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TRIETHYLCHOLINE INHIBITION OF PHOSPHATIDYLCHOLINE SYNTHESIS IN SYNAPTOSOMES AND MOUSE LEUKEMIC CELLS L1210 AND ACCELERATED SYNAPTOSOMAL UPTAKE OF CHOLINE AND TRIETHYLCHOLINE IN THE PRESENCE OF ACETYLCHOLINE*

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Abstract—Triethycholine and choline were incorporated into lipid fractions of L1210 mouse leukemic cells or isolated guinea pig cerebral cortex synaptosomes. Chromatography revealed that the incorporations were between 50 and 70 per cent into phosphatidyl triethylcholine or phosphatidylcholine respectively. Triethylcholine was a potent inhibitor of choline incorporation into lipid in either the L1210 cells or synaptosomes, while choline severely inhibited triethylcholine incorporation. Acetylcholine in the presence of 10^{-5} M eserine increased synaptosome lipid incorporation of choline 170 per cent and triethycholine 200 per cent. It is suggested that the lipid choline of synaptosomes represents a storage pool of choline for acetylcholine synthesis.

THIS LABORATORY initially reported that isolated synaptosomes incubated with choline incorporated choline into lipid-soluble fractions as well as into storage (synaptic) vesicles as free choline and hypothesized that the lipid choline might in fact function as a source of choline for acetylcholine synthesis in certain circumstances.[1,2] The fact that choline is incorporated into lipid by isolated synaptosomes has been confirmed by others,[3] although Miller and Dawson[4] have brought attention to the fact that contamination by the endoplasmic reticulum could account for a percentage of the capacity of synaptosomes to synthesize phospholipids. Furthermore this laboratory has shown that triethylcholine is incorporated into isolated synaptosomes and at certain low concentrations potentiates choline uptake by synaptosomes (at high concentrations triethylcholine inhibited total choline uptake), but does not potentiate the incorporation of choline into lipid by the isolated synaptosomes.[5,6] This last finding is of importance since recently many workers have reported that acetylcholine (similar in structure to triethylcholine) enhances turnover of the phosphorus moiety of phospholipids and may increase phospholipid or lipid synthesis in nervous tissue.[7–10] Also hemicholinium-3

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bromide (HC-3), which has pharmacological properties similar to those of triethylcholine (TEC), has been shown to stimulate the incororation of choline into membrane phosphatidylcholine in nerve ending fractions of brain tissue *in vivo*.[7] The present report gives evidence that TEC inhibits choline uptake into synaptosomes, phosphatidylcholine synthesis in both synaptosomes, and a non-neural lymphoma cell line, L1210, and is incorporated into a compound with a chromatographic R_f identical with lecithin. Furthermore, data are presented which indicate that acetylcholine in the presence of eserine enhances choline and triethylcholine uptake by synaptosomes and the incorporation of choline and triethylcholine into synaptosomal lipid.

MATERIALS AND METHODS

Biochemicals. TEC was a gift from Ward Blenkensop & Co., Ltd., London, England; all other biochemicals were purchased from Sigma Chemical Corp., St. Louis, Mo., U.S.A. Distilled, glass-distilled, deionized water was used for all experimental procedures.

Particle isolation. Synaptosomal fractions were prepared as described previously[1,11 14] by the method of Gray, Whittaker *et al.*[15–18] at 0–4° from guinea pig cerebral cortices (brain stem transected between superior and inferior colliculi). The cortical fractions were freshly prepared and experiments were performed immediately after isolation. The synaptosome fraction "B" was the fraction used in all experiments referred to herein and the terminology used is that of Gray, Whittaker *et al.*[15 18]

L1210 cells. The L1210 cell line was carried in mice as previously described[19] by i.p. inoculation of 10^6 cells.

Incubation mixtures. For the synaptosome measurements, the synaptosomes were suspended in 0·1 M Tris buffer, pH 7·6, at between 0·5 and 2·0 mg of synaptosome protein/100 μl and the required amount of radioactive precursor and inhibitor was added. This incubation system was routinely incubated for 1 hr at 37° and lipid-soluble radioactivity determined as given below. In previous publications[2,6] this medium is referred to as "Tris medium".

For L1210 measurements, cells were harvested from mice, washed once with saline and immediately resuspended in Fischer's medium[20] at between 0·5 and 2·0 mg of L1210 protein/100 μl and the required amount of radioactive precursor and inhibitor was added. This incubation system was incubated for 1 hr at 37° and lipid-soluble radioactivity was determined as given below.

In experiments designed to measure the effects of acetylcholine on lipid synthesis with choline-^{14}C as precursor in the synaptosomes, 10^{-5} M eserine was present as an acetylcholinesterase inhibitor[12] to prevent hydrolysis of acetylcholine.

Protein. Total protein was determined by the method of Lowry *et al.*[21] utilizing bovine serum albumin as standard.

Radioactively labeled precursors. TEC (for specific activity see below) was tritium labeled by a catalytic exchange process by New England Nuclear Corp. Thirty-six mg of TEC (supplied by Ward Blenkensop & Co., Ltd., London, England) was dissolved in 0·2 ml of H_2O containing 25 Ci of tritium gas and 300 mg of 5 per cent rhodium on alumina. The reaction mixture was stirred for 2 days at room

temperature. Unreacted tritium was removed with 10 ml of H_2O and the solvent was removed by vacuum distillation. Purity of ^{3}H-TEC was determined by either paper electrophoresis or thin-layer chromatography as given below. Choline-methyl-^{14}C chloride (3·0 Ci/mole) was purchased from New England Nuclear Corp.

Paper electrophoresis. [^{3}H]TEC was subjected to electrophoresis on Beckman paper strips (S and S 2043A mg 1) in acetic acid–formic acid solution at pH 2 for 1 hr (voltage constant, 17 V/cm) in a Durrum V-type cell[22] using crystalline TEC as standard. The movement of TEC was determined by staining with iodine vapor. The radioactivity in the paper strips was determined by counting on a Baird strip scanner, or by cutting up the paper and counting in a liquid scintillation counter.

Thin-layer chromatography. The sample was assayed on cellulose-based polyethylene chromatogram sheets, without fluorescent indicator (Eastman Kodak), in butanol-ethanol–acetic acid–water, 8:2:1:3[23] by ascending chromatography for 3 hr at 25° using crystalline TEC as standard. The resulting chromatograms were developed by staining in iodine vapor. The radioactivity was counted using a strip scanner or a liquid scintillation counter as described above.

Purification of [^{3}H]TEC. The [^{3}H]TEC supplied by New England Nuclear Corp. had a sp. act. of 7·5 mCi/mg. The [^{3}H]TEC was purified by subjecting the compound to thin-layer chromatography as described above. The material with an R_f value of 0·66, the same as TEC standard, was removed by scraping approximately 1 cm^2 from the chromatogram, and 0·2 ml of water was added. The resulting suspension was filtered through a sintered glass filter, washed with 0·2 ml of water and the filtrate was chromatographed a second time. The material running to the same R_f value as TEC was again collected, water was added, and the suspension filtered. The resulting solution (12 ml vol.) was the purified [^{3}H]TEC used through the experiments. A portion of this material was electrophorezed on paper at pH 2·0 for 1 hr in the formic acid–acetic acid buffer system (see above). All the radio-activity moved to the same position as the standard TEC (5·9 cm/hr). No radioactivity was observed in any other position on the electrophoresis strip.

Determination of specific activity of [^{3}H]TEC. The thin-layer chromatographic technique was used as a semi-quantitative assay procedure to determine the specific activity of the purified [^{3}H]TEC. Varying amounts of TEC in a volume of 10 μl vol. were chromatographed with 10 μl of [^{3}H]TEC in the system as described above. Iodine and/or Dragendorff's reagent was used to stain the TEC spots, and an assessment of the amount of TEC in 10 μl of the radioactive material was obtained.[23] The concentration of the purified [^{3}H]TEC was found to be $1{\cdot}25 \times 10^{-4}$ M.

One ml of the purified [^{3}H]TEC was found to contain 0·43 mCi of radioactivity and the sp. act. of the [^{3}H]TEC was found to be 3·44 Ci/m-mole $\pm$ 0·09.

Extraction for lipid or chloroform-soluble material. Chloroform-soluble material was determined by centrifuging the synaptosomes or L1210 cells (2000 *g*, 10 min) out of the assay mixure, adding 1 ml of H_2O and boiling this mixture for 1 min, cooling to the temperature of melting ice and adding 2 ml of chloroform–methanol (2:1, v/v). Experiments measuring total free choline before and after this procedure indicated that this procedure caused no release of free choline from complex choline compounds. 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 water was added. After remixing and centrifuging as indicated, 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 TEC. 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 or heated at pH 4 (adjusted with 0·1 N HCl) for 10 min at 80° to release bound choline or TEC. 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 TEC standards, radioactivity was determined in the strips by counting on a Baird strip scanner.

Chromatography on silicic-acid impregnated paper. Lipid extracts were chromatographed by ascending chromatography in the DAW system (diisobutyl ketone–acetic acid–water; 40:25:1) as previously described.[24,25] Standards were visualized by staining with Rhodamine 6G.

Two-dimensional thin-layer chromatography. The system described by Abdel-Latif and Smith[3] on Silica gel G for two-dimensional TLC was also utilized to identify the macromolecules labeled with the radioactive precursors.

RESULTS AND DISCUSSION

Incorporation of [^{3}H]TEC into a lipid-soluble fraction of synaptosomes and inhibition of this incorporation by choline. The data of Fig. 1 demonstrate that [^{3}H]TEC was incorporated into a lipid-soluble fraction of guinea pig cerebral cortex synaptosomes (under the conditions of assay about 7000 cpm/mg synaptosomal protein/hr). Choline was a potent inhibitor of this synaptosome incorporation of [^{3}H]TEC; at 10^{-2} M the incorporation was inhibited by more than 80 per cent (Fig. 1).

Incorporation of [^{14}C]choline into a lipid-soluble fraction of synaptosomes and inhibition of this incorporation by TEC. As shown in Fig. 2, [^{14}C]choline was incorporated into isolated guinea pig cerebral cortex synaptosomes in a lipid-soluble form (the control incorporation was about 14,000 cpm/hr/mg synaptosome protein). This incorporation into lipid in the isolated synaptosome was substantially inhibited by TEC. At 10^{-2} M, TEC inhibited choline incorporation into lipid by the synaptosome greater than 60 per cent. Lower concentrations of TEC also inhibited the synaptosomal lipid incorporation of choline; even at 10^{-5} M TEC, slight inhibition occurred.

Incorporation of [^{3}H]TEC into a lipid-soluble fraction of L1210 mouse leukemic cells. The data of Fig. 3 demonstrate that [^{3}H]TEC was incorporated into a lipid fraction of L1210 cells and that the amount incorporated was related to the amount of [^{3}H]TEC present in the incubation mixture. At 10 μl of [^{3}H]TEC, about 6000 cpm/mg protein/hr were incorporated into lipid in the L1210 cells, while at 30 μl 18,000 cpm/mg protein/hr were incorporated.

Incorporation of [^{14}C]choline into a lipid-soluble fraction of L1210 mouse leukemic cells in the presence of TEC. [^{14}C]choline was readily incorporated into a lipid

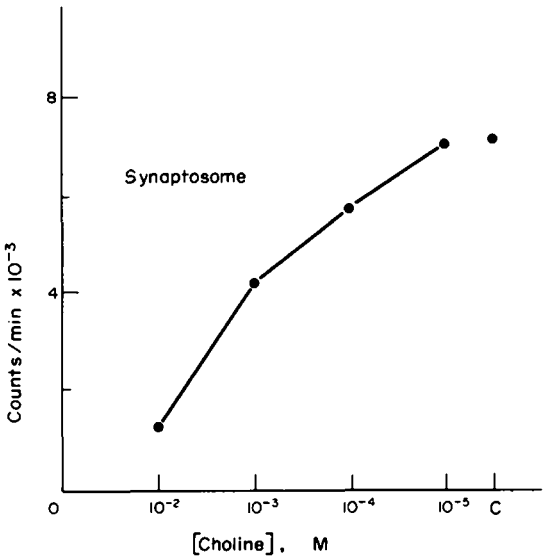

FIG. 1. Incorporation of [^{3}H]TEC into a lipid fraction of isolated synaptosomes from guinea pig cerebral cortex in the presence of choline. Experiments were performed as given in Materials and Methods. [^{3}H]TEC was present at 10 μl of a 0·43 mCi/ml solution of [^{3}H]TEC (3·44 Ci/m-mole) in a final volume of 0·21 ml. C refers to control, in which no choline was present. Choline concentration is final concentration. Means from six independent determinations. Data are for 1 mg of synaptosomal protein.

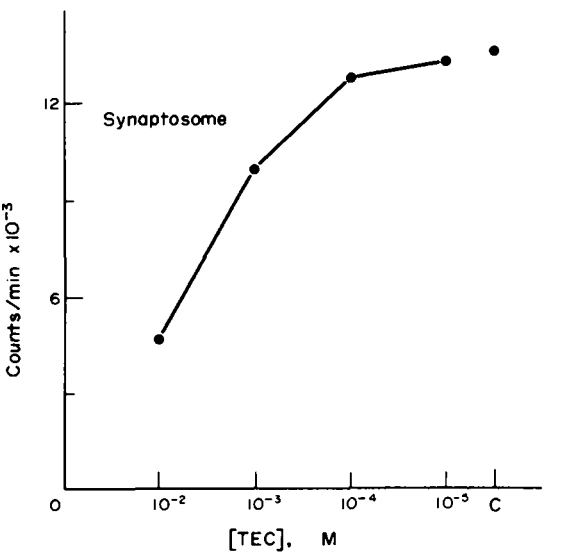

FIG. 2. Incorporation of [^{14}C]choline into a lipid fraction of isolated synaptosomes from guinea pig cerebral cortex in the presence of TEC. Experiments were performed as given in Materials and Methods. [^{14}C]choline was present as 0·5 μCi of [^{14}C]choline (167 nmoles; 3·0 Ci/mole) in a final volume of 0·51 ml. C refers to control, in which no TEC was present. TEC concentration is final concentration. Means from six independent determinations. Data are for 1 mg of synaptosomal protein.

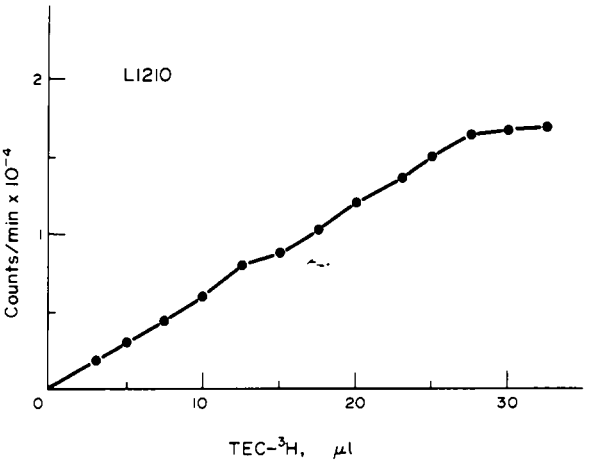

FIG. 3. Incorporation of [^{3}H]TEC into a lipid fraction of L1210 mouse leukemic cells as a function of the amount of [^{3}H]TEC present in the incubation mixture. Experiments were performed as given in Materials and Methods. [^{3}H]TEC (3·44 Ci/m-mole) was present in the amount indicated of a 0·43 mCi/ml solution. Final volume was always 0·13 ml. Each point is the mean of six determinations, and the data are for 1 mg of L1210 protein.

fraction of L1210 cells (1600 cpm/mg L1210 protein/hr) and this incorporation was markedly decreased by TEC, as shown in Fig. 4. At 10^{-2} M TEC, [^{14}C]choline incorporation into the L1210 lipid fraction was inhibited to 10 per cent the control level; inhibition occurred even at 10^{-5} M TEC (approximately 15 per cent inhibition).

Incorporation of [^{3}H]TEC into a lipid fraction of L1210 cells in the presence of choline. The data of Fig. 5 indicate that at levels of 10^{-2} to 10^{-6} M choline [^{3}H]TEC incorporation into L1210 lipid was severely inhibited. At 10^{-2} M choline [^{3}H]TEC incorporation into L1210 lipid was inhibited almost 90 per cent.

Identification of products L1210 and guinea pig cerebral cortex synaptosome incubations with [^{14}C]choline or [^{3}H]TEC. The data in Fig. 6 indicate that with the synaptosome incubation 50 per cent and with the L1210 incubation about 60 per cent of the [^{14}C]choline radioactivity that was lipid soluble was recovered as phosphatidylcholine, with the remainder of the material at the origin in the silicic acid-impregnated paper chromatography system. Similarly, with the synaptosomes and the L1210 cells, 60 per cent of the [^{3}H]TEC material migrated essentially with the lecithin standard, indicating that [^{3}H]TEC was probably substituted for choline in this molecule to produce phosphatidyl triethylcholine. Utilizing the two-dimensional TLC system of Abdel-Latif and Smith[3] for all four systems described herein, (i.e. [^{3}H]choline and [^{3}H]TEC incorporation into synaptosomes and L1210 cells), the lipid-soluble radioactivity migrated about 70 per cent with phosphatidyl-choline, 2 per cent with sphingomyelin and 3 per cent with lysophosphatidylcholine. The material remaining at the origin was not identified. Hydrolysis of the total chloroform soluble extract of any of the four systems yielded recoveries approaching 100 per cent for the radioactive precursor, i.e. [^{14}C]choline was recovered 100 per cent as [^{14}C]choline and [^{3}H]TEC was recovered 100 per cent as [^{3}H]TEC.

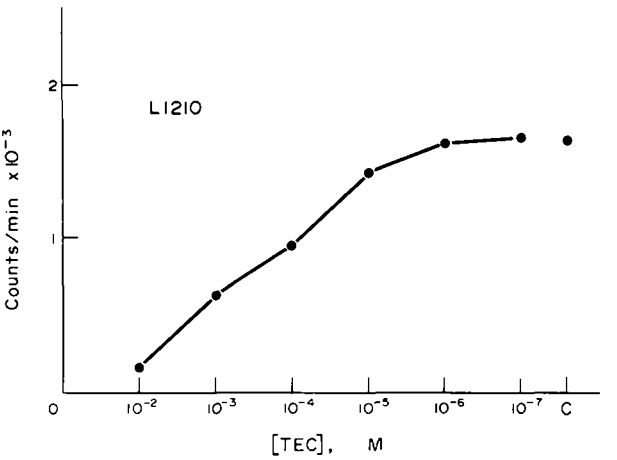

FIG. 4. Incorporation of [^{14}C]choline into a lipid fraction of L1210 mouse leukemic cells in the presence of TEC. Experiments were performed as given in Materials and Methods. [^{14}C]choline was present as 0·5 μCi of [^{14}C]choline (167 nmoles; 3·0 Ci/mole) in a final volume of 0·13 ml. C refers to control, in which no TEC was present. TEC concentration is final concentration. Means from six independent determinations. Data are for 1 mg of L1210 protein.

Furthermore, hydrolysis of the phosphatidylcholine radioactive portions of the silicic acid-impregnated paper chromatograms yielded either 100 per cent [^{14}C] choline or 100 per cent [^{3}H]TEC in either the synaptosome or L1210 systems.

Accelerated incorporation of [^{3}H]TEC or [^{14}C]choline into a lipid-soluble extract of guinea pig cerebral cortex synaptosomes in the presence of acetylcholine. The data of Fig. 7 clearly demonstrate that acetylcholine accelerated the incorporation of both [^{3}H]TEC and [^{14}C]choline into lipid fractions of synaptosomes. In the presence of 10^{-4} M ACh, [^{3}H]TEC incorporation was more than 200 per cent

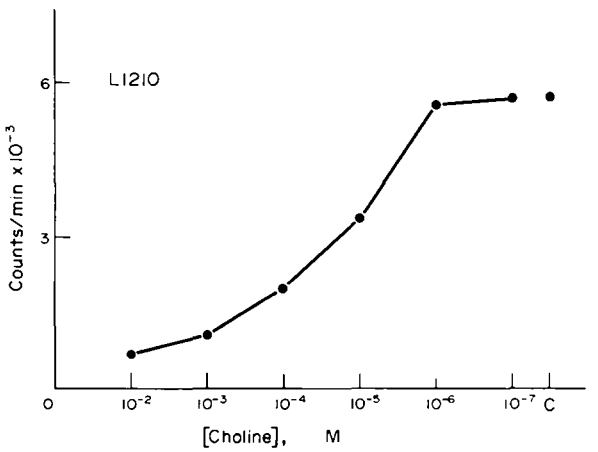

FIG. 5. Incorporation of [^{3}H]TEC into a lipid fraction of L1210 mouse leukemic cells in the presence of choline. Experiments were performed as given in Materials and Methods. [^{3}H]TEC was present as 10 μl of [^{3}H]TEC (0·43 mCi/ml; 3·44 Ci/mole) in a final volume of 0·13 ml. C refers to control, in which no choline was present. Choline concentration is final concentration. Means from six independent observations. Data are for 1 mg of L1210 protein.

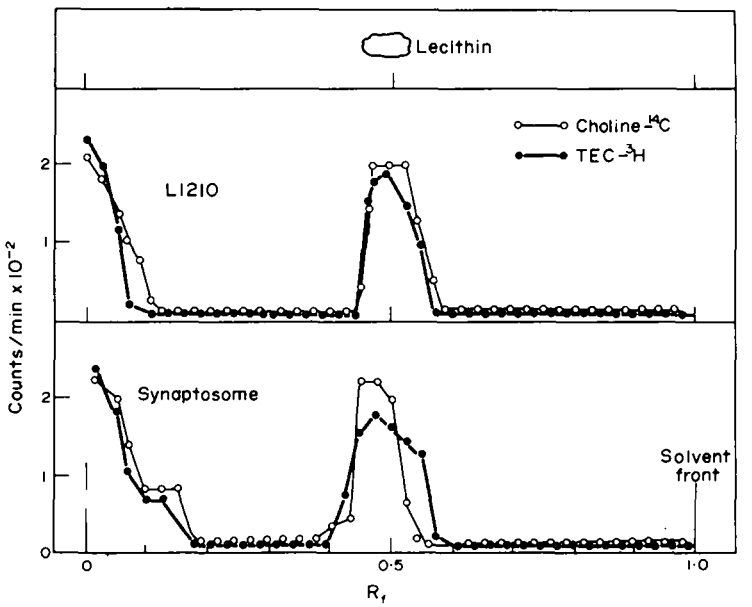

FIG. 6. Chromatography on silicic acid-impregnated paper of products of L1210 and synaptosome incubations with [^{14}C]choline or [^{3}H]TEC. Ascending chromatography was carried out for 18 hr as given in Materials and Methods. The lower panel indicates lipid fractions for control incubations with synaptosomes, as given in Figs. 1 and 2. The middle panel indicates lipid fractions for control incubations with L1210 cells, as given in Figs. 4 and 5. The top panel indicates the mobility of standard lecithin in the system stained with rhodamine 6G.

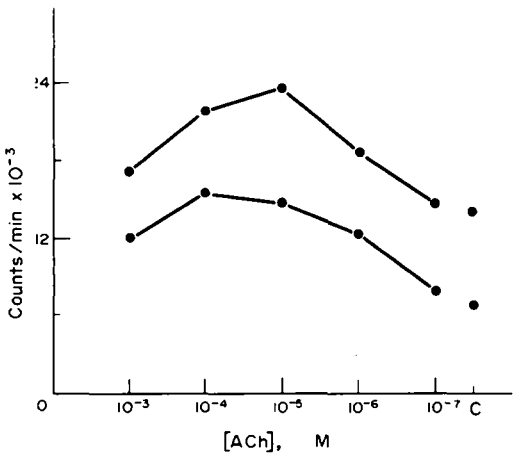

FIG. 7. Incorporation of [^{14}C]choline and [^{3}H]TEC into a lipid fraction of synaptosomes from guinea pig cerebral cortex in the presence of ACh and 10^{-5} M eserine. Experiments were performed as given in Materials and Methods. [^{14}C]choline (3·0 Ci/mole) was present as 0·5 μCi of [^{14}C]choline in a final volume of 0·13 ml. [^{3}H]TEC (3·44 Ci/m-mole) was present at 20 μl of a 0·43 mCi/ml solution of [^{3}H]TEC in a final volume of 0·21 ml. C refers to controls, in which no acetylcholine was present. Acetylcholine concentration is final concentration. Means from six independent observations. Data are for 1 mg of synaptosome protein. Eserine (10^{-5} M) was present in all controls and experimental assays. The top curve and control are for [^{14}C]choline incorporation, while the lower curve and control are for [^{3}H]TEC incorporation into lipid fraction.

of the control level, while at 10^{-5} M acetylcholine in the presence of 10^{-5} M eserine, the [^{14}C]choline incorporation was more than 170 per cent of the control level. Silicic acid-impregnated paper chromatography indicated that the increased synthesis was primarily in the phosphatidyl choline or phosphatidyl triethylcholine portion of the chromatogram. In the L1210 cells, ACh caused no increased synthesis of lipid choline or TEC.

The fact that choline is incorporated into lipid and phosphatidylcholine in the synaptosome may indicate that such choline represents a possible storage pool for ACh synthesis. Furthermore, in cases in which high levels of ACh are necessary for nervous transmission, the present results indicate that increased movement of choline into lipid in the synaptosomes could occur, perhaps as a storage mechanism; whether or not a similar mechanism occurs *in vivo* is not known. Finally, hydrolysis of ACh by AChE to form choline might also provide material for incorporation into this possible storage pool for utilization at a later time or merely provide precursor for membrane lipid synthesis. That this acceleration of choline and TEC lipid synthesis by ACh is a mechanism peculiar to nervous tissue is demonstrated by the fact that it did not occur in the L1210 cells.

TEC may be a useful tool for studying membrane biogenesis, since it inhibits choline incorporation into phosphatidylcholine in L1210 cells. It could be very interesting to determine whether the phosphatidyl triethylcholine can be correctly inserted into biological membranes, and whether such insertion would affect other lipid or protein or glycoprotein insertions, or indeed would inhibit membrane synthesis *in toto*. Although a true sample of phosphatidyl triethylcholine was not available for identification in the present study, such an identification would be essential for further membrane studies. It is likely that the compound being synthesized is phosphatidyl triethylcholine although absolute identification requires the analytic comparison with authentic phosphatidyl triethylcholine.

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