc. Minces were preincubated as before and subsequently incubated an additional 30 minutes in Krebs containing 0.1mM $[^{14}\text{C}]\text{ACh}$ or $[^{14}\text{C}]\text{AHc}$ and subcellular fractions prepared as described previously.

Accumulation of Extracellular $[^{14}\text{C}]\text{ACh}$

The results shown in Table 6 indicate that after a 30 minute incubation in Krebs with $[^{14}\text{C}]\text{ACh}$, the total ACh content did not differ significantly between Krebs (15.8 nmoles/gm) and L. K. Krebs (12.6 nmoles/gm), pretreatments in the P_3 fraction. However, unlike that observed with $[^{14}\text{C}]\text{cho}$ -line, the depleted P_3 fraction of L. K. Krebs exposed minces did not contain a greater amount of $[^{14}\text{C}]\text{ACh}$ (1.8 nmoles/gm) when compared to the same fraction of contralateral minces (1.5 nmoles/gm) preincubated in Krebs media. The ratio of labelled to total ACh therefore, in the P_3 fraction of repleted tissues (0.20) did not differ significantly from the

P_3 fraction of nondepleted minces (0.14) (Fig. 4). The results in Figure 4 further indicate that the ratio of [^{14}C]ACh to total ACh in the P_3 fraction after either pretreatment did not exceed the ratios attained in the associated S_3 fractions.

Accumulation of Extracellular [^{14}C]AHC

Table 6 indicates that similar amounts of acetylated product (ACh and AHc) existed in the P_3 fractions between pretreatments. Additionally, as was observed when forebrain tissues were incubated with preformed ACh, the repleted P_3 fraction did not accumulate significantly more [^{14}C] AHc when compared to the P_3 fraction of nondepleted minces. Thus, the ratio of [^{14}C]AHc to ACh, in the P_3 fraction did not differ significantly between pretreatments (0.09 and 0.09) (Fig. 5). Similarly, the ratio of [^{14}C] AHc to ACh in the P_3 fraction (0.09) did not exceed that attained in the S_3 fraction (0.22) after L. K. Krebs preincubation. Somewhat different results were obtained when mouse brain hippocampal minces were incubated in media containing [^{14}C]AHc (0.1mM). Although the total amount of acetylated product did not differ significantly from the P_3 fraction of nondepleted minces (2.71 nmoles/gm), a greater amount of [^{14}C] AHc existed in the P_3 fraction of L. K. Krebs pretreated minces (0.65 nmoles/gm) than in the P_3 fraction after Krebs pretreatment (0.26 nmoles/gm) (Table 8). The resulting ratio of [^{14}C] AHc to ACh in the repleted P_3 fraction (0.17) exceeded that measured in

the same fraction of Krebs preincubated minces (0.11). However, it should be noted that the amount of preformed labeled product taken up into the P_3 fraction of L. K. Krebs preincubated minces was insufficient to refill the depleted transmitter pool.

Lithium pretreatment did not result in significantly greater amounts of [^{14}C] AHC existing in the S_3 fraction relative to Krebs preincubated minces. Similarly, the ratio of [^{14}C] AHC to ACh in the S_3 fraction of L. K. Krebs preincubated minces did not differ significantly from those preincubated in Krebs media (0.16 and 0.12, respectively, Table 8).

Depletion of P_3 ACh and Its Effect Upon the Transport of Extracellular Precursors and Products

Dowdall and Whittaker (1975) and Jenden et al. (1976) have reported that the pre-existing nerve terminal levels of ACh regulate the transport of extracellular choline into the nerve ending. Table 7 summarizes the effect the lithium-induced depletion of P_3 ACh has upon the transport of the extracellular precursors [^{14}C]choline and [^{14}C]homocholine and the preformed products [^{14}C] ACh and [^{14}C] AHC into forebrain or hippocampal minces.

The reduction of P_3 ACh in forebrain minces augmented the transport of the extracellular precursors [^{14}C]choline (84%) and [^{14}C]homocholine (76%) into the repleted P_3

fraction relative to the nondepleted Krebs preincubated tissue. Transport of either precursor into the S_3 fraction of forebrain minces was not altered by exposure to the lithium containing media; although greater amounts of newly synthesized [^{14}C] ACh existed in the S_3 fraction of L. K. Krebs, relative to Krebs, preincubated minces. Similarly, lithium pretreatment increased (124%) the amount of [^{14}C]homocholine transported into the repleted relative to the nondepleted P_3 fraction of hippocampal minces. However, L. K. Krebs pretreatment also increased (57%) the amount of [^{14}C] homocholine transported into the S_3 fraction when compared to Krebs nondepleted minces.

Conversely, in forebrain tissue, depletion of P_3 ACh did not result in a greater transport of extracellular preformed [^{14}C] ACh or [^{14}C] ACh into either the S_3 or P_3 fractions of L. K. Krebs preincubated minces relative to those minces preincubated in Krebs media. Similarly, in mouse hippocampal minces, L. K. Krebs pretreatment did not result in any greater accumulation of extracellular ^{14}C ACh into the S_3 fraction, when compared to the amount present in the S_3 fraction of nondepleted minces. However, the lithium induced depletion of P_3 ACh did result in significantly (150%) greater accumulation of extracellular ^{14}C ACh into the repleted fraction, although in amounts insufficient to replace the lost transmitter.

DISCUSSION

There is general agreement that ACh is distributed bimodally between the cytoplasm and vesicles within the nerve terminals of cholinergic neurons (DeRobertis et al., 1963; Whittaker et al., 1964). However, the extent of biochemical interdependence, as well as the functional significance, of each compartment is presently unknown. The purpose of this investigation was to determine whether vesicles, depleted of ACh, could be refilled with newly synthesized transmitter (or false transmitter) independently of the cytoplasm. Specifically, it was of interest to ascertain whether vesicular ACh is ultimately derived from a pool of ACh synthesized within the cytoplasm.

The results presented in Table 1, show that a Krebs medium containing lithium and elevated (32.7mM) K^+ selectively depletes the level of ACh in a crude vesicular (P_3) fraction without altering cytoplasmic (S_3) ACh levels in mouse forebrain minces. Furthermore, lowering the K^+ concentration (4.7mM) in the lithium Krebs medium did not enhance nor antagonize this differential effect upon nerve terminal stores of transmitter, indicating that the addition of K^+ does not alter the central metabolism of ACh.

The presynaptic transport of extracellular choline for ACh formation, is sodium dependent (Simon et al., 1975; Simon

and Kuhar, 1976), and thus, the reduction of P_3 ACh levels may, in part, be attributable to a lack of an adequate sodium ion concentration in the incubation medium. Treatment of forebrain minces in a low (28mM) sodium (lithium free) Krebs solution results in a 50% reduction of P_3 ACh levels, as compared to the P_3 fraction of minces incubated in a normal (145mM Na^+) Krebs medium. However, in addition, a 30% reduction of S_3 ACh content was observed after low sodium Krebs incubation; a result different from that observed when minces are incubated in the lithium Krebs media. Thus, qualitative, as well as quantitative, differences exist, pertaining to ACh metabolism, when minces are incubated in the two modified Krebs media.

The lithium-induced reduction of P_3 ACh content may be explained in the following way. According to Keynes and Swan (1959) lithium, like elevated K^+ , depolarizes nerve terminals. Incubation of brain minces in lithium Krebs, lithium high K^+ Krebs or high K^+ Krebs will release similar amounts of endogenous ACh from the P_3 fraction, thereby lowering its content. Exposure of minces to high K^+ Krebs augments the Ca^{2+} -dependent release of P_3 ACh while allowing extracellular choline to be transported into the presynaptic nerve terminal, acetylated and released. A net synthesis of ACh thus occurs during tissue incubation in high K^+ Krebs media (Salehmoghaddam and Collier, 1976). Lithium, however, in addition to depolarizing nerve terminals, also blocks choline transport presynaptically (Simon and Kuhar,

1976) thereby limiting the availability of precursor and thus preventing the repletion of P_3 stores of transmitter with newly synthesized ACh. It is important to note that lithium does support ChAT activity (Potter *et al.*, 1968). The selective depletion of P_3 ACh levels may reflect a more immediate requirement by this compartment, relative to the S_3 fraction, for extracellular choline.

Minces, preincubated in the L. K. Krebs medium with subsequent exposure to Krebs containing [14 C]choline, demonstrated a higher ratio of labelled to total ACh in the repleted P_3 fraction (0.63) relative to that attained by the S_3 (0.33) fraction. Similarly, incubation of depleted forebrain minces in the presence of the labelled choline analog [14 C]homocholine, produced a greater ratio of labelled ACh to ACh in the repleted P_3 fraction (7.26) relative to the S_3 fraction (0.44). Thus it appears that depleted vesicular stores of transmitter are refilled with newly synthesized product formed in close association with the P_3 fraction rather than through mobilization of preformed cytoplasmic ACh. Qualitatively analogous results were obtained when hippocampal minces were incubated in the presence of [14 C]homocholine. The lithium-induced depletion of P_3 ACh resulted in a higher ratio of labelled ACh (1.50) to ACh appearing in this fraction when compared to the S_3 fraction (0.33).

Several investigators have reported that homocholine is not readily acetylated by cytoplasmic ChAT (Currier and

Mautner, 1974; Barker and MiHog, 1975; Collier et al., 1977). Thus it was unexpected that nearly equivalent amounts of newly synthesized [^{14}C] AHC were measured in the P_3 (4.7 nmloles/gm) and the S_3 (5.3 nmoles/gm) fractions of lithium pretreated minces. However, it is possible that the [^{14}C] AHC found associated with the S_3 fraction may have originated from that synthesized by a membrane-bound ChAT (located on or near the vesicular and/or neuronal membranes), which is released from the enzymes synthetic site into the cytoplasm.

Depletion of forebrain P_3 ACh content augments the transport of extracellular [^{14}C]choline (84%) and [^{14}C]homocholine (76%) into this compartment but not into the S_3 fraction suggesting that repletion of P_3 stores of ACh occurs independently of the S_3 fraction possibly through direct accumulation of extracellular precursor. Hippocampal minces depleted of P_3 ACh also demonstrated an enhanced uptake of [^{14}C]homocholine (126%) relative to the nondepleted P_3 fraction. However, 58% more [^{14}C]homocholine was transported into the S_3 fraction of L. K. Krebs preincubated minces relative to the same fraction of Krebs pretreated minces. The reason for the difference between forebrain and hippocampal minces regarding the transport of labelled homocholine into the S_3 fraction is not readily explainable. However, 2.5 times more [^{14}C] AHC was found in the S_3 fraction of L. K. Krebs pretreated hippocampal minces relative to the S_3 fraction of Krebs preincubated tissue.

Further evidence that the depleted P_3 fraction is preferentially repleted with newly synthesized product formed from extracellular precursor rather than preformed S_3 ACh, is derived from the observation that the total amount of acetylated product in the repleted P_3 fraction of forebrain minces incubated with $\frac{1}{4}$ ^{14}C homocholine (4.7 nmoles ^{14}C AHC/gm plus 2.6 nmoles ACh/gm) exceeded the amount of product in the lithium depleted P_3 fraction (2.5 nmoles ACh/gm) by the amount of newly formed ^{14}C AHC.

Forebrain minces, depleted of P_3 ACh and subsequently incubated in Krebs containing $\frac{1}{4}$ ^{14}C ACh, demonstrated ratio of ^{14}C ACh to total ACh in the repleted P_3 fraction that did not exceed that attained in the S_3 fraction (0.20 and 0.14, respectively). Similarly, incubation of lithium pretreated forebrain minces in Krebs containing ^{14}C AHC resulted in a ratio of ^{14}C AHC to ACh in the repleted P_3 fraction (0.09) that did not exceed the ratio attained in the S_3 fraction (0.09). It should be noted that 5.1 nmoles ^{14}C ACh/gm tissue was measured in the repleted P_3 fraction after incubation with ^{14}C choline but only 1.7 nmoles ^{14}C ACh/gm tissue was measured in the repleted P_3 fraction when minces were incubated with the preformed product. Similarly 4.7 nmoles ^{14}C AHC/gm tissue, formed from extracellular ^{14}C homocholine, was found in the repleted P_3 fraction whereas only 1.2 nmoles of labelled product/gm tissue was associated with this fraction after exposure to preformed ^{14}C AHC. Thus newly synthesized and not preformed product can replete a depleted P_3 fraction.

When L. K. Krebs pretreated hippocampal minces were post-incubated in Krebs containing [^{14}C] AHC, similar ratios of [^{14}C] AHC to ACh were attained in the S_3 and P_3 fractions (0.16 and 0.17, respectively). Furthermore, the total amount of preformed [^{14}C] AHC found in the repleted P_3 fraction (0.65 nmoles/gm) was substantially less than the amount present in this fraction when hippocampal minces were incubated with labelled homocholine (2.8 nmoles [^{14}C] AHC/gm). However a significantly greater amount of preformed [^{14}C] AHC was accumulated by the repleted P_3 fraction (0.65 nmoles/gm) when compared to the nondepleted P_3 fraction of Krebs preincubated minces (0.26 nmoles/gm). It appears from these results that a P_3 fraction depleted of ACh may accumulate preformed extracellular product directly although in amounts insufficient to replete the transmitter stores.

Collier et al. (1977), reported that preganglionic stimulation of the cat superior cervical ganglion resulted in increased tissue amounts of both unacetylated labelled homocholine and newly formed labelled AHC. The results of this study support this finding. In both forebrain and hippocampal minces, depletion of P_3 ACh resulted in substantially greater amounts of unacetylated [^{14}C] choline or [^{14}C]homocholine appearing in the repleted P_3 fraction. This result may indicate that acetylation, and not substrate availability, is rate limiting in the repletion of transmitter stores. A similar interpretation was given by Collier et al. (1977).

However, it is possible that the unacetylated precursor is sequestered in a location not accessible to ChAT. Kadota et al. (1974), reported that the monodisperse vesicle fraction of the nerve terminal contain primarily choline and little acetylated product. Until it is determined that the unacetylated precursors are not entering such a pool and are in fact associated with the synaptic vesicles of the P₄ fraction, it can not be concluded that acetylation is rate limiting.

The results of this study indicate that the replacement of lost transmitter from the P₃ or crude vesicular fraction occurs preferentially through the direct uptake and acetylation of extracellular precursor, independently of the S₃ or cytoplasmic compartment. Also that accumulation of preformed extracellular product is not a means of repletion of vesicular transmitter stores.

TABLE 1

Differential Effect of L. K. Krebs Treatment on the Acetyl Choline Content of S₃ and P₃ Fractions

Treatment	Subcellular Fractions	
	S ₃	P ₃
Krebs	11.8 ± 1.6 (12)	8.7 ± 0.9 (12)
Lithium Krebs	12.8 ± 2.5 (12)	2.5 ± 0.4*(12)

Mouse forebrains were incubated for 30 minutes in Krebs or L. K. Krebs. Values represent the Mean + S.E.M. *Results differ significantly from Krebs at $P < 0.05$ (Analysis of variance: one way classification).

TABLE 2

Differential Effect of Lithium Low K^+ Krebs Treatment on the Acetylcholine Content of S_3 and P_3 Fractions

Treatment	Subcellular Fractions	
	S_3	P_3
Krebs	10.8 \pm 1.4 (6)	7.2 \pm 1.4 (6)
Lithium Krebs	9.5 \pm 1.6 (6)	2.3 \pm 0.5* (6)

Mouse forebrain minces were incubated for 30 minutes in Krebs or a lithium low K^+ Krebs medium. Values represent the Mean \pm S.E.M. ()=experimental animals. *Results differ significantly from Krebs at $P < 0.05$. (Analysis of variance: one way classification).

TABLE 3

Differential Effect of L. K. Krebs Treatment on the Choline Content of S₃ and P₃ Fractions

Treatment	Subcellular Fractions	
	S ₃	P ₃
Krebs	85.6 ± 11.5 (12)	29.1 ± 4.6 (12)
Lithium Krebs	60.6 ± 8.4* (12)	25.8 ± 4.0 (12)

Mouse forebrain minces were incubated for 30 minutes in Krebs or L. K. Krebs. Values represent the Mean ± S.E.M. for 12 animals. *Results differ significantly from Krebs at P < 0.05 (Analysis of Variance: one way classification).

TABLE 4

Ability of a Depleted P₃ Fraction to be Refilled with Newly Synthesized ACh or ACh Independently of the S₃ Fraction

Incubation 1	Incubation 2	S ₃ (nmoles/gm wet wgt)				P ₃	
		¹⁴ C ACh	Total ACh	¹⁴ C ACh	Total ACh	¹⁴ C ACh	Total ACh
Krebs	Krebs + ¹⁴ C Choline	4.2 ± 0.6	15.1 ± 1.1	2.6 ± 0.2	10.5 ± 2.4		
L.K. Krebs	L.K. Krebs + ¹⁴ C Choline	4.9 ± 0.7	17.3 ± 1.3	5.1 ± 0.4	9.6 ± 1.7		
		¹⁴ C ACh	ACh	¹⁴ C ACh	ACh		
Krebs	Krebs + ¹⁴ C Homocholine	3.4 ± 0.2	17.4 ± 2.0	2.1 ± 0.2	7.2 ± 1.5		
L.K. Krebs	Krebs + ¹⁴ C Homocholine	5.3 ± 0.3*	13.2 ± 1.2	4.7 ± 0.3*	2.6 ± 0.3*		

Mouse forebrain minces were incubated for 30 minutes in Krebs or L. K. Krebs and then incubated an additional 30 minutes in Krebs containing either ¹⁴C choline (0.1mM) or ¹⁴C homocholine (0.1mM) and paraoxon (0.01μM). Results are expressed as the Mean ± S.E.M. for 12 brains. *Results differ significantly from Krebs at P<0.05.

TABLE 5

Accumulation and Acetylation of Choline and Homocholine by Subcellular Fractions of Mouse Forebrain.

Incubation 1	Incubation 2	S_3 (nmoles/gm wet wgt)				P_3
		^{14}C Choline	^{14}C ACh	^{14}C Choline	^{14}C ACh	
Krebs	Krebs + ^{14}C Choline	17.4 \pm 1.4	4.2 \pm 0.6	3.7 \pm 0.4	2.6 \pm 0.2	
L.K. Krebs	Krebs + ^{14}C Choline	18.7 \pm 0.9	4.9 \pm 0.7	5.9 \pm 0.4*	5.1 \pm 0.4*	
		^{14}C Homocholine	^{14}C AHc	^{14}C Homocholine	^{14}C AHc	
Krebs	Krebs + ^{14}C Homo-choline	11.5 \pm 0.6	3.4 \pm 0.2	4.1 \pm 0.2	2.1 \pm 0.2	
L. K. Krebs	Krebs + ^{14}C Homo-choline	11.7 \pm 0.7	5.3 \pm 0.3*	7.2 \pm 0.5*	4.7 \pm 0.3*	

Forebrain minces were preincubated for 30 min. in either Krebs or L. K. Krebs followed by a 30 min. incubation in Krebs containing ^{14}C choline or ^{14}C homocholine at 0.1mM concentrations and paraoxon (0.1uM). Results are expressed as the Mean \pm S.E.M. for 12 brains. *Results differ significantly from Krebs at $P < 0.05$ (Analysis of variance one way classification).

TABLE 6

Ability of a Depleted P₃ Fraction of Forebrain Minces to Accumulate the Preformed Acetylated Products ¹⁴C ACh and ¹⁴C AHc.

Incubation 1	Incubation 2	S ₃ (nmoles/gm wet wgt)				P ₃
		¹⁴ C ACh	Total ACh	¹⁴ C ACh	Total ACh	
Krebs	Krebs + ¹⁴ C ACh	11.9 ± 0.5	28.0 ± 2.5	1.5 ± 0.2	15.8 ± 3.3	
L.K. Krebs	Krebs + ¹⁴ C ACh	8.8 ± 0.4	23.8 ± 2.4	1.8 ± 0.3	12.6 ± 1.7	
		¹⁴ C AHc	ACh	¹⁴ C AHc	ACh	
Krebs	Krebs + ¹⁴ C AHc	3.2 ± 0.2	22.9 ± 3.4	1.2 ± 0.1	15.5 ± 2.6	
L.K. Krebs	Krebs + ¹⁴ C AHc	3.2 ± 0.1	16.1 ± 1.9	1.3 ± 0.1	15.4 ± 2.1	

Mouse forebrain minces were incubated initially in either a Krebs or L. K. Krebs medium and subsequently incubated in Krebs containing ¹⁴C ACh or ¹⁴C AHc and paraoxon (0.1μM). Results are expressed as the Mean ± S.E.M. for 12 animals.

TABLE 7

Differential Effect of L. K. Krebs Pretreatment on the Movement of Extracellular Precursors - ^{14}C Choline or ^{14}C Homocholine and Acetylated Products - ^{14}C ACh and ^{14}C AHc, into the S_3 and P_3 Fractions.

<u>Incubation 1</u>	<u>Incubation 2</u>	<u>Subcellular Fractions</u>	
	<u>Forebrain</u>	<u>S_3</u>	<u>P_3</u>
Krebs	Krebs + ^{14}C Choline	346.3 \pm 29.2	88.4 \pm 8.5 (12)
L.K. Krebs	Krebs + ^{14}C Choline	377.0 \pm 17.2	163.0 \pm 8.3* (12)
Krebs	Krebs + ^{14}C Homochol.	87.5 \pm 2.8	34.6 \pm 1.3 (16)
L.K. Krebs	Krebs + ^{14}C Homochol.	96.9 \pm 3.8	61.1 \pm 3.5* (16)
Krebs	Krebs + ^{14}C ACh	31.7 \pm 1.3	4.0 \pm 0.6 (12)
L.K. Krebs	Krebs + ^{14}C ACh	23.2 \pm 1.1*	4.0 \pm 0.7 (12)
Krebs	Krebs + ^{14}C AHc	33.6 \pm 1.8	12.6 \pm 1.1 (8)
L.K. Krebs	Krebs + ^{14}C AHc	34.4 \pm 0.9	13.9 \pm 0.6 (8)

<u>Incubation 1</u>	<u>Incubation 2</u>	<u>S_3</u>	<u>P_3</u>
	<u>Hippocampus</u>		
Krebs	Krebs + ^{14}C Homochol.	117.2 (2)	31.7 (2)
L.K. Krebs	Krebs + ^{14}C Homochol.	184.7 (2)	71.0 (2)
Krebs	Krebs + ^{14}C AHc	15.6 \pm 2.3	3.2 \pm 0.4 (3)
L.K. Krebs	Krebs + ^{14}C AHc	19.3 \pm 1.8	7.6 \pm 0.9* (3)

Mince were preincubated in Krebs or L.K. Krebs, and subsequently incubated an additional 30 min. in Krebs containing one of the following: ^{14}C Choline (0.1mM; sp.act.13.65 dpm/pmol), ^{14}C Homocholine (0.1mM, 5.1 dpm/pmol in forebrain exper. and 16.5 dpm/pmol in hippocampal studies), ^{14}C ACh (0.1mM, 2.65 dpm/pmol), ^{14}C AHc (0.1mM, 11.9 dpm/pmol). Results expressed as Mean \pm S.E.M. *Differ significantly from Krebs at $P < 0.05$ (Anal. of Var.: One way classification).

TABLE 8

Ability of a Depleted P₃ Fraction of Hippocampal Minces to be with Acetylhomocholine Formed from Extracellular Homocholine or Preformed Product

Treatment 1	Treatment 2	Subcellular Fractions							
		S ₃			P ₃				
		¹⁴ C AHc	ACh	R	¹⁴ C AHc	ACh	ACh	R	
Krebs	Krebs + ¹⁴ C Homocholine	1.1	16.3	0.07	0.06	4.2	0.14		
L.K. Krebs	Krebs + ¹⁴ C Homocholine	2.9	9.2	0.33	2.1	1.7	1.50		
		¹⁴ C AHc	ACh	R	¹⁴ C AHc	ACh	ACh	R	
Krebs	Krebs + ¹⁴ C AHc	1.3±0.2	11.4±2.5	0.12±0.02	0.3±.02	2.5±0.3	0.11±0.01		
L.K. Krebs	Krebs + ¹⁴ C AHc	1.6±0.2	11.8±3.1	0.16±0.04	0.65±0.08*	4.0±0.7	0.17±0.02*		

Mouse brain hippocampal minces were incubated initially in Krebs or L.K. Krebs and subsequently incubated in Krebs containing 0.1mM concentrations of labelled homocholine or labelled AHc. Results are expressed as Mean ± S.E.M. for incubation with labelled AHc (n=3). Incubation with labelled homocholine was performed twice and expressed as the Mean of 2 experiments. R = ratio of labelled AHc to ACh; all other values are as nmoles/gm wet wgt. *Results differ significantly at P<0.05.

FIGURE I

SUBCELLULAR FRACTIONATION SCHEME OF BRAIN TISSUE

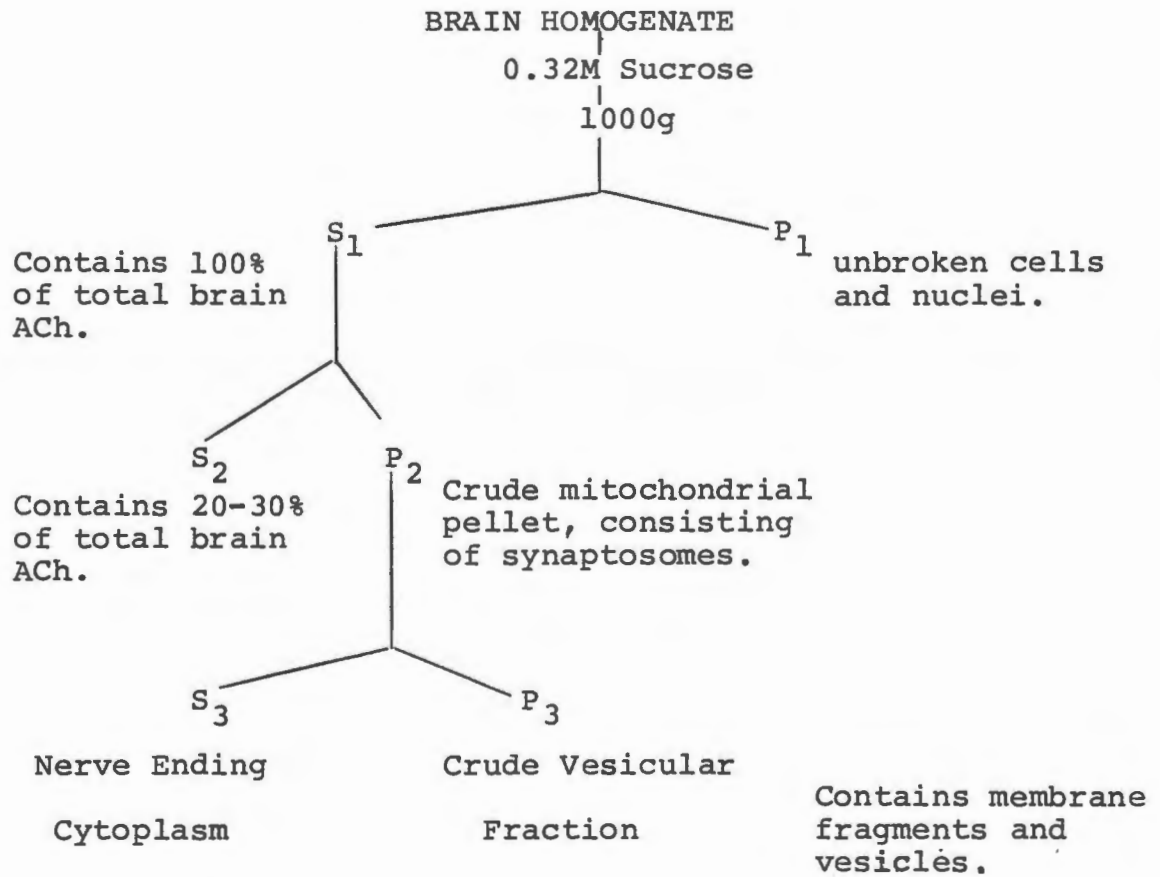
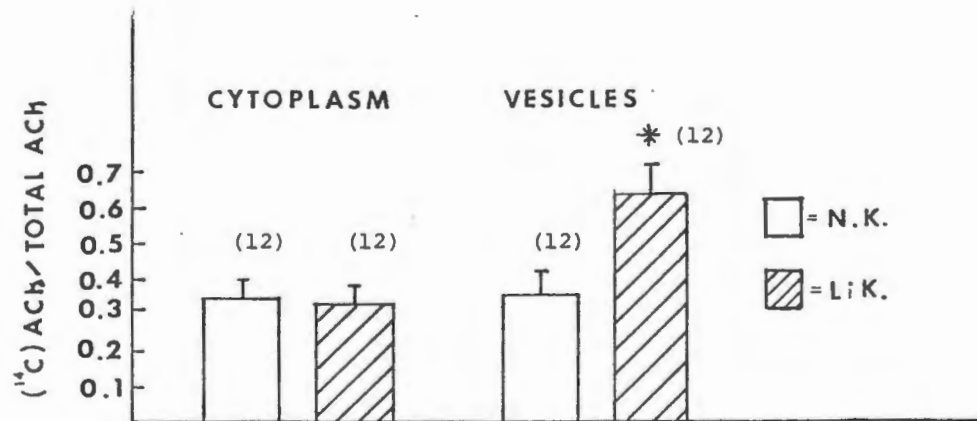


FIGURE II

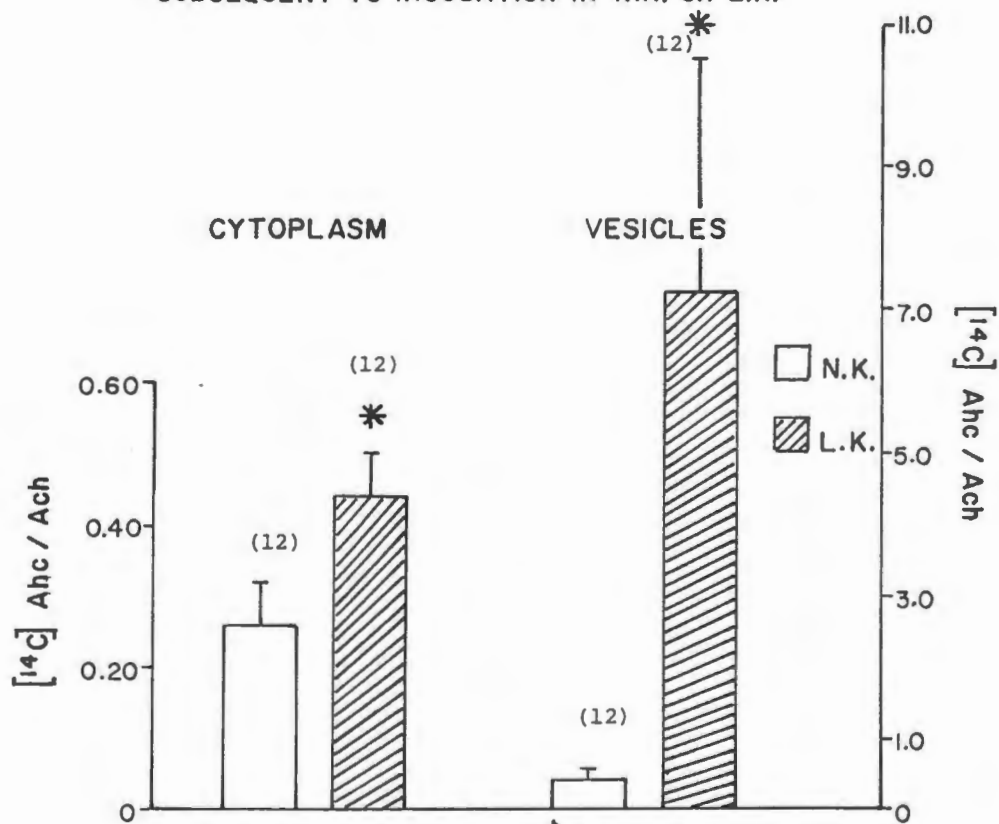
INCUBATION IN N.K. WITH 100 μ M (14 C) CHOLINE
AFTER PREINCUBATION IN N.K. OR Li⁺K.



Values represent the mean \pm S.E. for ratio of label to total ACh in subcellular fractions. An asterisk indicates a significant difference, $P < 0.05$ (Analysis of Variance: one way classification). () = experimental animals used.

FIGURE III

INCUBATION IN N.K. WITH $100\mu\text{M}$ [^{14}C] HOMOCHOLINE
 SUBSEQUENT TO INCUBATION IN N.K. OR L.K.



Values represent the Mean \pm S.E.M. for the ratio of labelled ACh to ACh in S_3 and P_3 subcellular fractions. An asterisk indicates a significant difference from Krebs at $P < 0.05$ (Analysis of Variance: one way classification). () = experimental animals used.

FIGURE IV

INCUBATION IN N.K. WITH 100 μ M (14 C) ACh
SUBSEQUENT TO INCUBATION IN N.K. OR LiK.

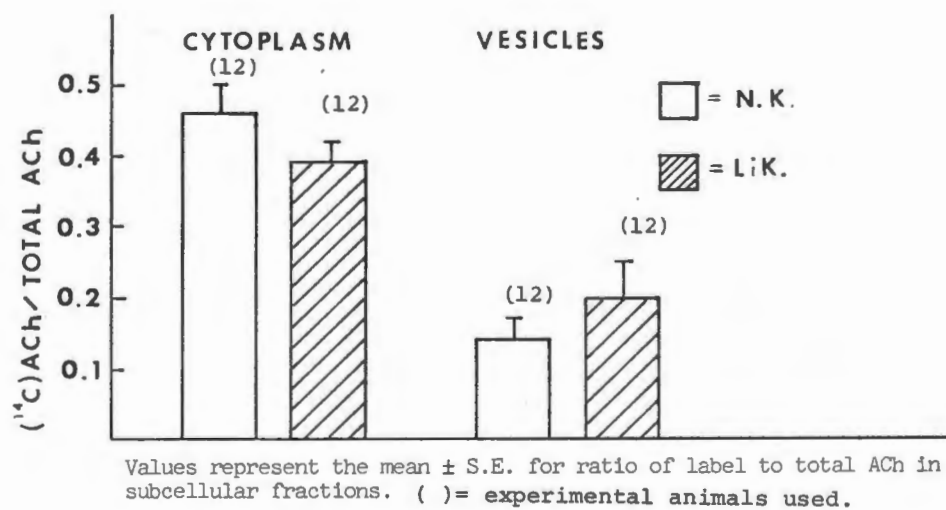
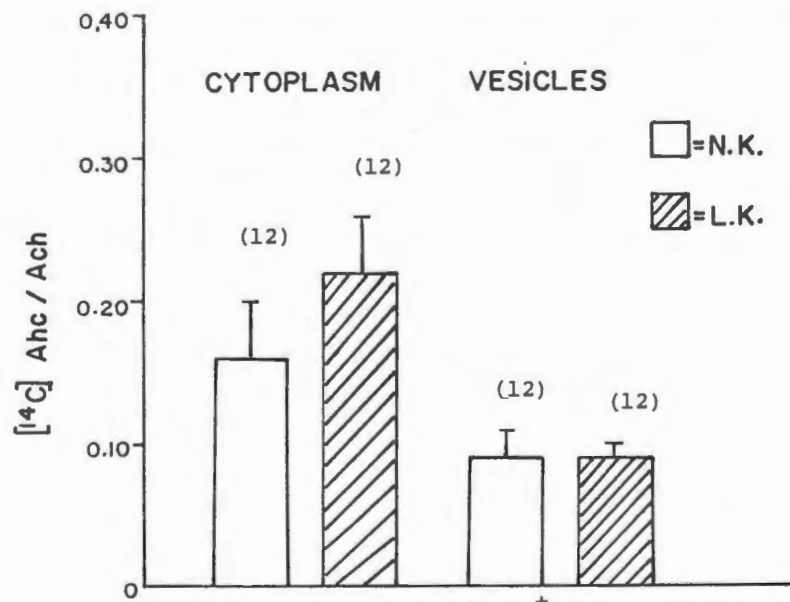


FIGURE V

INCUBATION IN N.K. WITH $100 \mu\text{M}$ $[^{14}\text{C}]$ Ahc
 SUBSEQUENT TO INCUBATION IN N.K. OR L.K.



Values represent the Mean \pm S.E.M. for the ratio of labelled AHc to ACh in S_3 and P_3 subcellular fractions. () = experimental animals used.

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