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ACCUMULATION AND METABOLISM OF CHOLINE AND HOMOCHOLINE BY MOUSE BRAIN SUBCELLULAR FRACTIONS

STEPHEN H. NELSON, CHRISTINA G. BENISHIN and PAUL T. CARROLL
Department of Pharmacology, University of Rhode Island, Kingston, RI 02881, U.S.A.

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Abstract—Minces of mouse forebrain were incubated in Krebs or a Krebs solution containing high K^+ (32.7 mM) and Li^+, instead of Na^+, for 30 min at 37°; subcellular fractions were prepared, and the levels of ACh in the S_3 and P_3 fractions were determined and compared for the two treatments. Incubation of minces in the Krebs solution with high K^+ and Li^+, instead of Na^+, depleted the ACh content of the P_3 fraction 70 per cent, without altering that of the S_3 fraction with respect to incubation of minces in normal Krebs. Subsequent incubation of the depleted minces in normal Krebs containing [^{14}C]choline (0.1 mM) and paraoxon (0.1 μM) refilled the depleted P_3 fraction with newly synthesized [^{14}C]ACh, and the ratio of [^{14}C]ACh to total ACh in this fraction (0.63) exceeded that of the S_3 (0.33). Incubation of depleted minces in normal Krebs solution containing the choline analog [^{14}C]homocholine (0.1 mM) and paraoxon (0.1 μM) also refilled the depleted P_3 fraction with newly synthesized [^{14}C]acetylhomocholine, and the ratio of [^{14}C]acetylhomocholine to ACh in this fraction (7.26) exceeded that of the S_3 (0.44). Refilling of the depleted P_3 fraction was due to an increase in the accumulation of precursor ([^{14}C]choline 84 per cent and [^{14}C]homocholine, 76 per cent) which occurred independently of the S_3. Incubation of depleted minces with either extracellular [^{14}C]ACh or [^{14}C]acetylhomocholine did not refill the depleted P_3 fraction with these compounds. These results suggest that ACh, lost from the crude vesicular fraction, can be replaced with newly synthesized ACh independently of the cytoplasm.

Acetylcholine (ACh) in brain tissue is stored in at least two subcellular pools within cholinergic nerve endings: the cytoplasm and the synaptic vesicles [1, 2]. The functional significance of this compartmentation to neurotransmission remains obscure. In the classical model of a cholinergic nerve ending, ACh is synthesized in the cytoplasm and then transferred to vesicles from where it is released [3, 4]. Unequivocal neurochemical evidence for this model is scarce and not all studies support it. In particular, acetylcholine transport into isolated vesicles above diffusional levels has not been shown [5, 6], and several recent studies report that the enzyme which synthesizes ACh, choline-*O*-acetyltransferase (EC 2.3.1.6, ChAT), not only exists in the cytoplasm [7, 8], but may also be associated with neuronal and/or vesicular membranes [9–15]. Thus, it is possible that transmitter synthesized and translocated into vesicles is that synthesized by ChAT associated with them, and not ACh synthesized in the neuronal cytosol.

The present experiments were designed to test this possibility by making use of the previously reported finding [16] that exposure of brain tissue to a Krebs medium containing elevated potassium and lithium, instead of sodium, caused depletion of fraction P_3 ACh, but not of S_3 ACh. If such tissue is subsequently exposed to [^{14}C]choline and allowed to replete its depleted P_3 ACh, then, by measuring the specific activity of S_3 and P_3 ACh, it should be possible to distinguish between cytoplasmic and vesicular synthesis of ACh. If the vesicular ACh is synthesized locally, its specific activity would be expected to exceed that of cytoplasmic ACh, and if vesicular ACh is derived from cytoplasmic ACh, it would not.

In the present experiments, the acetylation of homocholine and the subcellular distribution of acetylhomocholine were also measured, to determine whether they differ from choline. This analogue of choline is transported into rat brain synaptosomes and the cat superior cervical ganglion, and acetylated [17, 18]. It is released from the latter as acetylhomocholine during stimulation by a calcium-dependent process [18]. Homocholine differs from choline in that it is not acetylated by solubilized ChAT [18–22], but is acetylated by intact rat brain synaptosomes [18], possibly because homocholine is acetylated by ChAT associated with vesicular and/or neuronal membranes [22].

METHODS AND MATERIALS

Materials. Choline chloride [1,2^{14}C] (sp. act. 6.34 mCi/mmole), acetylcholine iodide [acetyl-1-^{14}C] (sp. act. 1.2 mCi/mmole), ATP [^{32}P] (sp. act. 17–35 Ci/mmole), toluene standard [^{14}C], acetyl CoA [acetyl-1-^{14}C] sp. act. adjusted to 2 mCi/mmole), and methyl iodide [^{14}C] (sp. act. 20.0 and 49.0 mCi/mmole) were obtained from the New England Nuclear Corp. (Boston, MA). Unlabeled methyl iodide and 3-dimethylamino-1-propanol and 3-heptanone were purchased from Aldrich Chemicals (Milwaukee, WI). Acetic anhydride was obtained from the Eastman Kodak Co. (Rochester, NY), acetyl CoA from CalBiochem (San Diego, CA), choline kinase (EC 2.7.1.32), AChE (EC 3.1.1.7) and para-

oxon from the Sigma Chemical Co. (St. Louis, MO), and sodium tetraphenylboron from the Fisher Scientific Co. (Medford, MA).

Preparation of homocholine iodide. Unlabeled methyl iodide (1.5 ml, 24.1 mmole) when added to 3-dimethylamino-1-propanol (3.5 ml, 29.6 mmole), immediately produced a white precipitate. The precipitate was washed (2 × 10 ml) with anhydrous ether and centrifuged at 3500 r.p.m. for 10 min at 4°. The supernatant fractions were decanted, and the product was dried *in vacuo* to afford 5.61 g (95%) of homocholine iodide, m.p. 196–198.5°; p.m.r. (DMSO-d_6) σ 4.75 (t,1,J = 5Hz, OH), 3.49 (M,4,–CH_2–CH_2–CH_2–), 3.14 [S,9,N(CH_3)$_3$] and 1.92 (P,2,–CH_2–CH_2–CH_2).

N-[*Me*^{14}C]Homocholine iodide was synthesized as described above except that 2 μl of [^{14}C]CH_3I were diluted with 20 μl of unlabeled CH_3I to a specific activity of 4.85 mCi/mmole. The labeled product was then dissolved in distilled water (pH 6.5) and stored at −20°. The specific activity was determined to be 2.30 mCi/mmole.

Preparation of acetylhomocholine iodide. Homocholine iodide (2.0 g; 8.1 mmole) was dissolved in 20 ml of acetic anhydride and refluxed for 4 hr while stirring under an atmosphere of N_2 (g), according to the method of Waltz *et al.* [23]. The excess anhydride was removed under reduced pressure to yield a brown precipitate. The solid was washed (2 × 10 ml) with anhydrous ethyl ether which removed a colored contaminant. The colorless product was recrystallized by dissolving it in a minimal amount of absolute ethanol and then adding ethyl ether to the cloud point. The supernatant fraction was decanted and the crystalline material was filtered and washed with anhydrous ether (5 × 2–3 ml). The product was dried *in vacuo* over P_2O_5 for 24 hr to provide 2.15 g (91.5%) of acetylhomocholine iodide: m.p. 96–97.5°; p.m.r. (DMSO-d_6) σ 4.12 (t,2,J = 6Hz,–CH_2–CH_2–O–), 3.51 (M,2,(CH_3)N–CH_2–CH_2–), 3.17 [S,9,N(CH_3)$_3$], 2.07 (S,3,O–CH_2–CH_3) and 2.07 (P,2,–CH_2–CH_2–CH_2–).

Anal. Calc. for $C_8H_{18}NO_2I$: C, 33.46; H, 6.32; N, 4.88. Found: C, 33.29; H, 6.16; N, 5.01.

N-[*Me*^{14}C]Acetylhomocholine iodide was synthesized in a similar manner, except that the specific activity of the undiluted methyl iodide was 49.0 mCi/mmole. The final recrystallized product, however, was dried by a stream of dry (H_2SO_4) nitrogen gas. The product was dissolved in distilled water (pH 6.5) and stored at −20°. The specific activity was determined to be 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 tetramethylsilane (TMS) as an internal standard. Descending paper chromatography with a solvent system of *n*-butanol–ethanol–1 N acetic acid–water (8:2:1:3) was used to ascertain homogeneity of [^{14}C]homocholine and [^{14}C]acetylhomocholine.

Choline-O-acetyltransferase activity in subcellular fractions. The extent of overlap between the S_3 and P_3 fractions was determined by measuring choline-*O*-acetyltransferase (EC 2.3.1.6) activity in the respective fractions by the procedure of McCaman and Hunt [24] as modified by Fonnum [25] and described by Spyker *et al.* [26]. Subcellular fractions were prepared as described below, and the choline-*O*-acetyltransferase, that was ionically bound to membranes (P_3) following hypo-osmotic rupture of the P_2 fraction and centrifugation, was removed using two 100 mM sodium phosphate buffer (pH 7.4) washes of the P_3 fraction. Approximately 84 per cent of the choline-*O*-acetyltransferase activity that was recovered from the P_2 fraction existed in the pooled wash and S_3 fractions, and approximately 15.6 per cent of the activity remained in the washed P_3 fraction.

Preparation of brain minces. Male (CD-1) albino mice were killed by cervical dislocation in a cold room (4°) where the brains (minus cerebellum, pons and medulla) were removed and sectioned through the median sagittal fissure. Each hemisphere was weighed and placed in several hundred milliliters (ice-cold) of the same ionic medium that was to be used for incubation. Following their removal, they were minced and maintained in a petri dish until the onset of incubation.

Tissue fractionation. The procedure of Gray and Whittaker [27] as modified by Collier *et al.* [28] and Salehmoghaddam and Collier [29] was used. Following incubation, minces were washed twice in 5 ml of 0.32 M ice-cold sucrose and then homogenized in 5 ml of 0.32 M sucrose at 840 r.p.m. using eight up and down strokes in a teflon-to-glass homogenizer. The homogenate was centrifuged (4°) at 1000 *g* for 10 min, and the pellet was discarded. The supernatant fraction (S_1) was centrifuged at 17,000 *g* for 15 min to prepare the P_2 fraction (nerve ending fraction). The S_2 fraction was discarded. The P_2 fraction was resuspended in 2 ml of ice-cold glass distilled H_2O (pH 4.0) containing paraoxon (0.1 μM) and homogenized at 500 r.p.m. using eight up and down strokes. This homogenate was centrifuged at 100,000 *g* for 90 min to yield a pellet (P_3) and a supernatant fraction (S_3). ACh in the P_3 fraction includes ACh in synaptic vesicles as well as any ACh included in other organelles. ACh in the S_3 fraction includes ACh in the cytoplasm of cholinergic nerve terminals as well as any ACh that has leaked from occluded stores during tissue preparation. The P_3 pellet was surface-washed twice with 8 ml of glass-distilled water to reduce overlap between it and the S_3 fraction.

Endogenous ACh assay. To determine the amount of ACh present in the S_3 fraction, a 10 μl aliquot was dried and then assayed by a modified method of Goldberg and McCaman [30]. In this procedure, the extraction of ACh by tetraphenylboron/3-heptanone (TPB/3-heptanone) was eliminated. Determination of ACh in the S_3 fraction was linear when 1–20 μl aliquots of the 2000 μl sample were used. Omission of AChE during the second stage of the Goldberg and McCaman procedure [30] resulted in values equivalent to blank, suggesting that only ACh was being determined.

To compare this procedure with that using the TPB/3-heptanone extraction, a 100 μl aliquot of the S_3 fraction was mixed with 300 μl TPB/3-heptanone (5 mg/ml). After thorough mixing and a 20-min centrifugation at 1000 *g*, a 200 μl aliquot of the

organic layer was removed, mixed with 100 μl of 1 N HCl, and centrifuged. The organic layer was then discarded, and a 25 μl aliquot of the aqueous layer was transferred to a new tube and dried. Determination of ACh in the cytoplasmic fractions of minces prepared from four brains that had been incubated with [^{14}C]choline (0.1 mM) and paraoxon (0.1 μM) in Krebs bicarbonate buffer produced values (17.6 ± 4.5 nmoles/g) similar to those obtained for the same samples using the modified procedure (18.9 ± 2.9 nmoles/g). In tissue samples incubated with either [^{14}C]homocholine or [^{14}C]acetylhomocholine, the tetraphenylboron extraction was employed. Homocholine did not interfere with the assay of ACh.

To extract the ACh present in the P_3 fraction, the 100,000 *g* pellets were transferred to ground glass homogenizers (Duall 20) containing 500 μl of formic acid–acetone (FA/A, 15:85, v/v) and allowed to stand for at least 20 min in the cold to permit complete extraction of ACh [31]. The samples were then centrifuged for 20 min at 1000 *g*, and a 10 μl aliquot of the supernatant fraction was transferred to a tube and dried. Determination of ACh was done directly on the dried samples and was linear when 1–10 μl aliquots of the 500 μl sample were used. Omission of AChE during the second stage of the Goldberg and McCaman assay yielded values equivalent to blank. To compare the modified procedure with that using TPB/3-heptanone extraction, a 50 μl aliquot of the FA/A was dried in a tube. Then, 50 μl of a 10 mM sodium phosphate buffer (pH 6.6) were added to the dried tube and subsequently mixed with 200 μl of TPB/3-heptanone (5 mg/ml). After a brief centrifugation, 150 μl of the organic phase were transferred to a tube containing 75 μl of 1 N HCl. After thorough mixing and a 10-min centrifugation at 1000 *g*, the organic layer was removed and a 25 μl aliquot of the aqueous layer was transferred to a new tube and dried. Determination of ACh in the P_3 fraction of six brain samples incubated with [^{14}C]choline (0.1 mM) in Krebs for 4 min yielded ACh values (8.8 ± 1.4 nmoles/g) similar to those (9.3 ± 1.4 nmoles/g, N = 8) using the modified procedure. In tissue samples incubated with either [^{14}C]homocholine or [^{14}C]acetylhomocholine, the tetraphenylboron extraction was employed. When the TPB/3-heptanone extraction is not used, the results are reliable for amounts of tissue below 2.5 mg, whereas greater amounts of tissue result in erroneously high values, as described in a recent report [32]. In each assay, ACh standards were assayed simultaneously with test samples and were used to quantitate the amount of ACh in the test sample. The addition of 100 pmole ACh standards to tissue samples prepared from minces that had been incubated in Krebs or lithium–Krebs solution indicated that neither tissue nor lithium interfered with the ACh determination.

In one group of samples incubated in normal Krebs or lithium–Krebs solution, paraoxon was omitted during the tissue preparation. Its absence resulted in a reduction of S_3 ACh content to non-detectable levels, whereas the ACh content of the P_3 fraction did not differ significantly from those samples in which paraoxon was included during the tissue preparation (data not shown).

Determination of labeled ACh and acetylhomocholine. To determine the amounts of labeled ACh and acetylhomocholine present in the S_3 and P_3 fractions, a 10 μl aliquot of the S_3 fraction and a 10 μl aliquot of the P_3 fraction (after homogenization in 500 μl FA/A) were dried, and the labeled substrates were converted to labeled phosphoryl substrates using choline kinase (EC 2.7.1.32), and were then separated from their respective acetyl derivatives using TPB/3-heptanone (75 mg/ml) as described previously [33, 34]. This procedure phosphorylates both [^{14}C]choline and [^{14}C]homocholine standards linearly from 25 to 200 pmoles, and virtually none of the phosphorylated substrates are extracted into the TPB/3-heptanone layer. The extraction of unphosphorylated [^{14}C]choline, [^{14}C]homocholine, [^{14}C]ACh and [^{14}C]acetylhomocholine from the aqueous phase into the TPB/3-heptanone phase is complete. To determine the amounts of non-extractable metabolites of [^{14}C]choline or [^{14}C]homocholine formed during incubation of brain tissue with these compounds, the same procedure as described above was employed except that choline kinase and ATP were omitted during the incubation of tissue samples. The amount of radioactivity present in the aqueous phase after TPB/3-heptanone addition was then determined.

Incubation procedures. Initially, brain minces were incubated in 10 ml of Krebs solution (mM concentration: NaCl, 117; KCl, 3.5; KH_2PO_4, 1.2; $CaCl_2$, 2.5; $MgSO_4 \cdot 7H_2O$, 1.2; $NaHCO_3$, 28.0; and glucose, 11.0) for two 15-min periods (fresh solution was added after the initial 15 min) while contralateral minces were incubated similarly in a medium in which LiCl replaced NaCl (mM concentration: LiCl, 117; KCl, 3.5; KH_2PO_4, 1.2; $CaCl_2$, 2.5; $MgSO_4 \cdot 7H_2O$, 1.2; $NaHCO_3$, 28.0; and glucose, 11.0). Following incubation, minces were washed twice with 5 ml of 0.32 M sucrose, and the amounts of ACh present in the subcellular fractions were determined. Since lithium has been reported to impair tissue respiration [35], potassium bicarbonate was substituted for sodium bicarbonate in all experiments, with the exception of the first set (Table 1),

Table 1. Differential effect of lithium-containing media on the levels of ACh in the S_3 and P_3 fractions*

Treatment	ACh (nmoles/g)	
	Subcellular fractions	
	S_3	P_3
(1) Krebs	10.8 ± 1.4 (6)	7.2 ± 1.4 (4)
(2) Lithium	9.5 ± 1.6 (6)	2.3 ± 0.5† (4)
(3) Krebs	11.8 ± 1.6 (12)	8.7 ± 0.9 (12)
(4) Lithium (32.7 mM K^+)	12.8 ± 2.5 (12)	2.5 ± 0.4† (12)

* Minces of mouse forebrain were incubated for 30 min in one of the ionic media and subcellular fractions were prepared. The ionic medium used in treatment 2 contained 117 mM Li^+ and 28 mM Na^+. The ionic medium used in treatment 4 contained 117 mM Li^+, 32.7 mM K^+ and no Na^+. Each value represents the mean ± S.E.M. The numbers in parentheses = the number of experimental animals used.

† Results differ significantly from Krebs treatments 1 or 3 at $P < 0.05$ (analysis of variance: one-way classification).

Table 2. Ability of a P_3 fraction with reduced ACh content to refill with newly synthesized ACh independently of the S_3 fraction*

Incubation 1	Incubation 2	S_3			P_3		
		[^{14}C]ACh	Total ACh	[^{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	0.35 ± 0.05	2.6 ± 0.2	10.5 ± 2.4	0.35 ± 0.07
Lithium (32.7 mM K^+)	Krebs + [^{14}C]choline	4.9 ± 0.7	17.3 ± 1.3	0.33 ± 0.05	5.1 ± 0.4†	9.6 ± 1.7	0.63 ± 0.09†‡

* Minces were preincubated for 30 min in either Krebs or a lithium solution (32.7 mM K^+, and Li^+ instead of Na^+) and then were incubated for another 30 min in Krebs solution with [^{14}C]choline (0.1 mM, sp. act. 13.95 d.p.m./pmole) and paraoxon (0.1 μM). Results are expressed as mean ± S.E.M. for twelve brains in nmoles/g wet weight. Ratios were determined by comparing the amount of [^{14}C]ACh to total ACh for subcellular fractions prepared from each individual brain.

† Results differ significantly from Krebs at $P < 0.05$ (analysis of variance: one-way classification).

‡ P_3 ratio differs from S_3 ratio at $P < 0.05$ (analysis of variance: one-way classification).

Table 3. Ability of a P_3 fraction with reduced ACh content to refill with newly synthesized acetylhomocholine independently of the S_3 fraction*

Incubation 1	Incubation 2	S_3			P_3		
		[^{14}C]Ah	ACh	[^{14}C]Ah/ACh	[^{14}C]Ah	ACh	[^{14}C]Ah/ACh
Krebs	Krebs + [^{14}C]homocholine	3.4 ± 0.2	17.4 ± 2.0	0.26 ± 0.06	2.1 ± 0.2	7.2 ± 1.5	0.46 ± 0.10
Lithium (32.7 mM K^+)	Krebs + [^{14}C]homocholine	5.3 ± 0.3†	13.2 ± 1.2	0.44 ± 0.06†	4.7 ± 0.3†	2.6 ± 0.3†	7.26 ± 3.2†‡

* Minces were preincubated for 30 min in either Krebs or a lithium solution (32.7 mM K^+, and Li^+ instead of Na^+) and then incubated another 30 min in Krebs solution with [^{14}C]homocholine (0.1 mM, sp. act. 5.1 d.p.m./pmole) and paraoxon (0.1 μM). Results are expressed as mean ± S.E.M. for twelve brains in nmoles/g wet weight. Ratios were determined by comparing the amount of [^{14}C]Ah ([^{14}C]acetylhomocholine) to ACh for subcellular fractions prepared from each individual brain.

† Results differ significantly from Krebs at $P < 0.05$ (analysis of variance: one-way classification).

‡ P_3 ratio differs from S_3 ratio at $P < 0.05$ (analysis of variance: one-way classification).

Table 4. Facilitated accumulation of cholinergic precursors in a P_3 fraction with reduced ACh content*

Incubation 1	Incubation 2	S_3 (d.p.m./mg)	P_3 (d.p.m./mg)
Krebs	Krebs + [^{14}C]choline	346.3 ± 39.2 (12)	88.1 ± 8.5 (12)
Lithium (32.7 mM K^+)	Krebs + [^{14}C]choline	377.0 ± 17.2 (12)	163.0 ± 8.3† (12)
Krebs	Krebs + [^{14}C]homocholine	87.5 ± 2.8 (16)	34.6 ± 1.3 (16)
Lithium (32.7 mM K^+)	Krebs + [^{14}C]homocholine	96.9 ± 3.8 (16)	61.6 ± 3.5† (16)

* Minces were preincubated for 30 min in either Krebs or a lithium solution (32.7 mM K^+, and Li^+ instead of Na^+) and then incubated another 30 min in Krebs solution with [^{14}C]choline (0.1 mM, sp. act. 13.95 d.p.m./pmole) and paraoxon (0.1 μM) or [^{14}C]homocholine (0.1 mM, sp. act. 5.1 d.p.m./pmole) and paraoxon (0.1 μM). Results are expressed as mean ± S.E.M. The numbers in parentheses = the number of experimental animals.

† Results differ significantly from Krebs at $P < 0.05$ (analysis of variance: one-way classification).

to maintain tissue respiration in the presence of lithium. This medium contained K^+ (32.7 mM) and LiCl instead of NaCl. The pH of the ionic media used for incubation remained at pH 7.4 ± 0.1 throughout the tissue incubation periods.

In other experiments (Tables 2–5), forebrain minces were preincubated in Krebs or lithium–Krebs solution (32.7 mM K^+ and Li^+ instead of Na^+) for a total of 30 min, washed twice with ice-cold Krebs [the second wash contained paraoxon (0.1 μM)], and then incubated for 30 min in Krebs containing paraoxon (0.1 μM) and one of the following compounds: [^{14}C]choline (0.1 mM), [^{14}C]homocholine (0.1 mM), [^{14}C]ACh (0.1 mM) or [^{14}C]acetylhomocholine (0.1 mM). Following the second incubation, the minces were washed twice with 5 ml of ice-cold 0.32 M sucrose, and the ratio of labeled ACh or labeled acetylhomocholine to ACh was determined in the subcellular fractions. It should be noted that the nmoles of [^{14}C]ACh formed in the tissue from [^{14}C]choline represent a minimum amount, since production of choline by the tissue during incubation can dilute added [^{14}C]choline [36].

The concentrations of [^{14}C]choline (0.1 mM) and [^{14}C]homocholine (0.1 mM) used in the present experiments are of sufficient magnitude to support both high and low affinity choline transport systems in minces of mouse brain [33].

In those experiments in which minces were incubated with [^{14}C]homocholine, and the ratios of [^{14}C]acetylhomocholine to ACh were determined for the S_3 and P_3 fractions for individual brains, the amounts of ACh present in the S_3 and P_3 fractions were estimated by subtracting the amount of [^{14}C]acetylhomocholine (determined as described previously) from the amount of total acetylated product ([^{14}C]acetylhomocholine and ACh) measured by the procedure of Goldberg and McCaman [30].

ACh release. Minces of mouse forebrain were prepared as described before except that the washing solution contained paraoxon (0.1 μM). Minces were then incubated for 30 min at 37° under 95% O_2–5% CO_2 at 90 cycles/min in 3 ml of one of the following solutions: Krebs, lithium–Krebs solution (replacement of NaCl with LiCl), high K^+ (32.7 mM) Krebs or high K^+ (32.7 mM) Krebs with Li^+ instead of Na^+. At the end of the incubation period, the samples were chilled and centrifuged, and an aliquot of the supernatant fraction was used for the determination of ACh. The supernatant fraction (100 μl) was mixed with 300 μl of TPB/3-heptanone (5 mg/ml) and centrifuged. Then 200 μl of the organic phase were transferred to a tube containing 100 μl of 1 N HCl. After thorough mixing and centrifugation, the organic layer was discarded and a 30 μl aliquot of the 1 N HCl solution was transferred to a new tube and dried. The ACh contents of these samples were determined by the method of Goldberg and McCaman [30]. Standards ranging from 200 to 2000 pmoles were added to 100 μl aliquots of each of the ionic media.

RESULTS

Selective reduction of P_3 ACh. We reported previously that incubation of forebrain minces in a medium containing high K^+ (32.7 mM) and Li^+ instead of Na^+ selectively reduced P_3 ACh approximately 70 per cent without altering the S_3 ACh with respect to incubation of minces in a normal Krebs medium [16]. Results shown in Table 1 indicate that incubation of forebrain minces in a medium containing low K^+ (4.7 mM) and Li^+ instead of Na^+ also selectively reduced P_3 ACh approximately 70 per cent without altering S_3 ACh.

The amounts of ACh released from forebrain minces incubated either in a medium containing high K^+ (32.7 mM) and Li^+ instead of Na^+ or in a medium containing low K^+ (4.7 mM) and Li^+ instead of Na^+ were similar (29.9 ± 1.8 nmoles/g/30 min, N = 8, and 33.7 ± 4.8 nmoles/g/30 min, N = 12, respectively). These amounts are significantly lower than the amounts of ACh released from forebrain minces incubated in either a Krebs medium (54.4 ± 6.1 nmoles/g/30 min, N = 12) or a high K^+ (32.7 mM) Krebs medium (132.0 ± 10.8 nmoles/g/30 min, N = 8).

Can a P_3 fraction depleted of its ACh content be refilled with newly synthesized ACh independently of the S_3 fraction? The results presented in Table 2 indicate that, when forebrain minces were preincubated in a medium containing high K^+ and Li^+

instead of Na^+ in order to deplete the P_3 ACh and were then subsequently incubated in a Krebs medium containing [^{14}C]choline in order to replete it, a higher ratio of labeled to total ACh was attained in the repleted P_3 fraction (0.63) than in the non-depleted P_3 fraction of minces preincubated in Krebs (0.35). The ratio of labeled to total ACh in the repleted P_3 fraction (0.63) exceeded that in the S_3 fraction (0.33). Additionally, the total amount of ACh in the repleted P_3 fraction (9.6 ± 1.7 nmoles/g) was similar to the total amount of ACh present in the non-depleted P_3 fraction (10.5 ± 2.4 nmoles/g).

Greater amounts of both unacetylated [^{14}C]choline and [^{14}C]ACh were found in the repleted P_3 fraction (5.9 ± 0.4 and 5.1 ± 0.4 nmoles/g, respectively) than in the non-depleted P_3 fraction (3.7 ± 0.4 and 2.6 ± 0.2 nmoles/g, respectively). Conversely, the amounts of [^{14}C]choline and [^{14}C]ACh found in the S_3 fraction of minces preincubated in high K^+ and Li^+ instead of Na^+ (18.7 ± 0.9 and 4.9 ± 0.7 nmoles/g, respectively) did not exceed the amounts found in the S_3 fraction of minces preincubated in Krebs (17.4 ± 1.4 and 4.2 ± 0.6 nmoles/g, respectively).

Can a P_3 fraction depleted of its ACh content be refilled with newly synthesized acetylhomocholine independently of the S_3 fraction? The results shown in Table 2 suggest that the P_3 fraction depleted of its ACh content was refilled with newly synthesized ACh independently of the S_3 fraction. To test whether a P_3 fraction depleted of its ACh content can also be refilled with newly synthesized acetylhomocholine independently of the S_3 fraction, forebrain minces were preincubated in a medium containing high K^+ and Li^+ instead of Na^+ and subsequently incubated in a Krebs medium containing [^{14}C]homocholine. The results shown in Table 3 indicate that a substantially higher ratio of labeled acetylhomocholine to ACh was attained in the repleted P_3 fraction (7.26) than was attained in the non-depleted P_3 fraction (0.46). The ratio of labeled acetylhomocholine to ACh in the repleted P_3 fraction (7.26) also greatly exceeded that of the S_3 fraction (0.44). The amount of ACh in the repleted P_3 fraction (2.6 ± 0.3 nmoles/g) was similar to the amount of ACh in the depleted P_3 fraction (2.5 ± 0.4 nmoles/g, see Table 1). The total amount of acetylated product in the repleted P_3 fraction (4.7 ± 0.3 nmoles/g of [^{14}C]acetylhomocholine plus 2.6 ± 0.3 nmoles/g of ACh) was not significantly below the total amount of acetylated product in the non-depleted P_3 fraction (2.1 ± 0.2 nmoles/g of [^{14}C]acetylhomocholine plus 7.2 ± 1.5 nmoles/g of ACh).

Greater amounts of both unacetylated [^{14}C]homocholine and [^{14}C]acetylhomocholine existed in the repleted P_3 fraction (7.2 ± 0.5 and 4.7 ± 0.3 nmoles/g, respectively) than in the non-depleted P_3 fraction (4.1 ± 0.2 and 2.1 ± 0.2 nmoles/g, respectively). Conversely, only the amount of [^{14}C]acetylhomocholine present in the S_3 fraction of minces preincubated in high K^+ and Li^+ instead of Na^+ (5.3 ± 0.3 nmoles/g) exceeded the amount present in the S_3 fraction of minces preincubated in Krebs (3.4 ± 0.2 nmoles/g), whereas the amounts of unacetylated homocholine present in the S_3 fraction of the two sets of pretreated minces were similar (11.5 ± 0.6 vs 11.7 ± 0.7 nmoles/g). Preincubation of minces in the medium containing high K^+ and Li^+ instead of Na^+ resulted in a higher ratio of labeled acetylhomocholine to total ACh in the S_3 fraction (0.44) as compared with minces preincubated in Krebs (0.26).

Does selective reduction of P_3 ACh content facilitate accumulation of cholinergic precursors in this fraction independently of the S_3? Some studies suggest that ACh levels may regulate the transport of extracellular choline into cholinergic nerve terminals [37, 38]. Another study indicates that pre-ganglionic stimulation augments accumulation of the precursors choline and homocholine in the superior cervical ganglion [18]. The results presented in Table 4 show that preincubation of minces in a medium containing high K^+ and Li^+ instead of Na^+ in order to selectively deplete P_3 ACh content augmented the accumulation of both extracellular [^{14}C]choline (84 per cent) and [^{14}C]homocholine (76 per cent) in the P_3 fraction when minces were subsequently incubated in a Krebs medium containing these compounds. Conversely, the pretreatment did not facilitate the accumulation of either precursor in the S_3 fraction. This pretreatment also did not enhance the accumulation of [^{14}C]choline by the P_1 or S_2 fractions (N = 4, data not shown).

Can a depleted P_3 pool of ACh be refilled with preformed extracellular ACh or acetylhomocholine? The results shown in Tables 2 and 3 indicate that a depleted P_3 pool of ACh can be refilled with either ACh newly synthesized from extracellular choline or acetylhomocholine newly synthesized from extra-

Table 5. Inability of a P_3 fraction with reduced ACh content to refill with either extracellular [^{14}C]ACh or [^{14}C]acetylhomocholine*

Incubation 1	Incubation 2	[^{14}C]ACh or [^{14}C]acetylhomocholine/total ACh S_3	P_3
Krebs	Krebs + [^{14}C]ACh	0.46 ± 0.04 (12)	0.14 ± 0.03 (12)
Lithium (32.7 mM K^+)	Krebs + [^{14}C]ACh	0.39 ± 0.03 (12)	0.20 ± 0.05 (12)
Krebs	Krebs + [^{14}C]acetylhomocholine	0.16 ± 0.04 (8)	0.09 ± 0.02 (8)
Lithium (32.7 mM K^+)	Krebs + [^{14}C]acetylhomocholine	0.22 ± 0.04 (8)	0.09 ± 0.01 (8)

* Minces were preincubated in Krebs or lithium solution (32.7 mM K^+, and Li^+ instead of Na^+) and then incubated another 30 min in Krebs solution with either [^{14}C]ACh (0.1 mM, sp. act. 2.65 d.p.m./pmole) and paraoxon (0.1 μM) or [^{14}C]acetylhomocholine (0.1 mM, sp. act. 11.9 d.p.m./pmole) and paraoxon (0.1 μM). Results are expressed as mean ± S.E.M. The numbers in parentheses = the number of experimental animals.

cellular homocholine. To test whether the preformed products, ACh and acetylhomocholine, can also refill a depleted P_3 pool of ACh, forebrain minces were preincubated in the medium containing high K^+ and Li^+ instead of Na^+ to deplete vesicle-bound ACh and were then subsequently incubated in Krebs with either [^{14}C]ACh or [^{14}C]acetylhomocholine. These results (Table 5) indicate that similar ratios of labeled to total ACh are attained in the initially depleted P_3 fraction (0.20 ± 0.05) and in the non-depleted P_3 fraction (0.14 ± 0.03). The ratios of acetylhomocholine to total ACh attained in the initially depleted P_3 fraction (0.09) and in the non-depleted P_3 fraction (0.09) were the same. In these experiments, the ratios of labeled ACh or labeled acetylhomocholine to total ACh attained in the P_3 fraction initially depleted did not exceed those ratios present in the cytoplasm.

DISCUSSION

The aim of the present experiments was to initially deplete the ACh content of a P_3 fraction of mouse forebrain independently of the S_3 fraction by first incubating brain minces in a Krebs medium containing high K^+ and Li^+ instead of Na^+ and then to subsequently incubate the minces in a Krebs medium containing either [^{14}C]choline or [^{14}C]homocholine to determine whether the depleted P_3 fraction could be replenished with either newly synthesized [^{14}C]ACh or [^{14}C]acetylhomocholine independently of the S_3 fraction.

Results obtained in the present study confirm previously reported results that the P_3 fraction of ACh can be depleted independently of the S_3 fraction by incubating brain minces in a Krebs medium containing Li^+ instead of Na^+ [16]. In addition, they indicate that the presence of elevated potassium in the lithium-containing Krebs is not essential to this selective depletion. A higher ratio of labeled to total ACh was attained in the P_3 fraction of minces preincubated in a Krebs medium containing high K^+ and Li^+ instead of Na^+ and subsequently incubated in a Krebs medium containing [^{14}C]choline than was attained in the S_3 fraction; thus, the ACh, synthesized to replace that lost from the P_3 fraction, appears to occur in close association with this fraction rather than by exchange with the S_3 fraction. A similar result was obtained when the choline analog homocholine was used in this model. The results obtained in this study, however, do not support or refute the possibility that homocholine is exclusively acetylated by choline-*O*-acetyltransferase closely associated with vesicles and not with the cytoplasm. Additionally, they do not support the study indicating that acetylhomocholine formation preferentially occurs in non-vesicle stores of transmitter [39], since the results in the present study indicate that the ratio of S_3 to P_3 [^{14}C]acetylhomocholine is similar to that of [^{14}C]ACh, rather than greater.

Forebrain minces depleted of P_3 ACh independently of S_3 ACh accumulated more extracellular [^{14}C]choline (84 per cent) and more [^{14}C]homocholine (76 per cent) than did non-depleted minces; enhanced accumulation of precursor did not occur in the S_3 fraction, suggesting that a P_3 fraction depleted of its ACh content may acquire extracellular precursor directly. Furthermore, the enhanced accumulation of the two precursors, [^{14}C]choline and [^{14}C]homocholine, resulted in higher ratios of labeled to total ACh and of labeled acetylhomocholine to ACh in the repleted P_3 fraction (0.63 and 7.26, respectively) than in the non-depleted P_3 fraction (0.35 and 0.46, respectively), indicating that the depleted P_3 fraction may refill with newly synthesized transmitter preferentially over preformed ACh. Additionally, the total amount of acetylated product present in the repleted P_3 fraction of minces postincubated with [^{14}C]homocholine (2.6 ± 0.3 nmoles/g of ACh plus 4.7 ± 0.3 nmoles/g of [^{14}C]acetylhomocholine) only exceeds the amount of transmitter present in a depleted P_3 fraction (2.5 ± 0.4 nmoles/g of ACh) by the amount of newly synthesized acetylhomocholine present there.

The ratios of labeled to total ACh and of labeled acetylhomocholine to ACh in the P_3 fraction of forebrain minces initially depleted and subsequently incubated in Krebs with either [^{14}C]ACh or [^{14}C]acetylhomocholine (0.20 ± 0.05 and 0.09 ± 0.01, respectively) were not higher than those in the P_3 fraction of non-depleted minces (0.14 ± 0.03 and 0.09 ± 0.02, respectively). The amounts of preformed products acquired by a depleted P_3 fraction (1.7 and 1.2 nmoles/g of [^{14}C]ACh and [^{14}C]acetylhomocholine, respectively) also did not replete the depleted P_3 fraction, whereas the precursors, [^{14}C]choline and [^{14}C]homocholine, did refill a depleted P_3 fraction with acetylated products (5.1 and 4.7 nmoles/g, respectively). Accumulation of extracellular ACh by brain tissue, unlike that of extracellular choline, is not believed to be specific for cholinergic nerve terminals [40, 41] and thus the distributions of ACh and acetylhomocholine following incubation of minces with these compounds cannot meaningfully be compared between the S_3 and P_3 fractions.

Greater amounts of both unacetylated precursor and acetylated products are found in the repleted P_3 than in the non-depleted P_3 fraction, a result which is somewhat similar to that obtained in the superior cervical ganglion in which pre-ganglionic stimulation not only increases the amount of acetylhomocholine but also of unacetylated homocholine as compared with the unstimulated control ganglion [18]. These authors suggested that acetylation might be rate limiting. In the present experiments, it cannot be ascertained whether the increased amounts of [^{14}C]choline and [^{14}C]homocholine associated with the repleted P_3 fraction were available for acetylation or were stored in the same releasable pool as the products, making the results difficult to interpret.

Depletion of P_3 ACh did not significantly augment accumulation of either [^{14}C]choline or [^{14}C]homocholine by the S_3 fraction of forebrain minces, although an increased amount of [^{14}C]acetylhomocholine, but not of [^{14}C]ACh, was found in the S_3 fraction. The reason for this difference is unclear.

Forebrain minces incubated in the two lithium-containing media not only released similar amounts of ACh (29.9 ± 1.8 vs 33.7 ± 4.8 nmoles/g/30 min)

but also exhibited similar magnitudes of selective decrease in the ACh content of the P_3 fraction (70 per cent) suggesting that the presence of high K^+ in the lithium-containing medium did not disrupt ACh metabolism. Incubation of brain tissue in a high K^+ Krebs medium also lowers the ACh content of the P_3 fraction [29] but, as we have shown, results in a substantially greater release of ACh (132 ± 10.8 nmoles/g/30 min) than does incubation of brain tissue in a high K^+ Krebs medium with Li^+ instead of Na^+ (29.9 ± 1.8 nmoles/g/30 min). This difference in ACh release for the two media may be explained in the following way. Both Li^+ [42] and high K^+ are believed to act as depolarizing agents and, thus, brain tissue incubated in Li^+ Krebs, Li^+ high K^+ Krebs or high K^+ Krebs may release similar amounts of preformed ACh from the P_3 fraction and thereby lower its ACh content. This lowering of P_3 ACh during incubation in high K^+ Krebs may allow more extracellular choline to enter cholinergic nerve endings and be released as newly synthesized ACh into the medium. Some investigators report that a reduction in tissue level of ACh facilitates extracellular choline transport [37, 38] and that extracellular choline can be utilized to support the Ca^{2+}-dependent, K^+-induced release of newly synthesized ACh [33].

When brain tissue is incubated in either a Li^+ low K^+ or a Li^+ high K^+ medium, similar amounts of preformed ACh may be released into the respective media from the P_3 fraction, but the presence of Li^+ will block the transport of extracellular choline into the tissue [43, 44] and thereby reduce the K^+-induced release of newly synthesized ACh released during a 30 min incubation while supporting choline-*O*-acetyltransferase activity [45].

Another study using somewhat different experimental conditions reported that substitution of Li^+ for Na^+ in a high K^+ medium significantly reduced the amount of newly synthesized ACh released from brain tissue [46]. In our experiments the release of ACh from forebrain minces incubated in either a Li^+ low K^+ medium or a Li^+ high K^+ medium (approximately 30 nmoles/g/30 min) exceeded the amount of ACh depleted from the P_3 fraction by these incubation conditions (approximately 5–6 nmoles/g); some of the ACh being released must, therefore, originate from continued turnover of some other ACh store.

Some of the reduction of the P_3 ACh content caused by the two Li^+-containing media may be due to the absence of Na^+ in these media rather than to the presence of Li^+. Other investigators have reported that incubation of brain tissue in a Na^+-free medium depletes brain ACh content [47] and that subsequent incubation of brain tissue in normal Krebs restores it [28]. Additionally, choline transport is Na^+-dependent [43, 44] and thus the lack of Na^+ in the Li^+-containing media may deplete P_3 ACh.

In summary, these results suggest that the extracellular precursors, choline and homocholine, may be directly accumulated by a crude vesicular fraction of mouse forebrain, independently of the cytoplasm, and may be utilized to replace lost ACh. Additionally, they suggest that the extracellular products, ACh and acetylhomocholine, may not be capable of replacing ACh lost from a crude vesicular fraction.

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