Reprinted from Nature, Vol. 290, No. 5805, pp. 417-418, April 2 1981

Choline biosynthesis by a preparation enriched in synaptosomes from rat brain

Jan K. Blusztajn & Richard J. Wurtman

Laboratory of Neuroendocrine Regulation, Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, USA

It is widely held that brain cells are unable to synthesize choline *de novo*, and that the only source of this compound for brain acetylcholine or membrane biosynthesis is the choline or choline-containing phospholipids taken up from the circulation[1]. This notion has been difficult to reconcile with observations[2-4] that there is a net efflux of choline from the brain. Recently we[5] and others[6,7] have demonstrated that various preparations of mammalian brain contain enzymes, the phosphatidylethanolamine *N*-methyltransferases (PeMT), which catalyse the synthesis of phosphatidylcholine (PC), using *S*-adenosylmethionine (SAM) as a methyl donor for the stepwise methylation of phosphatidylethanolamine (PE). The highest specific activity of PeMT was present in synaptosomal preparation[5,6]. We now report that rat brain synaptosomal preparations can also metabolize the PC generated by PeMT to liberate free choline.

Male Sprague–Dawley rats (180–220 g, Charles River) were housed under light (Vita-Lite, Duro-Test) for 12 h daily and had free access to water and food (Charles River Rat Chow). Animals were decapitated, their brains quickly dissected and synaptosomal preparations of whole brains (excluding cerebellum) were obtained as described previously[5]; their identity was confirmed by electron microscopy. Synaptosomal preparations were incubated in Krebs–Ringer bicarbonate buffer (*p*H 7.4) at 37 °C. Heat-treated synaptosomes (100 °C, 20 s) were used to generate blank values. Phosphatidyl-*N,N*-dimethylethanolamine (P2) (Gibco) was used as PeMT substrate, and [*Me*-^{3}H]*S*-adenosylmethionine (10 mCi μmol^{-1}, NEN) as the methyl donor. Enzymatic reactions were stopped by addition of the appropriate extraction solution. Phospholipids were extracted and purified as described before[5], and the ^{3}H-methyl present in newly formed ^{3}H-PC was quantified by liquid scintillation spectrophotometry. The choline, including [*Me*-^{3}H]choline, was extracted from the incubation media with 0.5% tetraphenylboron (Aldrich) in heptanone (Aldrich), and back-extracted from the heptanone phase into 0.4 M hydrochloric acid. The aqueous layer was then dried under vacuum, and the dry residue redissolved in ethanol and subjected to paper electrophoresis[8] and TLC. As described below, both procedures were needed to reduce background radioactivity. The radioactivity in segments of the chromatograms was counted by liquid scintillation spectrophotometry. The overall recovery of choline was calculated by adding ^{14}C-choline as an internal standard to synaptosomal incubations. The identity of the ^{3}H-choline formed enzymatically was established by subjecting the water-soluble products of the methylation reactions, prepurified by TLC, to phosphorylation by choline kinase and purifying the products by paper electrophoresis. Total concentrations of choline and PC in the incubations were assessed by radioenzymatic assay of choline[9], using choline kinase and [γ-^{32}P]ATP; the PC was purified by TLC and acid hydrolysed to choline before assay.

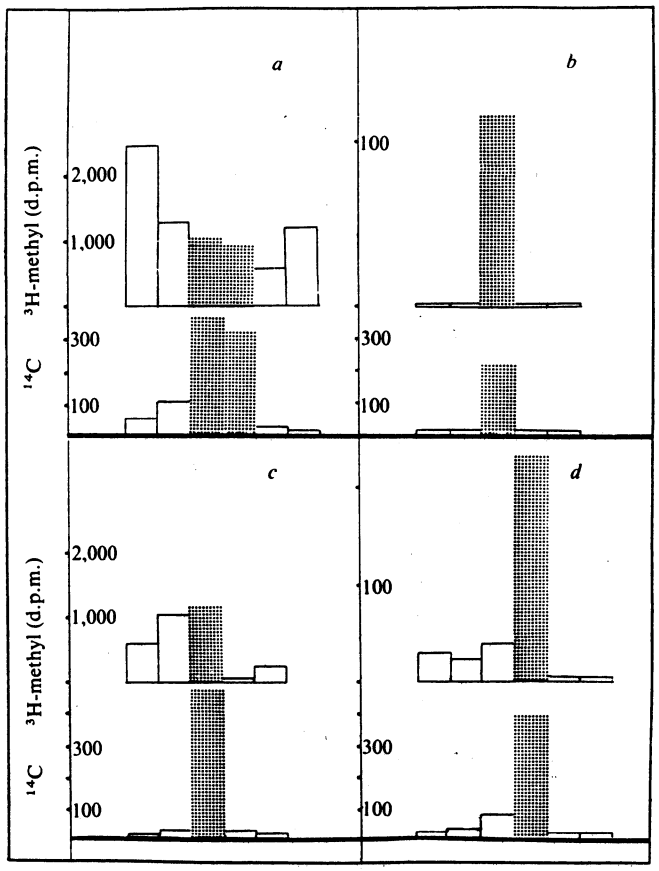

Fig. 1 Purification of newly synthesized choline. Synaptosomal preparations (0.5 mg protein) were incubated at 37 °C for 30 min in 0.1 ml Krebs–Ringer bicarbonate buffer (*p*H 7.4) containing: 4.5 μM [*Me*-^{3}H]SAM (10 mCi μmol^{-1}), 50 μg of ultrasound-dispersed P2, and 1,700 d.p.m. of [*Me*-^{14}C]choline (60 mCi mmol^{-1}). The reaction was stopped by addition of 0.3 ml of 0.5% tetraphenylboron in heptanone. The choline in the heptanone was back-extracted to 0.3 ml of 0.4 M HCl, which was then dried under vacuum. The dry residue was then redissolved in 0.05 ml of ethanol and applied to cellulose TLC plates (*a*) or to filter paper for electrophoresis (*c*). The TLC solvent system contained: *n*-butanol/ethanol/acetic acid/water 40:10:1:15 (by volume). The electrophoretic conditions were: 0.75 M acetic acid, 0.75 M formic acid *p*H 2.0, constant voltage of 17 V cm^{-1} for 40 min. Each sample also contained 5 μg of choline chloride carrier. The plates and papers were stained for choline[16], and segments staining positively were extracted with 50% ethanol in 0.2 M HCl. The extracts were then concentrated and rechromatographed as described above, except that TLC-derived material (*a*) was subjected to electrophoresis (*b*), while electrophoresis-derived material (*c*) was subjected to TLC (*d*). *a*, *b*, *c* and *d* show distributions of radioactivity on chromatograms prepared as described above. Shaded areas indicate segments which stained positively for choline. Lower panels show the distribution of ^{14}C-choline standards and provide estimates of choline recovery. Incubations using heat-treated synaptosomal preparations yielded flat background radioactivity patterns in *b* and *d*.

Table 1 Phosphorylation of newly synthesized choline by choline kinase

	Choline (d.p.m.)	Phosphorylcholine (d.p.m.)
Control	257 ± 22	0 (background)
Choline kinase	31 ± 7	323 ± 51

Incubations, extractions and TLC prepurification of newly synthesized choline were carried out as in Fig. 1 legend. Choline was extracted from TLC plates with 50% ethanol in 0.2 M HCl and the extracts were then dried under vacuum. The dry residues were resuspended in 0.1 ml of medium containing 12 mM glycylglycine, 8 mM dithiothreitol, 18 mM $MgCl_2$, 1.2 mM ATP and 0.05 U choline kinase (*p*H 7.4), and incubated for 15 min at 30 °C. Reaction was stopped with 0.1 ml ethanol and the mixtures concentrated and subjected to paper electrophoresis (as described in Fig. 1 legend). Segments were visualized for choline[16] and phosphorylcholine[17] and then the radioactivity counted. Data are expressed as means ± s.d.

Identification of newly synthesized choline proved relatively difficult. It was not until we combined TLC and paper electrophoresis that we could see a sharp ^{3}H-methyl radioactive peak that co-migrated with authentic nonradioactive and ^{14}C-choline (Fig. 1). The heat-treated preparation of synaptosomes produced no radioactive product that would co-migrate with authentic choline in either of these chromatographic systems. After subjecting the ^{3}H-methyl material that had co-chromatographed with authentic choline to phosphorylation by choline kinase, virtually none of the radioactivity co-chromatographed with choline (Table 1). The radioactivity could, however, be recovered from material co-migrating with phosphorylcholine, as would be expected if the ^{3}H-methyl material were authentic choline.

The rate of incorporation of ^{3}H-methyl groups into choline depended on the concentration of PeMT substrate present—addition of increasing amounts of P2 to incubation mixtures (Fig. 2) caused parallel increases in the apparent rates of synthesis of PC and choline until saturation occurred at ~500 μg P2 per ml. This increase in apparent synthesis rate was more pronounced for ^{3}H-PC, where the ratio of rate of ^{3}H-methyl incorporation when 1 mg per ml of P2 was present to incorporation without added P2 was 5.7; the same ratio for ^{3}H-choline was only 1.5. Addition of dimethylethanolamine (30 mM final concentration) did not enhance the synaptosomal formation of choline (data not shown). These results indicate that the ^{3}H-choline must have been formed by degradation of newly synthesized PC, or, less likely, by action of some as yet undiscovered enzyme on a degradation product of P2.

The SAM concentration at which our assay was carried out (4.5 μM) is below the apparent K_m for PeMT[5,6]. However, at this SAM concentration the reaction proceeded linearly with time for up to 50 min. The incorporation of ^{3}H-methyl into ^{3}H-PC proceeded at the rate of 1.3 pmol per mg protein per 30 min, and into ^{3}H-choline at 0.4 pmol per mg protein per 30 min (Table 2). The choline newly formed during this interval would represent 88 p.p.m. of the total choline in the synaptosomal preparation, whereas the newly formed PC would represent only 1.9 p.p.m. of total PC (Table 2). Thus the relative enrichment of the choline pool was 47-fold greater than that of PC. These results suggest that there may exist specific physical domains of phospholipids within synaptosomal membranes, such that the PE methylation and PC degradation occur in the same domain. This would explain why up to 23% of the [*Me*-^{3}H]choline present in the incubations could be recovered as free choline molecules and not covalently bound in the form of PC, while free choline constituted <1% of the total amount of

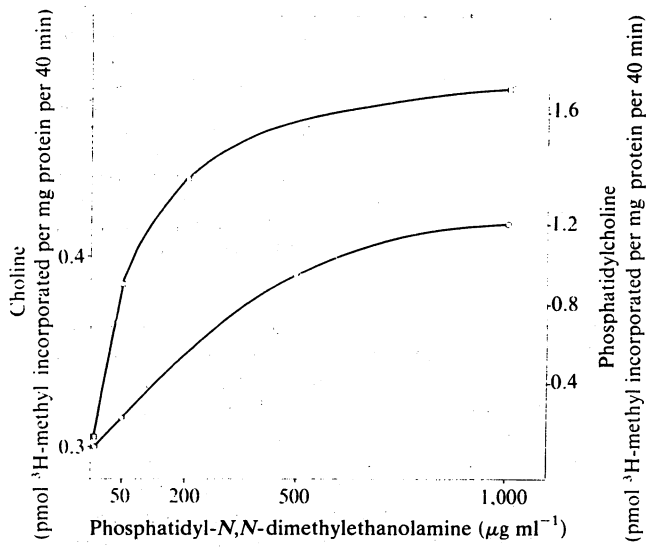

Fig. 2 Effects of P2 on PC (□) and choline (○) formation. Incubations were carried out for 40 min as described in Fig. 1 legend. Extractions and purification of products were performed as described in ref. 5 and Fig. 1 legend.

Table 2 Comparison of synthesis of phosphatidylcholine and choline by rat brain synaptosomal preparations

	Phosphatidylcholine	Choline
^{3}H-methyl incorporation (pmol per mg protein per 30 min)	1.323 ± 0.219	0.396 ± 0.086
Concentration (nmol per mg protein)	708 ± 29	4.5 ± 0.82
Specific activity (p.p.m.)	1.87 ± 0.37	88 ± 34
$\frac{I_{Ch}}{I_{PC}}$	0.30	
$\frac{SA_{Ch}}{SA_{PC}}$	47.3	

Incubation, extraction and purification conditions were as described in Fig. 1 and Table 1 legends. Free ^{3}H-choline and ^{3}H-choline hydrolysed off ^{3}H-PC were determined by radioenzymatic assay[9]. Data are expressed as means ± s.d. ^{3}H-methyl incorporations are I_{PC} and I_{Ch}, specific activities are SA_{PC} and SA_{Ch} for phosphatidylcholine and choline, respectively.

choline (that is free plus PC-bound) in the synaptosomal preparations (Table 2). Other studies have suggested the existence of two PC pools in synaptosomes, one with a $t_{1/2}$ of 2 days and another with $t_{1/2}$ of 52.5 days (ref. 10). Our results indicate the existence of yet another pool, which turns over much more rapidly, and which contains PC molecules derived from the PeMT pathway.

The brain is reported to contain several enzyme systems able to catalyse the degradation of PC to liberate free choline: phospholipases A_1 and A_2 and lysophospholipase[11] generate glycero-3-phosphorylcholine, which can subsequently be hydrolysed in a one-step[12] or two-step process[13] to yield free choline; phospholipase D can catalyse PC hydrolysis to liberate free choline directly[14]; lysosphospholipase D could liberate choline from lysophosphatidylcholine formed by the action of phospholipase A_2 (ref. 15). Our data do not indicate which, if any, of these enzyme systems liberate ^{3}H-choline from ^{3}H-PC formed in synaptosomal preparation by the transmethylation pathway—our present techniques are insufficient to detect other [*Me*-^{3}H]choline containing species, which might provide clues.

It seems clear that previously, by measuring only phospholipid products, we[5] and others[6,7] have underestimated the contribution of PeMT to brain choline. It is entirely possible that other compounds, such as glycero-3-phosphorylcholine and phosphorycholine, also accumulate in our experimental system as intermediates in the genesis of free choline; thus we may still be underestimating the total rate of choline production in the brain. The absolute contribution of *de novò* synthesis to total brain choline and the extent to which this source provides precursor molecules for acetylcholine formation await further study.

These studies were supported in part by a grant from the National Institute of Mental Health. We thank Mr Robert Scheinman for technical assistance.

Received 15 September 1980; accepted 20 January 1981.

1. Ansell, G. B. & Spanner, S. in *Cholinergic Mechanisms and Psychopharmacology* (ed. Jenden, D. J.) (Plenum, New York, 1977).
2. Aquilonius, S. M., Ceder, G., Lying-Tunell, U., Malmund, H. O. & Schubert, J. *Brain Res.* **99**, 422–430 (1975).
3. Choi, R. L., Freeman, J. J. & Jenden, D. J. *J. Neurochem.* **24**, 735–741 (1975).
4. Dross, K. & Kewitz, H. N.-S. *Archs Pharmac.* **275**, 91–106 (1972).
5. Blusztajn, J. K., Zeisel, S. H. & Wurtman, R. J. *Brain Res.* **179**, 319–327 (1979).
6. Crews, F. T., Hirata, F. & Axelrod, J. *J. Neurochem.* **34**, 1491–1498 (1980).
7. Mozzi, R. & Porcellati, G. *FEBS Lett.* **100**, 363–366 (1979).
8. Potter, L. T. & Murphy, W. *Biochem. Pharmac.* **16**, 1386–1388 (1967).
9. Goldberg, A. M. & McCaman, R. E. *J. Neurochem.* **20**, 1–8 (1973).
10. Pasquini, J. M., Krawiec, L. & Soto, E. F. *J. Neurochem.* **21**, 647–653 (1973).
11. Cooper, M. F. & Webster, G. R. *J. Neurochem.* **17**, 1543–1554 (1970).
12. Webster, G. R., Marples, E. A. & Thompson, R. H. S. *Biochem. J.* **65**, 374–377 (1957).
13. Abra, R. M. & Quinn, P. J. *Biochim. biophys. Acta* **380**, 436–441 (1975).
14. Saito, M. & Kanfer, *J. Archs Biochem. Biophys.* **169**, 318–323 (1975).
15. Wykle, R. L. & Schremmer, J. M. *J. biol. Chem.* **249**, 1742–1746 (1974).
16. Beiss, U. J. *Chromatography* **13**, 104–110 (1964).
17. Wade, H. E. & Morgan, D. M. *Nature* **171**, 529–530 (1953).