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SYNAPTIC VESICLES

INCORPORATION OF CHOLINE BY ISOLATED SYNAPTOSOMES AND SYNAPTIC VESICLES*

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Abstract—Isolated synaptosomes from guinea pig brain homogenates incorporated choline-^{14}C when incubated either in a medium with an external energy source or in one containing only Tris buffer. The incorporation was divisible into three fractions: total, TCA-insoluble, and chloroform-soluble. Incorporation of choline-^{14}C into the chloroform-soluble fraction was affected by the cations in the incubating medium: Na, K and Mg were inhibitory. Conversely, incorporation of acetylcholine-^{14}C into the chloroform-soluble fraction did not occur in the presence or absence of these ions. When the isolated synaptosomes were labeled with choline-^{14}C and the subsynaptosomal localization of the choline determined, the synaptic vesicle fraction contained the highest choline-^{14}C activity. When the various subsynaptosomal fractions were isolated and incubated with choline-^{14}C, the isolated synaptic vesicles incorporated choline-^{14}C to the greatest extent; extremely high incorporation occurred in both the "total" and chloroform-soluble fractions.

ACETYLCHOLINE (ACh), the transmitter at cholinergic peripheral synapses and in the Renshaw cell, is also considered to be a mediator of neurotransmission in many other central synapses. ACh is synthesized in the nerve endings where it is then stored so that it is available for release in response to nerve impulses. Choline is essential for the synthesis of ACh by choline acetyltransferase (ChAc) and evidence is available indicating that at the nerve terminals there is a specific choline transport mechanism[1,2] which is inhibited by prejunctional blocking drugs.[3, 4]

With the advent of methods for the isolation of nerve ending particles (synaptosomes) and synaptic vesicles,[5, 6] the exchangeability of the bound acetylcholine of synaptosomes and synaptic vesicles has been studied.[7] The subcellular localization of choline and acetylcholine in mouse brain cortex slices has been shown[8] to be similar, with 30 per cent of the choline and acetylcholine in the slices localized in the fractions containing nerve endings and mitochondria.

Recently a report has appeared[9] defining the uptake of choline-^{14}C into synaptosomes *in vitro*. This report is concerned with the transport of choline across the synaptosomal membrane and not with its subsynaptosomal fate; furthermore the uptake of choline by isolated synaptic vesicles was not studied.

The present investigation reports the uptake of choline by isolated synaptosomes and synaptic vesicles from guinea pig brain homogenates and the influence of cations

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on the uptake process. Evidence is presented for the incorporation of choline into lipid by these isolated particles. The incorporation of acetylcholine-^{14}C was also investigated and was found to differ both quantitatively and qualitatively from the incorporation of choline into these nerve-ending particles.

MATERIALS AND METHODS

Particle isolation. Synaptosomes and synaptic vesicles were prepared exactly by the method of Whittaker *et al.*[5, 6, 10] at 0–4° from guinea pig cerebral cortex (brain stem transected between superior and inferior colliculi). Guinea pigs weighing 300 g and which had been starved 16 hr before sacrifice were the source of cerebral cortex. Bands of sucrose gradients were diluted 2:1 with 0·1 M Tris buffer, pH 7·6, and centrifuged at 100,000 *g* for 30 min to remove sucrose. In each experiment assays were carried out in triplicate. All data in the tables are given as averages of these multiple observations; in most instances the values had a range of ±20 per cent. Routinely, 12 guinea pigs were used per experiment giving a yield of between 25 and 30 g wet weight of cortex. The procedures are outlined in schematic form in Fig. 1.

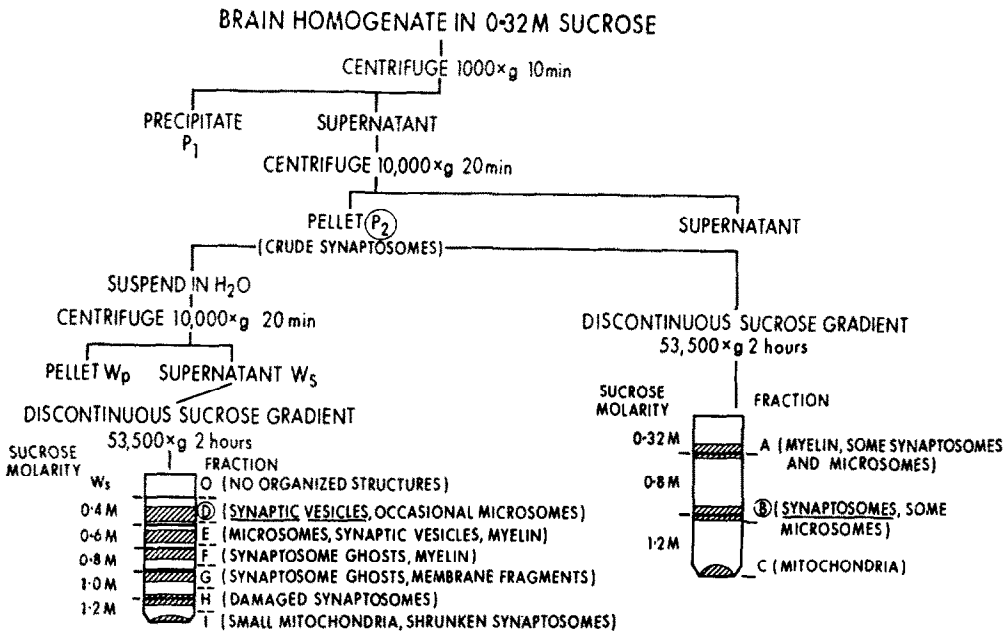

FIG. 1. Schematic representation of procedures used for the isolation of synaptosomes and synaptic vesicles. Procedures used and nomenclature are entirely those of Whittaker *et al.*[6, 10] All manipulations were carried out at the temperature of melting ice or 4°.

All experiments were done on the preparations immediately after isolation. In some instances choline or acetylcholine was incorporated before subsynaptosomal fractionation, while in other instances incorporation was measured after fractionation.

Source of choline-^{14}C and acetylcholine-^{14}C. Choline-methyl-^{14}C chloride (3·0c/mole) and acetyl-1-C^{14} choline iodine (2·0c/mole) were purchased from New England Nuclear.

Incubation mixtures. Two incubation mixtures were used. One mixture consisted

of only the 0·1 M Tris buffer, pH 7·6, in which the particles were resuspended plus 0·5 μc of choline-^{14}C (167 mμmoles) or acetylcholine-^{14}C (250 mμmoles) to a final volume of 0·510 ml; this mixture is referred to as the Tris medium. The other mixture contained, in addition, 10 mM $MgCl_2$, 10 mM sodium phosphate (pH 7·6), 5 mM phosphoenol pyruvate, 20 μg of pyruvic kinase [rabbit skeletal muscle, 1 mg of protein converts 60 μmoles of phospho(enol) pyruvate to pyruvate per min], 10 mM ATP, 2 mM EDTA, 22·5 mg/ml of a complete amino acid mixture,[11] 0·900 ml of the Tris suspension, and 0·154 M KCl to a final volume of 1·710 ml; this mixture is referred to as the complete medium. Routinely, incubations were carried out at 37° for 1 hr; the assay tubes were then immediately brought to the temperature of melting ice.

Radioactive measurements. Three types of assay for radioactivity in every case were employed: total incorporation, TCA-insoluble, and chloroform-soluble by a method similar to that described by Bosmann *et al.*;[12] no quench corrections were made.

Total incorporations. Total incorporation by the particles was measured in each instance after appropriate incubation by immediately centrifuging the assay tubes for 4 min at 5000 *g*. The supernatant was discarded and the pellet was washed by resuspension with a vortex mixer three times with 0·1 M Tris buffer, pH 7·6. The resultant pellet was dissolved in 0·2 ml of 1 N NaOH at 70°, plated on a glass filter, and the radioactivity determined in a Beckman liquid scintillation counter. The radioactivity assayed by this procedure represents total incorporation: all exchangeable material, all transported material, and all metabolized material.

TCA-insoluble material. TCA-insoluble material was determined after incubation by immediately precipitating the material with a volume of 30% TCA, equal to the incubation volume, washing the resulting pellets three times with 10% TCA and extracting once with ethanol: diethyl ether (2:1, v/v). The extracted pellet was dissolved in NaOH and counted. The radioactivity in this fraction represents material incorporated into or metabolized into protein.

Chloroform-soluble material. Chloroform-soluble material was determined by boiling the assay mixture for 1 min, cooling to the temperature of melting ice and adding 2 ml of chloroform : methanol (2:1, v/v). After centrifuging at 1000 *g* for 2 min to break the emulsion the entire aqueous layer plus the precipitate at the interface was removed by aspiration and a further 0·5 ml of deionized water was added. The aqueous layer was again carefully removed and discarded and the resulting chloroform extract was plated on a glass filter and counted. The chloroform-soluble material presumably represents lipid.

Identification of choline and acetylcholine. To determine the form of the radioactivity being assayed, after incorporation, samples were homogenized with 30 strokes in a tight-fitting Dounce homogenizer and either assayed directly by paper electrophoresis[13] or heated at pH 4 (adjusted with 0·1 N HCl) for 10 min at 80° to release bound choline or acetylcholine. Acetylcholine has been shown to withstand this heating process.[7, 10] After electrophoresis on paper strips (Schleicher & Schuell 2043A; Beckman Instrument Co.) in an inverted-V-type Durrum cell (Beckman Instrument Co.), in acetic acid/formic acid solution at pH 2 for 1 hr at a constant voltage 17 V/cm with appropriate choline and acetylcholine standards, radioactivity was determined in the strips by counting on a Baird strip scanner.

Protein. Total protein was determined by the method of Lowry *et al.*[14] Crystalline bovine serum albumin was used as a standard.

RESULTS

Incorporation of choline-^{14}C by isolated synaptosomes. Table 1 gives the results of incorporation of choline-^{14}C by isolated P_2 (Fig. 1) fraction. The total incorporation of choline by the P_2 fraction incubated in either the complete medium or the Tris medium was essentially the same; the incorporation into TCA-insoluble material was very low in each medium representing less than 10 per cent of the total incorporation. The chloroform-soluble incorporation with the complete media was low and represented less than 10 per cent of the total incorporation; with the Tris media very high incorporation was obtained representing more than 65 per cent of the total incorporation. This was a consistent observation: at all times with the Tris medium the counts in the chloroform-soluble fraction were four to eight times greater than with the complete medium.

TABLE 1. INCORPORATION OF CHOLINE INTO ISOLATED SYNAPTOSOMES*

System	Total incorporation	TCA-insoluble	Chloroform-soluble
Complete	17,000	400	1300
Tris	20,000	1600	13000

* Fraction P_2 was isolated and incubated with the indicated medium for 1 hr at 37° and the incorporation determined. Data given as cpm per mg protein.

TABLE 2. INCORPORATION OF CHOLINE BY SYNAPTOSOMES AND FRACTIONATION INTO SUB-SYNAPTOSOMAL PARTICLES*

Fraction	Total incorporation	TCA-insoluble	Chloroform-soluble
O (No organized structures)	300	80	30
D (Synaptic vesicles)	3000	300	200
E (Some synaptic vesicles)	2500	400	300
F (Synaptosome ghosts-membranes)	2200	300	200
G (Synaptosome ghosts)	1100	500	200
H (Damaged synaptosomes)	1000	400	500
I (Small mitochondria)	1000	600	200

* Data given as cpm per mg protein. Fraction P_2 was incubated for 1 hr with the complete medium. Twelve samples were incubated, pooled and then fractionated as illustrated in Fig. 1. After removal from the gradient and isolation of fractions 0 and D–I the radioactive measurements were made as given in Methods.

Subsynaptosomal localization of incorporated choline-^{14}C. When fraction P_2 was prelabeled in the complete medium with choline-^{14}C and fractionated on a discontinuous sucrose gradient, the incorporated choline-^{14}C was found mainly in fraction D, E, and F (Fig. 1) with the highest incorporation (although this may not be statistically significant) in fraction D, the synaptic vesicle fraction (Table 2). The cpm per mg protein given in Table 2 are much lower than in Table 1, indicating that a large portion of the radioactivity in the P_2 synaptosomes is solubilized during the rupturing and gradient procedures. This choline-^{14}C is most probably present as choline and not in macromolecular form and not membrane enclosed, making it soluble during the fractionation procedure. Conversely, the choline-^{14}C activity in the fractions given

in Table 2 must represent exchangeable or incorporated choline in the case of the membrane fractions (O, F, G) and membrane-bound transported choline-^{14}C as well as exchangeable or incorporated in the case of the particle fractions (D, E, H, I). The low activity in the chloroform-soluble fractions in Table 2 is consistent with the low incorporation found when the synaptosomes are incubated in the complete medium but presumably represents low but real incorporation of choline-^{14}C into lipid.

Identification of radioactive product. Six assay tubes of the isolated P_2 fraction were incubated in the Tris medium and six assay tubes in the complete medium in the usual manner. The "total incorporation" procedures were then performed on three of the six tubes and the resultant material pooled. The material from the pooled-washed fraction after centrifugation for "total incorporation" was homogenized and heated to release any bound choline or acetylcholine as described in Methods. After removal of insoluble protein from the heated "total incorporation" samples by centrifugation, the supernatants were spotted on paper and electrophoresed at pH 2 for 1 hr in a formic acid–acetic acid buffer system. In each instance all the radioactivity was recovered as choline when compared with a coelectrophoresed standard; no radioactivity coelectrophoresed with the acetylcholine standard or in any other position on the electrophoresis strip.

Incorporation of choline by subsynaptosomal particles. The incorporation and exchangeability of choline in "purified" synaptosomes and subsynaptosomal particles were studied with the Tris medium and the incorporation is shown in Table 3.

TABLE 3. INCORPORATION OF CHOLINE BY "PURE" SYNAPTOSOMES AND SUBSYNAPTOSOMIC PARTICLES*

Fraction	Total incorporation	TCA-insoluble	Chloroform-soluble
A (Myelin fragments)	42,000	11,000	1600
B ("Pure" synaptosomes)	36,000	1700	5000
C (Mitochondria)	17,000	400	900
W_s (Membranes-synaptic vesicles)	12,000	300	1100
W_p (Mitochondria)	62,000	400	1500
O (No organized structures)	31,000	5600	29,000
D (Synaptic vesicles)	431,000	23,000	278,000
E (Some synaptic vesicles)	53,000	500	4100
F (Synaptosome ghosts)	52,000	1100	7500
G (Synaptosome ghosts)	86,000	1500	5800
H (Damaged synaptosomes)	36,000	300	2200
I (Small mitochondria)	28,000	7700	21,000

* Fractionations were exactly as given in Fig. 1. After isolation of fractions, they were incubated in the Tris medium for 1 hr at 37°. The incubation contained the fraction to be tested in 0·5 ml of 0·1 M Tris at pH 7·6 plus 0·5 μc of choline-^{14}C. Data given as cpm per mg protein.

All fractions had high total incorporation and, as was true with the P_2 fraction in the Tris medium incubation (Table 1), high incorporation into the chloroform-soluble fraction occurred. Fraction B consisting of "purified" synaptosomes had a total incorporation of 36,000 cpm/mg protein and a chloroform-soluble incorporation of 5000 cpm/mg protein (Table 3). By far the greatest incorporation per milligram protein of choline-^{14}C in the total, TCA-insoluble and chloroform-soluble fractions was with

the D fraction, the synaptic vesicle fraction. Extremely high total incorporation of 431,000 cpm/mg protein and chloroform-soluble incorporation of 278,000 cpm/mg protein was found with this synaptic vesicle fraction. Other isolated fractions which had high total incorporations were E (some synaptic vesicles) and F and G (synaptosome ghosts). High chloroform-soluble incorporation occurred in fraction 0 (no organized structures), possibly indicating that lipid fragments containing exchangeable choline occur in this fraction; this is consistent with the fact that this fraction is of the lowest density in the sucrose gradient. The high TCA-insoluble incorporation in some of the fractions (notably A, D, and I) may represent choline-^{14}C in large proteo-lipid complexes not solubilized during the ethanol : ether wash.

Incorporation of choline-^{14}C into different fractions of synaptosomes. Since a consistent observation of this study was the high incorporation into chloroform-soluble material with the Tris medium but not with the complete medium, the components of the complete medium were selectively omitted and the incorporation by the isolated P_2 fraction studied. Elimination of the energy producing components (ATP, pyruvic kinase, phosphoenol pyruvate) of the complete medium did not affect the total incorporation of choline-^{14}C nor did this procedure affect the incorporation into the chloroform-soluble fraction or the TCA-insoluble fraction. The effects of the removal of the components of the incubation medium on incorporation of choline-^{14}C are shown in Table 4.

TABLE 4. INCORPORATION OF CHOLINE INTO DIFFERENT FRACTIONS OF SYNAPTOSOMES WITH DIFFERING INCUBATION MEDIA*

System	Total incorporation	TCA-insoluble	Chloroform-soluble
Complete	17,000	400	1300
—Na	23,000	1500	4300
—K	22,000	700	2000
—EDTA	16,000	1000	2500
—Na, —K	17,000	1800	6000
—Na, —K, —EDTA	21,000	1600	2200
—Mg	22,000	2500	2400
—Mg, —Na, —K	18,000	100	3600
—Mg, —Na, —K, —EDTA	28,000	800	1200

* Complete media as given in Methods. In each instance the indicated component was omitted and replaced with an equal volume of distilled water. Data given as cpm per mg protein. Isolated P_2 fractions were incubated for 1 hr at 37° and analyzed as given in Methods.

When the Na and K components of the complete medium were omitted the incorporation into the chloroform-soluble fraction was increased more than 4-fold. Thus, Na and K each inhibit the uptake of choline-^{14}C into the chloroform-soluble material but Na was far more inhibitory than K; when Na was omitted from the incubation medium the incorporation was raised more than 3-fold, when K was omitted the increased incorporation was less than 2-fold. When the EDTA component of the complete medium was omitted along with the Na and K components the chloroform-soluble incorporation was increased less than 2-fold. Thus, Na and K were inhibitors to the incorporation into the chloroform-soluble fraction, but EDTA was necessary, perhaps to chelate endogenous Na and K. Mg also has an inhibitory effect on choline-^{14}C incorporation into the chloroform-soluble fraction.

Na and K also had an inhibitory effect on the incorporation of choline-^{14}C into the TCA-insoluble fraction and EDTA was also found to be necessary in the absence of Na and K for optimal uptake of choline-^{14}C. When the Mg was omitted from the incubation system the incorporation of choline-^{14}C into the TCA-insoluble fraction was increased more than 6-fold and therefore Mg has a greater inhibitory effect on the incorporation of choline-^{14}C into this fraction. Removal of Na, K, Mg and EDTA from the incubation medium did cause an increase in the total incorporation of choline-^{14}C into the synaptosomes.

Incorporation of acetylcholine-^{14}C. When acetylcholine-^{14}C was substituted for choline-^{14}C in the system, the P_2 fraction incorporation was much lower on a protein basis than for choline-^{14}C. In the complete medium the uptake of ACh was 18 per cent that of choline whereas in the Tris medium the ACh uptake was 43 per cent in comparison to choline (see Tables 1 and 5). The incorporation was higher in all three

TABLE 5. INCORPORATION OF ACETYLCHOLINE-^{14}C BY ISOLATED SYNAPTOSOMES USING COMPLETE AND TRIS MEDIA AS GIVEN IN METHODS*

System	Total incubation	TCA-insoluble	Chloroform-soluble
Complete	3100	30	90
Tris	8500	170	140

* In each case the isolated P_2 fraction was incubated with the indicated medium for 1 hr at 37° and the incorporation determined. Data given as cpm per mg protein.

methods of assay with the Tris medium, probably because of the smaller total volume with the Tris; but most important and significant was the low incorporation into the chloroform-soluble fraction with the Tris medium (Table 5). Less than 3 per cent of the ACh incorporated was taken up into the chloroform-soluble fraction. Thus acetylcholine, unlike choline, is not incorporated into lipid in the absence of Na and K. Of other neurohumoral transmitters studied, some followed the behaviour of choline in this respect whereas others followed the behaviour of acetylcholine.*

DISCUSSION

The results presented herein demonstrate that synaptosomes, synaptic vesicles and other subsynaptosomal particles when incubated with choline-^{14}C at 37° for 1 hr incorporate choline into TCA-insoluble and chloroform-soluble fractions as well as into the "total incorporation" fraction. When choline-^{14}C is incorporated by isolated synaptosomes the highest amount of radioactivity is found in the synaptic vesicle fraction.

Marchbanks[9] recently investigated the uptake of choline-^{14}C into synaptosomes *in vitro* but did not investigate the subsynaptosomal fate of the choline. Marchbanks suggested it was possible that mitochondria also take up choline with approximately the same kinetics as synaptosomes. The present work shows that the incorporation of choline into pure synaptosomes is therefore the most specific subcellular fraction for the uptake of choline and synaptic vesicles are the most specific subsynaptosomal

* B. A. Hemsworth and H. B. Bosmann, unpublished observation.

fraction for the uptake of choline. Potter[2] has investigated choline uptake into nerve ending fractions of rat brain; he observed no facilitated uptake into nerve ending ghosts. Potter[2] did not fully investigate the uptake of choline by synaptosomes and subsynaptosomal particles and the experiments reported here are an extension of the work initially reported by Marchbanks[9] and by Potter.[2] In the present work we have shown that with the Tris incubation medium incorporation of choline into synaptosomal ghosts occurs. Furthermore, choline is taken up into the chloroform-soluble fraction although this uptake is very much less than the total uptake by the synaptosomal ghosts.

The present work demonstrates that there is a high incorporation of choline into the chloroform-soluble fraction of synaptosomes. The nature of this incorporation has not been investigated in the present experiments and no attempt has been made to ascertain whether the choline incorporation represents exchange, transport across membranes into membrane-bound particles, or synthesis of new material. The "total incorporation" of choline presented in this paper is not dependent on an energy-producing system or on specific ions. However, the incorporation of choline-^{14}C into lipid by synaptosomes which occurs in the "Tris system" is a function of the ions in the incubating media; incorporation of choline-^{14}C into the chloroform-soluble fraction is inhibited in the presence of Na and K and Mg ions, both individually and together.

Marchbanks[9] and Potter[2] investigated the effect of Na and K on the total incorporation of choline into nerve-ending particles and found that omission of Na from the medium decreased choline influx. Omission of K and Mg was shown by these workers to have little influence on choline uptake. Our results are in agreement with the work of Marchbanks[9] and Potter[2] in that removal of K and Mg had little effect on the total incorporation into synaptosomes and subsynaptosomal particles but these ions did selectively influence to a great extent the incorporation of choline into the chloroform-soluble fraction. Our results also show that removal of Na from the incubation medium has little effect on the total incorporation but increased uptake of choline into the chloroform-soluble fraction.

It is apparent that some cations are necessary for the chloroform-soluble incorporation of choline into synaptosomes because when Na, K and Mg were all omitted from the incubation medium an optimal incorporation of choline into this chloroform-soluble fraction did not occur. It has been demonstrated by Burton and Howard[15] that K increases the uptake of choline into nerve-ending fractions and lowers uptake into synaptic vesicles illustrating different binding capacities under the influence of different cations. It is tempting to speculate that the ion concentrations in the immediate environment of the particles may function as a control mechanism determining whether choline-^{14}C is incorporated into lipid (chloroform-soluble material), perhaps for storage, or whether choline-^{14}C is in a soluble pool available for acetylation. In this regard it is of interest that acetylcholine-^{14}C, when incubated with isolated synaptosomes in the "Tris medium," was not incorporated into the chloroform soluble material.

Marchbanks[9] has found that choline-^{14}C influx into synaptosomes has two components, one linear and one saturable; it is possible that the linear component may represent membrane transport while the saturable component may represent incorporation into lipid as reported herein.

Schuberth and Sundwall[8] also investigated the subcellular localization of choline taken up by mouse brain slices and showed that 30 per cent of the radioactivity in the slices was localized in the nerve-ending and mitochondrial fraction. They also showed that choline and ACh, which were specifically bound to nerve-ending particles, had a similar subcellular distribution. Our results demonstrate that ACh had a much lower incorporation into synaptosomes than choline on a protein basis when each was tested at the same concentration and specific activity. These results are in agreement with Burton and Howard[15] who showed that synaptic vesicles incorporated choline to a greater extent than ACh. One of the interesting features of these experiments is that ACh, unlike choline, was not incorporated into lipid.

Collier and Lang[16] and Collier and MacIntosh[17] in two recent papers have indicated that in the perfused superior cervical ganglia of the cat choline was incorporated into acetylcholine, phosphorylcholine, and phospholipid. On the basis of rate studies these authors indicated that free choline liberated from phosphorylcholine or phospholipid was unlikely to be an important source of choline for acetylcholine synthesis. In the present communication choline was also found to be incorporated into lipid but it is not known whether this material represents an available pool of choline for acetylcholine synthesis.

In the present investigation all the choline taken up into the synaptosomal fraction was identified as choline. No bound ACh which would be resistant to hydrolysis by cholinesterase was observed in the synaptosomal fraction. This does not necessarily mean that no ACh is formed within the nerve-ending fractions as the cholinesterases within the vesicle preparations would be expected to hydrolyze any free ACh formed during the incubation.

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