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Conversion of ethanolamine, monomethylethanolamine and dimethylethanolamine to choline-containing compounds by neurons in culture and by the rat brain

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The incubation of neurons from chick embryos in primary culture with [^{3}H]ethanolamine revealed the conversion of this base into monomethyl, dimethyl and choline derivatives, including the corresponding free bases. Labelling with [*methyl*-^{3}H]monomethylethanolamine and [*methyl*-^{3}H]dimethylethanolamine supported the conclusion that in chick neuron cultures, phosphoethanolamine appears to be the preferential substrate for methylation, rather than ethanolamine or phosphatidylethanolamine. The methylation of the latter two compounds, in particular that of phosphatidylethanolamine, was seemingly stopped at the level of their monomethyl derivatives. Fetal rat neurons in primary culture incubated with [^{3}H]ethanolamine showed similar results to those observed with chick neurones. However, phosphoethanolamine and phosphatidylethanolamine and, to a lesser extent, free ethanolamine, appeared to be possible substrates for methylation reactions. The methylation of water-soluble ethanolamine compounds *de novo* was further confirmed by experiments performed *in vivo* by intraventricular injection of [^{3}H]ethanolamine. Phosphocholine and the monomethyl and dimethyl derivatives of ethanolamine were detected in the brain 15 min after injection.

INTRODUCTION

Nervous tissue requires choline (Cho) for the general cellular demands of phosphatidylcholine (PtdCho) and sphingomyelin biosynthesis. In addition, there is a tissue-specific requirement for Cho by cholinergic neurons, for acetylcholine formation. The supply of Cho may be a regulatory mechanism controlling the synthesis of the neurotransmitter, and several sources of Cho have been identified. Quantitatively, the most important supply of Cho is dietary, with transport into the brain from the circulation across the blood–brain barrier [1–3], and with an efficient uptake mechanism into glial cells and neurons (for reviews see [4–6]). Another source of Cho is by the catabolism of Cho phospholipids [7,8] which may also partially contribute to the artero-venous difference in Cho concentrations in rat brain [9–11]. This degradation of PtdCho may be catalysed by phospholipase D activity which is present in rat brain synaptosomes, and it liberates Cho from endogenous PtdCho, thus supplying this acetylcholine precursor [12].

PtdCho synthesis by nervous tissue occurs by the classical *de novo* or Kennedy pathway, which has as consecutive substrates Cho, *P*Cho and CDP-Cho. More recently, it has been shown that nerve cell cultures [13] and rat brain synaptosomal preparations [14,15] have some capacity to synthesize PtdCho by the stepwise methylation of phosphatidylethanolamine (PtdEtn). The Cho phospholipids produced by this or any of the other mechanisms may then lead to free Cho which can eventually be used as substrate for acetylcholine synthesis [11,16].

It has also been suggested that the direct stepwise methylation of unesterified Etn to yield unesterified Cho may occur in rat brain tissue [10]. This would imply that monomethylethanolamine (MeEtn) and dimethylethanolamine (Me_2Etn), the presumed intermediates, should also be found in this tissue. Isolated neurons from chick embryos in primary cultures, when incubated in the presence of radioactive Etn, were shown to produce labelled *P*Cho, CDP-Cho and PtdCho [17]. In addition, [^{3}H]MeEtn and [^{3}H]Me_2Etn were both converted to their corresponding phosphorylated derivatives and phospholipids by fetal rat brain aggregating cell cultures [18].

These results suggested that methylation of PtdCho precursors may not be restricted to the level of the phospholipids. We wished to determine whether the methylation of water-soluble precursors could contribute to the labelling of Cho phospholipids and therefore represent a source of Cho in nerve tissue.

The models which were chosen for this investigation were primary cultures of neurons from rat and chick embryo cortical hemispheres incubated with [^{3}H]Etn, [*methyl*-^{3}H]MeEtn or [*methyl*-^{3}H]Me_2Etn for time periods varying from 15 to 240 min. The amount of radioactivity incorporated in the water-soluble free bases, their corresponding phosphorylated derivatives and their phospholipids was determined and provided evidence for their formation under these conditions. Experiments performed in the rat brain *in vivo* with intraventricular injections of [^{3}H]Etn supported the results observed with the nerve cell cultures.

Abbreviations used: Etn, ethanolamine; Cho, choline; Ptd, 1,2 diacyl-*sn*-glycero-3-phospho (phosphatidyl).

MATERIALS AND METHODS

Materials

[*methyl*-^{3}H]MeEtn and [*methyl*-^{3}H]Me_2Etn were synthesized in this laboratory. [1-^{3}H]Etn (specific activity 30 Ci/mmol), [*methyl*-^{3}H]Cho (specific activity 15 Ci/mmol) and *P*[*methyl*-^{14}C]Cho (specific activity 50–60 mCi/mmol) were purchased from Amersham and further purified by t.l.c. before use. *P*MeEtn and *P*Me_2Etn were prepared from PtdMeEtn and PtdMe$_2$Etn respectively as explained below. Etn, MeEtn, Me_2Etn, *P*Etn, Cho, *P*Cho, PtdCho, PtdEtn and poly-L-lysine (type VII) were obtained from Sigma. The silica gel 60 plates and chromatography solvents were obtained from Merck. Media for tissue culture were purchased from Flow, fetal calf serum from Gibco and plastic Petri dishes from Falcon. Phosphatases were purchased from Boehringer and phospholipase C (from *Bacillus cereus*) from Sigma. The chemically defined medium was Dulbecco's modified basal Eagle's medium containing 5 mg of insulin/l, 15 mg of putrescine/l, 100 mg of transferrin/l, 10^{-12} M-progesterone, 2×10^{-8} M-oestradiol and 2×10^{-8} M-sodium selenite. The added compounds were obtained from Sigma.

Synthesis of [^{3}H]MeEtn, [^{3}H]Me_2Etn, *P*MeEtn and *P*Me_2Etn

[^{3}H]MeEtn and [^{3}H]Me_2Etn were chemically synthesized by the published procedure [19]. *P*MeEtn and *P*Me_2Etn were obtained in this laboratory according to the method of Hanahan *et al.* [20] for the preparation of diacylglycerols, with some modifications. These compounds were produced by the hydrolytic action of phospholipase C on PtdMeEtn and PtdMe$_2$Etn and were isolated by the addition of 5 vol. of chloroform/methanol (2:1, v/v) to the incubation tubes. The aqueous phase, which contains *P*MeEtn and *P*Me_2Etn, was evaporated to dryness, and the residues were dissolved in 0.5 M-HCl and quantified by determination of the phosphorus content [21].

Culture conditions

Chick neurons. These were obtained from dissociated cerebral hemispheres of 8-day-old chick embryos using the technique of Pettmann *et al.* [22]. Briefly, after dissociation of the hemispheres through a nylon sieve (48 μm mesh) in 5 ml of Dulbecco's medium containing 20% fetal calf serum, (1–2) $\times 10^6$ cells/ml were seeded on plastic Petri dishes (60 mm diam.) precoated with poly-L-lysine (10 mg/l). The media were replaced after three days and again after five days. The experiments were performed after 6 days in culture. Under these conditions, cultures contain exclusively (98%) neurons, which develop synapses after 3 days of culture and reach full maturation by the 6th day [22].

Rat neurons. Neurons were prepared from the cortices of 13–14-day-old rat fetuses (Wistar) according to the technique of Gensburger *et al.* [23] with some modifications. The cortices were mechanically dissociated in Eagle's basal medium containing 20% fetal calf serum by repeated passage through a 1 mm diam. needle, and seeded on poly-L-lysine-precoated Petri dishes. After 2 h of incubation, the medium was removed and replaced by freshly prepared chemically-defined medium. The medium was changed after 3 and 6 days and the cultures were used after 7 days. The purity of the cultures and the basic characteristics of these neurons have been reported elsewhere [23].

Experiments *in vivo*

A 5 μl sample containing 50 μCi of [^{3}H]Etn was injected with a microsyringe into the third ventricle of 300 g Wistar rats following the co-ordinates: AP (−2) ML (+2) DV (+4).

At fixed time points, the animals were killed by focused microwave irradiation (10 kW, 1.6 s at 75% maximum power; Buschner) and the brains (including brain stem and cerebellum) were dissected out and homogenized in water (10 ml/g).

Incubation conditions

Chick embryo neurons were incubated with either 10 μM-[^{3}H]Etn (10 μCi/ml of growth medium), with (91 μM-[^{3}H]MeEtn (0.875 μCi/ml) or with 10 μM-[^{3}H]Me_2Etn (0.23 μCi/ml). Rat neurons were incubated with 10 μM-[^{3}H]Etn (10 μCi/ml of growth medium). The medium was discarded at the end of the incubation, and the cultures were washed 3 times with 5 ml of 154 mM-NaCl solution prewarmed at 37 °C, scraped into 3 ml of water and the cells were homogenized with a Polytron instrument. The homogenate was lyophilized and the lipids were extracted according to the technique of Folch-Pi *et al.* [24]. The extract was first washed with 1 ml of water and the aqueous and organic phases were separated by centrifugation (2000 *g*, 10 min). The organic phase was washed once more with the Folch upper phase (chloroform/methanol/water, 3:48:47, by vol.) and, after centrifugation, the aqueous phases were pooled. The aqueous and organic phases were evaporated under a stream of N_2 and the dried material was dissolved in 100 μl of 0.5 M-HCl (aqueous phase) or 100 μl of chloroform/methanol (2:1) (organic phase). Aliquots of each solution were subjected to t.l.c. as described below.

Separation of water-soluble compounds

This separation was accomplished by bi-dimensional t.l.c. chromatography. Silica t.l.c. plates were activated at 110 °C for 30 min and run overnight with butanol/methanol/conc. HCl/water (5:5:1:1, by vol.) and then dried. Aliquots of each sample (usually 10 μl) were applied together with 5 μl of a mixture of standards containing (per ml) 30 mg of Etn, 2 mg of MeEtn, 2 mg of Me_2Etn, 2 mg of Cho, 30 mg of *P*Etn, 30 mg of *P*Cho, 1 mg of *P*MeEtn and 1 mg of *P*Me_2Etn. The first solvent system was butanol/methanol/conc. HCl/water (10:10:1:1, by vol.). After approx. 6 h, the plates were dried and the migration in the second dimension carried out overnight with a solvent containing 0.15 M-NaCl/methanol/ammonia (13:7:1, by vol.). The plates were then dried and the various substances were revealed by using iodine vapour. After evaporation of the iodine, the spots were scraped off and radioactivity counted after addition of 0.5 ml of water and 10 ml of Biofluor. The R_F values of the different compounds are presented in Table 1.

Identification of the water-soluble compounds

The various products of the cultured cells were found to co-migrate with authentic standards. In order to confirm the identity of the phosphorylated MeEtn and Me_2Etn, they were subjected to hydrolysis.

After incubation of neurons with 10 μM-[^{3}H]Etn

Table 1. Relative R_F values of water-soluble compounds and phospholipids

Relative R_F values were evaluated with respect to *P*Etn for the water-soluble compounds and to PtdEtn for phospholipids. The values were representative of an average bi-dimensional separation performed as explained in the text. LPE, lyso-PtdEtn; LPC, lyso-PtdCho; SM, sphingomyelin.

Compound	R_F First dimension	R_F Second dimension
Water-soluble:		
Etn	0.98	0.54
MeEtn	0.83	0.31
Me_2Etn	0.58	0.37
Cho	0.41	0.28
*P*Etn	1	1
*P*MeEtn	1	0.79
PMe_2Etn	0.67	0.85
*P*Cho	0.48	0.83
Phospholipids:		
PtdEtn	1	1
PtdMeEtn	0.91	0.88
PtdMe$_2$Etn	1.31	0.82
PtdCho	0.67	0.76
LPE	0.65	0.88
LPC	0.30	0.50
SM	0.28	0.35

Table 2. Identification of phosphorylated water-soluble compounds

After incubation of chick neurons in culture with 10 μM-[^{3}H]Etn (10 μCi/ml), the extraction and t.l.c. separation of water-soluble compounds was performed as described in the Materials and methods section. The phosphorylated compounds (*P*Etn, *P*MeEtn, *P*Me_2Etn, *P*Cho) were revealed by iodine vapours, scraped and eluted with 5 ml of 0.5 M-HCl. Each sample was then divided into two batches and lyophilized. HCl (0.5 M, 0.1 ml) was added to one batch, considered as the control, while 0.5 ml of 6 M-HCl was added to the other. Hydrolysis of the phosphorylated compounds was performed for 18 h at 110 °C. Hydrolysates were brought to dryness under a stream of N_2 and redissolved in 0.1 ml of 0.5 m-HCl. Control and hydrolysates were then chromatographed on silica gel plates with the mixture 0.15 M-NaCl/methanol/ammonia (13:7:1, by vol). The phosphorylated compounds and the corresponding free bases were revealed, scraped and the radioactivity measured as described in the text. Values represent the means ± S.D. from four separate Petri dishes.

	Control (c.p.m.)	Hydrolysate (c.p.m.)
*P*Etn	1955610 ± 95070	20885 ± 1764
Etn	17562 ± 2804	1578012 ± 160294
*P*MeEtn	21194 ± 1210	197 ± 23
MeEtn	157 ± 15	18282 ± 3262
PMe_2Etn	926 ± 122	300 ± 24
Me_2Etn	325 ± 64	1442 ± 254
*P*Cho	9012 ± 1236	709 ± 102
Cho	571 ± 46	9722 ± 1024

(10 μCi/ml) for 4 h, the water-soluble products were separated by t.l.c. as described above and the scraped silica gel eluted with 0.5 M-HCl and divided into two aliquots. After lyophilization, one sample was resuspended in 0.5 M-HCl and the other in 6 M-HCl, and both were heated at 110 °C for 18 h. HCl was removed by evaporation and the dry material was resuspended in 0.5 M-HCl. Both samples were separated by t.l.c. as described above. The results shown in Table 2 indicate that after hydrolysis of the isolated phosphorylated compounds the radioactivity present in the corresponding free base was increased by an amount roughly equivalent to that lost from the phosphorylated derivative. Similar results (not shown) were obtained after incubation of cells with [^{3}H]MeEtn and [^{3}H]Me_2Etn. In a similar manner, radioactivity co-migrating with *P*Cho was converted to free Cho by incubation with both alkaline and acid phosphatases.

The identity of the products was also examined in the experiments where neurons were incubated with [^{3}H]MeEtn and [^{3}H]Me_2Etn as precursors. These results showed that only the methylated and phosphorylated derivatives of these compounds were found after chromatographic separation (see Figs. 2 and 3).

Separation of phospholipids

This was accomplished by bi-dimensional t.l.c. employing chloroform/methanol/ammonia (13:7:1, by vol.) as solvent in the first dimension and butanol/acetic acid/water (3:1:1, by vol.) in the second dimension. The R_F values of the individual compounds which were identified by comigration with standards are shown in Table 1. After drying the plates, the individual compounds were revealed with iodine vapour, scraped and the radioactivity counted after addition of 0.5 ml of water and 10 ml of Biofluor.

Protein determination

Protein was measured according to the procedure of Lowry *et al.* [25]. After 6 days in culture, the average protein content of chick neurons was 450 ± 33.9 μg/Petri dish (mean ± S.D.; n = 180) and that of rat neurons was 250 μg ± 31.1/Petri dish (n = 90)

Phosphorus determination

Phosphorus was quantified according to Rouser *et al.* [21].

Statistics

Student's t test was used when significance was sought.

RESULTS

Incubation of [^{3}H]Etn with chick neurons

When chick neurons were incubated with [^{3}H]Etn, the principal radioactive product was *P*Etn, which increased linearly up to 2 h and then reached a plateau (Fig. 1*a*). The amount of free [^{3}H]Etn increased, and plateaued by 1 h and was much lower than the amount of [^{3}H]*P*Etn at all time points. After a 30 min lag period, the label present in PtdEtn increased linearly during the course of the experiment.

The rate of appearance of radioactivity in the phospholipids PtdMeEtn, PtdMe$_2$Etn and PtdCho is presented in Fig. 1(*b*). The labelling of the latter two phospholipids

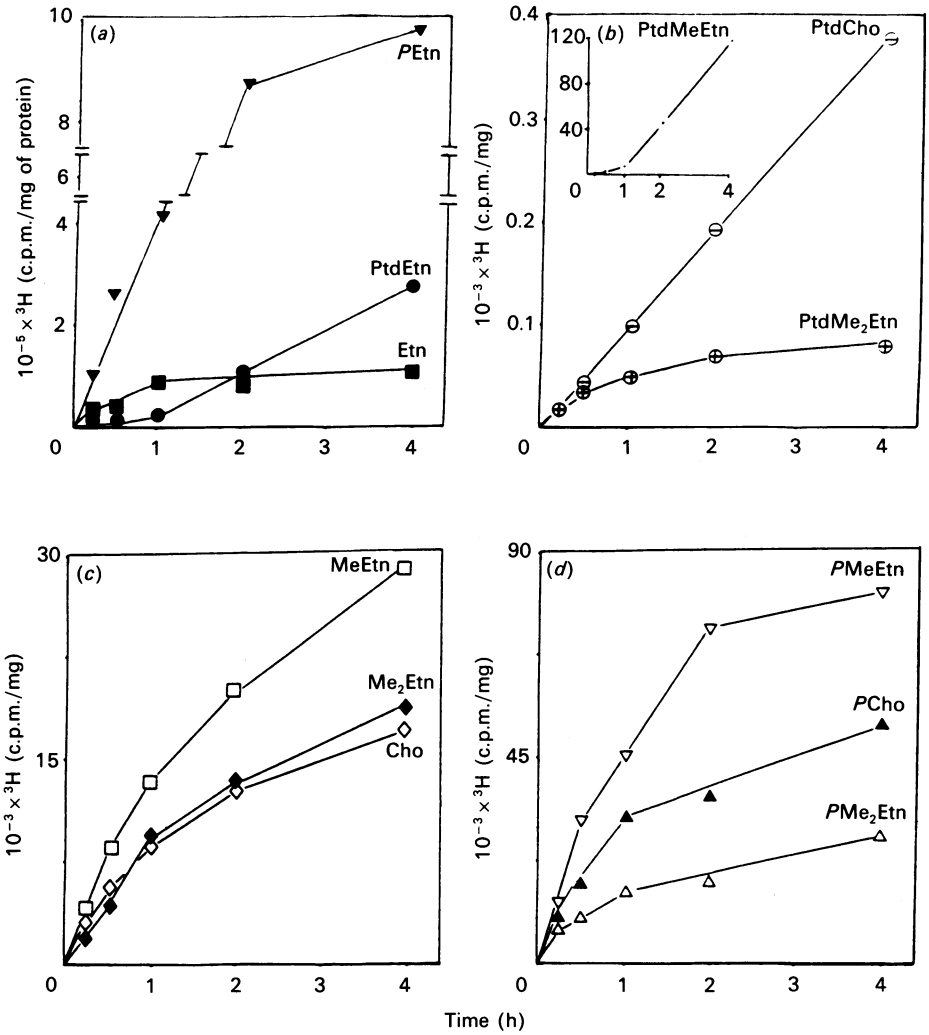


Fig. 1. Metabolism of [^{3}H]Etn in chick neurons in culture

E_8 chick neurons grown in culture in the absence of supportive cells were obtained according to [22] and were incubated with [^{3}H]Etn as described in the text. At the indicated time points, neurons were collected and various compounds isolated by t.l.c. Symbols: ■, Etn; ▼, *P*Etn; ●, PtdEtn; ⊕, $PtdMe_2Etn$; ⊖, PtdCho; · —, PtdMeEtn (inset); □, MeEtn; ◆, Me_2Etn; ◇, Cho; ▽, *P*MeEtn; △, PMe_2Etn; ▲, *P*Cho. Values represent the means of four samples; the S.D. did not exceed 10%.

was no greater than 1.5 and 3.7% of that of PtdEtn and PtdMeEtn respectively.

The incorporation of the label from Etn into MeEtn was nearly twice that appearing in Me_2Etn and Cho, which have superimposable patterns of labelling (Fig. 1*c*). The label found in MeEtn and in both Me_2Etn and Cho represented 28 and 17% respectively of that present in Etn. These percentages are greater than those found in the corresponding phosphatidyl derivatives.

Large quantities of radioactivity were present in the phosphorylated esters of the bases derived from Etn, and after 30 min of incubation there was more label in *P*MeEtn than in PMe_2Etn or *P*Cho (Fig. 1*d*), and this difference persisted during the experiment. In accordance with the observations with the free bases (Fig. 1*c*), there was a notable difference between the *P*MeEtn and *P*Cho labelling patterns (Fig. 1*d*). This observation supports the possibility of a progressive methylation of aqueous soluble compounds. Moreover, the radioactivity from [^{3}H]Etn incorporated into the individual phospholipids (Fig. 1*b*), especially at the early stages of incubation, was very low compared with the radioactivity in the corresponding water-soluble free bases (Fig. 1*c*) and phosphorylated bases (Fig. 1*d*). This suggests that the water-soluble materials were not derived from the phospholipids. Therefore it seems reasonable to propose that most of the methylation is occurring at the level of the free bases or their corresponding phosphorylated derivatives rather that at the level of the phospholipid.

Incubation of chick neurons with [^{3}H]MeEtn

The labelling patterns for MeEtn, Me_2Etn, Cho and their corresponding phosphorylated derivatives and phospholipids derived from [^{3}H]MeEtn are presented in Figs. 2(*a*)–2(*c*). The principal labelled compounds are the phosphorylated derivatives of the free bases, which appear to increase linearly with time (Figs. 2*a* and 2*c*), whereas the amount of radioactivity found in the free bases is much lower at all time points examined.

Labelled free Me_2Etn and free Cho produced from [^{3}H]MeEtn are nearly undetectable at 15 min (Fig. 2*b*) and only trace amounts are present at 4 h. However,

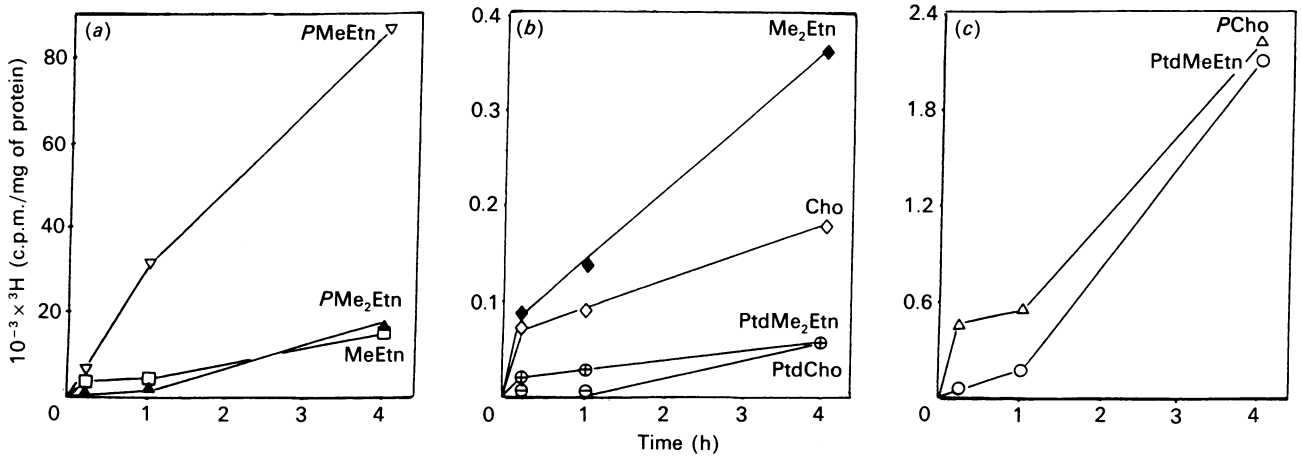


Fig. 2. Metabolism of [^{3}H]MeEtn in chick neurons in culture

Neurons were incubated with [*methyl*-^{3}H]MeEtn and at fixed time points the metabolites were extracted and isolated as described in the text. □, MeEtn; ▽, *P*MeEtn; ▲, *P*Me$_2$Etn; ◆, Me$_2$Etn; ◇, Cho; ⊕, PtdMe$_2$Etn; ⊖, PtdCho; △, *P*Cho; ○, PtdMeEtn. Values represent the means of four samples; the S.D. did not exceed 10%.

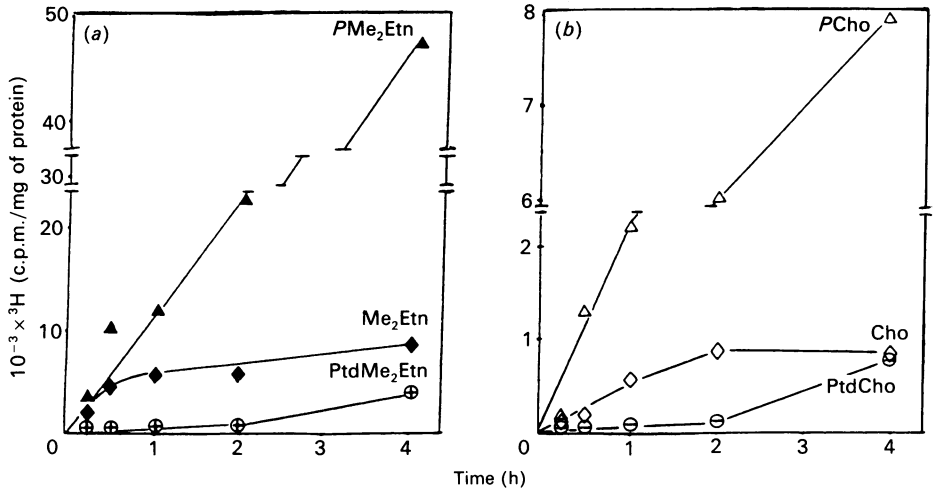


Fig. 3. Metabolism of [^{3}H]Me$_2$Etn in chick neurons in culture

Neurons were incubated with [*methyl*-^{3}H]Me$_2$Etn and at fixed time intervals the metabolites were extracted and isolated as described in the text. ◆, Me$_2$Etn; ▲, *P*Me$_2$Etn; ⊕, PtdMe$_2$Etn; ◇, Cho; △, *P*Cho; ⊖, PtdCho. Values are means of four samples; the S.D. did not exceed 10%.

there are appreciable quantities of label in *P*Me$_2$Etn and *P*Cho, corresponding to about 10 and 6% respectively of that found present in *P*MeEtn (Figs. 2*a* and 2*c*). This suggests that methylation occurs at the level of the phosphorylated bases preferentially to methylation of the free bases. PtdCho was nearly undetectable for the first hour of incubation and only small amounts of PtdMe$_2$Etn were found (Fig. 2*b*). These observations indicate that there is only a minimal contribution of phospholipid methyltransferase activity for the conversion of PtdMeEtn to PtdMe$_2$Etn and PtdCho.

Incubation of chick neurons with [^{3}H]Me$_2$Etn

The appearance of label in Me$_2$Etn, Cho, *P*Me$_2$Etn, *P*Cho, PtdMe$_2$Etn and PtdCho from [^{3}H]Me$_2$Etn as a function of time in chick neuronal cultures is shown in Figs. 3(*a*) and 3(*b*). The principal radioactive material produced is *P*Me$_2$Etn. The ratio of *P*Me$_2$Etn/Me$_2$Etn for the first three time intervals is about 2, and it continues to rise for the next 3 h to about 5.4. This is similar to the result obtained with [^{3}H]Etn and [^{3}H]MeEtn. The formation of radioactive PtdMe$_2$Etn is slight for the first hour of incubation and gradually increases with time (Fig. 3*a*). The ratio of radioactivity in Cho to that in Me$_2$Etn is nearly constant at 1:10. In contrast, the conversion of *P*Me$_2$Etn to *P*Cho progressively increases from 6.5% at 15 min to 17.4% at 240 min. The values at 1, 2 and 4 h suggest that the possible conversion of PtdMe$_2$Etn to PtdCho is about 10–16%, while the conversion of *P*Cho to PtdCho does not exceed 10% (Fig. 3*b*).

Incubation of rat neurons with [^{3}H]Etn

There was a marked difference in the principal labelled material derived from [^{3}H]Etn between the cultures of rat and chick neurons. *P*Etn was the principal labelled

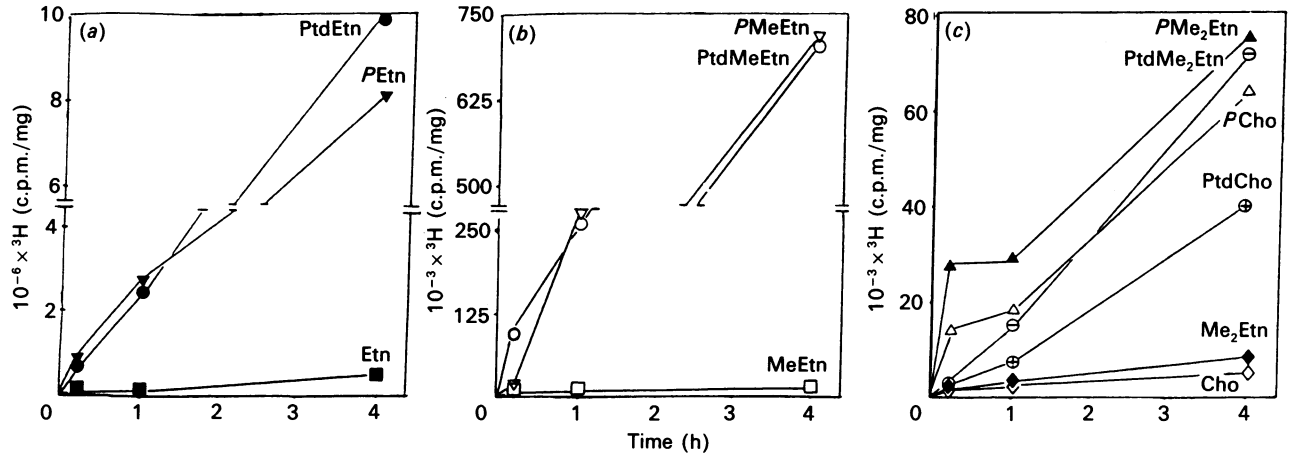


Fig. 4. Metabolism of [^{3}H]Etn in fetal rat neurons in culture

Neurons from 14-day-old fetuses were grown in the absence of supportive cells according to [23] with some modifications, incubated with [^{3}H]Etn and at fixed time intervals the various metabolites extracted and isolated as described in the text. ■, Etn; ▼, *P*Etn; ●, PtdEtn; □, MeEtn; ▽, *P*MeEtn; ○, PtdMeEtn; ◆, Me_2Etn; ▲, *P*Me_2Etn; ⊖, PtdMe$_2$Etn; △, *P*Cho; ⊕, PtdCho. Values are the means of four samples; the S.D. did not exceed 10%.

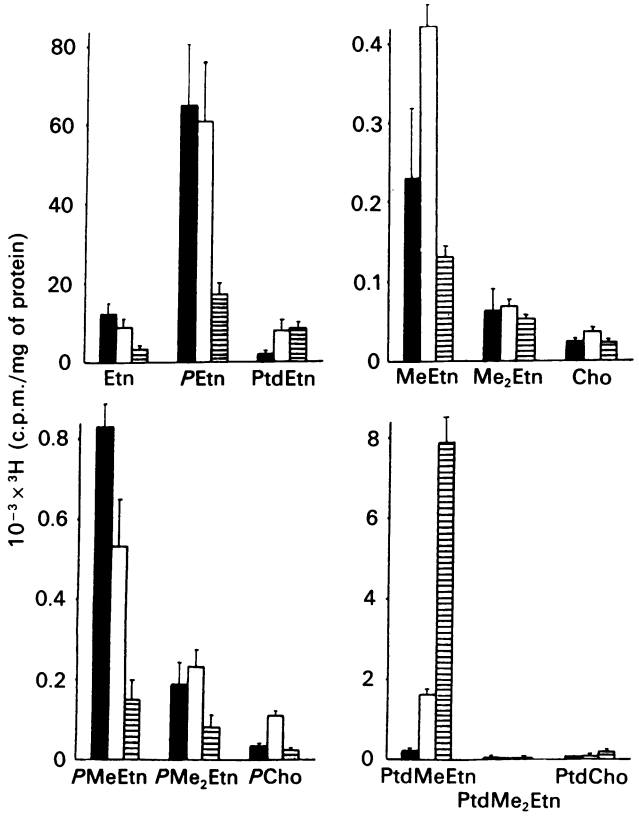


Fig. 5. Metabolism of [^{3}H]Etn in the rat brain

Wistar rats were injected intraventricularly with [^{3}H]Etn and the metabolites examined 15 min (■), 60 min (□), or 24 h (▤) after injection as explained in the text. Values are the means of four samples ± S.D.

product at all time intervals in chick neurons, with considerably less PtdEtn (Fig. 1). In contrast, the amounts of label incorporated into *P*Etn and PtdEtn by the rat neurons were nearly equivalent during the course of these experiments (Fig. 4*a*). With chick neurons, the conversion of *P*Etn to PtdEtn increased from 3 to 30% (Fig. 1*a*), that of *P*MeEtn to PtdMeEtn increased from 0.6 to 2.5% (Figs. 1*b* and 1*d*) and that of *P*Me_2Etn to PtdMe$_2$Etn increased from 0.5 to 9% (Figs. 1*b*, 1*f* and 2) during the time course of the experiment. The corresponding values for these conversions were between 88 and 140% (Figs. 4*a*–4*c*) with the rat neurons. There was a nearly identical rate of label appearance in PtdMeEtn and *P*MeEtn with the rat neurons (Fig. 4*b*), a similar result to that given by the corresponding Etn derivatives (Fig. 4*a*). These relatively similar rates were not seen with either the Me_2Etn or the Cho derivatives (Fig. 4*c*).

Metabolism of [^{3}H]Etn in the rat brain *in vivo*

It was of interest to determine whether the incorporation of [^{3}H]Etn into the various derivatives containing additional methyl groups observed with the cultured neurons could be demonstrated in rats *in vivo*. Animals were injected intraventricularly with [^{3}H]Etn, and at 0.25, 1 and 24 h post-injection, brain tissue was removed and the label appearing in the water-soluble and the lipid-soluble compounds was estimated. These results support the observations obtained with rat neuron cultures, showing a similar labelling pattern for the free bases and their phosphorylated metabolites (Figs. 5*a*–5*d*).

In addition to the expected derivatives of Etn, there was reasonable incorporation of [^{3}H]Etn into *P*MeEtn, PtdMeEtn, *P*Me_2Etn and *P*Cho. Lesser amounts of radioactivity appear in the free bases and very little in PtdMe$_2$Etn or PtdCho.

DISCUSSION

The possible methylation of free Etn or Etn-containing compounds in the nervous tissue has been considered since Kewitz and coworkers found that more Cho was leaving rat brain than entering it [9,10]. This arteriovenous difference was attributed to Cho release from the Cho-containing phospholipids [15], perhaps from a pool arising by stepwise methylation of Etn phospholipids

[13,14]. The suggestion was also made that free Etn might be directly methylated to yield free Cho [9]. The possible methylation of *P*Etn or CDP-Etn was offered as a potential explanation of labelling of hepatic lipids *in vivo* [26]. However, there were no attempts to determine if predictable methylated intermediates became labelled. A trial to confirm this observation was unsuccessful and it was suggested that impurities present in the material injected or incomplete separation of the various metabolites contributed to the original observations [27]. The inability to demonstrate the existence of the suspected methylated intermediates has hampered any evaluation of the potential physiological role of such a metabolic pathway.

The production of free Cho in the brain at the expense of Cho phospholipids by the activity of phospholipase is a mechanism available in synaptosomes for acetylcholine synthesis [12,16]. The supply of Cho derived (1) from the circulation, (2) by its liberation from phospholipids, or (3) by the conversion of Etn-containing compounds, could produce one of the precursors for the synthesis of the neurotransmitter.

It has been speculated that a possible interference with these sources for Cho might contribute to the pathology of diseased cholinergic neurons [15]. Labelling patterns in cultured neurons now provide a tool for investigating these pathways.

Labelling *in vitro*

The results demonstrate that the addition of ^{3}H-labelled precursors to cultured neurons leads to the conversion of Etn, MeEtn and Me_2Etn into their phosphorylated water- and lipid-soluble compounds and eventually into Cho, *P*Cho and PtdCho. A casual perusal of the results might suggest that neurons in culture have the capability to sequentially methylate Etn to Cho, *P*Etn to *P*Cho and PtdEtn to PtdCho. A closer examination of the data reveals the existence of preferential routes for these conversions. In chicken neurons, the incorporation of MeEtn into Me_2Etn was very low or nil. The appearance of labelled PtdMe_2Etn from MeEtn was undetectable for the first hour and was still very low even at 4 h of incubation. However, there appeared to be reasonable labelling of *P*Me_2Etn at all time periods.

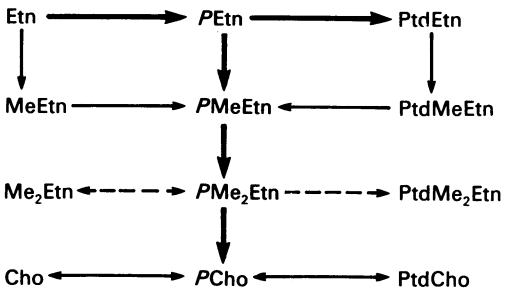
Estimations of the ratios of incorporation obtained with [^{3}H]Me_2Etn as substrate reveal differences compared with those with [^{3}H]MeEtn. The ratios of derivatives of Cho and Me_2Etn show reasonable conversions of the free bases, the phosphorylated bases and the phospholipids (Table 3). The ratios for MeEtn and Etn, their phosphorylated bases and their phospholipids after incubation with [^{3}H]Etn show reasonable conversions. This leads to the conclusion that, in these chicken neuronal cultures, Etn is rapidly phosphorylated to *P*Etn and converted to PtdEtn by the classical *de novo* pathway. *P*Etn may also be methylated to *P*MeEtn, *P*Me_2Etn and *P*Cho. It is less likely that free Etn is eventually methylated to provide free Cho because of a block in the conversion of MeEtn into Me_2Etn. PtdEtn can be converted to PtdMeEtn but not to PtdMe_2Etn or PtdCho for the same reasons (see Scheme 1). The free Me_2Etn and Cho appearing after incubation with [^{3}H]Etn might be hydrolysis products of their phosphorylated derivatives.

The ratios of the various derivatives of Etn in the case of rat neurons (Table 4) suggests that all possible

Table 3. Ratios of incorporation of ^{3}H-labelled bases in chick neurons in culture

The ratios were obtained from the data shown in Figs. 1 (*), 2 (**) and 3 (***).

Time (min)	MeEtn* / Etn	Me_2Etn** / MeEtn	Cho*** / Me_2Etn***
15	0.107	0	0.11
30	0.164	–	0.069
60	0.159	0.023	0.1
120	0.135	–	0.155
240	0.284	0.24	0.095
	PMeEtn* / ***P***Etn	***P***Me_2Etn** / ***P***MeEtn	***P***Cho*** / ***P***Me_2Etn
15	0.103	0.1	0.065
30	0.117	–	0.12
60	0.151	0.065	0.17
120	0.085	–	0.24
240	0.085	0.188	0.174
	PtdMeEtn* / PtdEtn	PtdMe_2Etn** / PtdMeEtn	PtdCho*** / PtdMe_2Etn
15	0.436	0	0.286
30	0.391	–	0.36
60	0.352	0	0.092
120	0.429	–	0.151
240	0.365	0.028	0.169



Scheme 1.

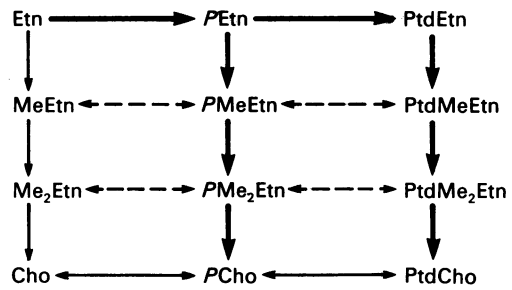
pathways eventually leading to PtdCho may occur, although the methylation of *P*Etn to *P*Cho seems more prominent (Scheme 2). However, the conversion of Etn to MeEtn is quite small as compared with the others.

The reason for the apparent differences between the chick and rat brain neuronal cultures is not readily apparent. Preliminary results have shown that when chick neurons were grown in the absence of serum, using the same chemically defined medium employed for the rat neurons, the overall metabolism of Etn increased in general and resembled that of rat neurons. The apparent difference may then depend on the poorer support that chemically prepared medium gives to the development of rat neurons.

Table 4. Ratios of incorporation of [^{3}H]Etn in rat neurons in culture

Ratios were calculated from the data shown in Fig. 4.

Time (min)	MeEtn / Etn	Me_2Etn / MeEtn	Cho / Me_2Etn
15	0.06	0.44	0.56
60	0.042	0.84	0.65
240	0.026	0.57	0.64
	*P*MeEtn / *P*Etn	*P*Me_2Etn / *P*MeEtn	*P*Cho / *P*Me_2Etn
15	0.1	0.276	0.5
60	0.091	0.103	0.68
240	0.099	0.095	0.85
	PtdMeEtn / PtdEtn	PtdMe$_2$Etn / PtdMeEtn	PtdCho / PtdMe$_2$Etn
15	0.039	0.05	1.53
60	0.115	0.048	0.54
240	0.072	0.089	0.56



Scheme 2.

Metabolism of [^{3}H]Etn *in vivo*

The results obtained with intraventricular injections of [^{3}H]Etn showed that the rat brain, as suggested by Kewitz and coworkers [9,10], has the capacity to synthetize free Cho *de novo* by the stepwise methylation of Etn, *P*Etn and PtdEtn. The data do not indicate, however, any preferential pathway utilized by the nervous tissue to produce the methylated derivative *in vivo* and appear similar to those found in the rat neurons in culture.

The purpose of these experiments was to show that the pathways described in the cultures were also present *in vivo*. The detection of the intermediate methyl derivatives of Etn, *P*Etn and PtdEtn strongly suggests that this is the case.

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