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EVIDENCE FOR THE FORMATION OF 2-METHYLTHIAMINE IN NERVE TISSUE

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Abstract—Rats and cats were injected with [^{3}H]choline, [^{14}C]choline or [^{35}S]thiamine. Choline and thiamine metabolites were extracted from different tissues by ion-pair extraction. Different chromatographic techniques and the use of two separate precursors—choline and thiamine—showed that a fraction of the radioactivity was incorporated into a [^{3}H]2-methylthiamine-like substance. A relationship was found between the amounts of radioactive acetylcholine and the 2-methylthiamine-like substance formed.

An unidentified polar metabolite of choline has been detected in cat ventral roots during studies on the synthesis of acetylcholine (ACh). This metabolite (compound A) has chemical characteristics in common with 2-methylthiamine. The importance of thiamine as a co-enzyme in carbon-group transfer and decarboxylation reactions is well-documented. The diphosphate form of thiamine is the active co-factor. There is evidence which suggests that thiamine plays a unique role in the function of excitable membranes in addition to its role as the co-enzyme in intermediary metabolism. Von Muralt [1] found a release of thiamine from stimulated nerves, an observation confirmed and extended by Cooper and Pincus [2]. In the main, it was the non-phosphorylated form of thiamine which was released. Thiamine has also been shown to reverse the decrease in excitability with time normally observed in the voltage-clamped node of Ranvier [3].

The present paper discusses the possibility that the formation of 2-methylthiamine may reflect cholinergic nicotinic receptor activity. The suggested inter-relationship between thiamine and nicotinic receptor activity could explain one role of thiamine in excitable membranes.

MATERIAL AND METHODS

In vivo *administration of precursors*

The spinal cords of 17 anaesthetized cats (Nembutal, 30 mg/kg i.p.) were exposed over the lumbar and sacral regions. The radioactive precursors (choline chloride, (N-$C^3H_3)_3$); choline chloride, (N-($^{14}CH_3)_3$); [^{35}S]thiamine hydrochloride; betaine chloride, (N-$^{14}CH_3)_3$) and adenosyl-L-(S-$^{14}CH_3$)methionine) were dissolved (one at a time) in 2 μl of 0,9% NaCl and injected into the region of ventral horn cells using the technique described by Ochs & Burger [4]. The dorsal root ganglia were injected with 20 μCi of [^{3}H]choline (dissolved in 2 μl of 0,9% NaCl).

In vitro *incubations with precursors*

With tissue. Ventral roots (0.1 g) were homogenized with 25 strokes with a glass pestle or chopped with scissors in 15 mM sodium phosphate buffer, pH 7.9 (5% w/v). Incubation (0.3 ml) was carried out at 37° for 30 min in one of the following media.

1. 150 mM sodium phosphate buffer, pH 7.9
   0.03, 0.3 or 3.0 μM thiamine
   30 μM [^{3}H]choline
   (1 μCi) or 30 μM [^{3}H]ACh (0.1 μCi) and 10^{-5} M eserine sulphate
2. 150 mM sodium phosphate buffer, pH 7.9
   0.03, 0.3
   or 3.0 μM thiamine
   30 μM adenosyl-L-methionine (S-$^{14}CH_3$) (0.36 μCi)

Five and four experiments respectively were carried out in each group.

Without tissue. Twenty nmoles of ACh were incubated with 1 nmole of [^{35}S]thiamine in 0.1 ml of 0.1 M Tris buffer, pH 7.9 or pH 9, at 70° for 60 min (10 experiments). Blank incubations were performed at pH 3.0 (10 experiments).

Extraction of tissue samples

Thirty min after injection of isotopes (40 min in the case of rat tissues), the ventral roots were homogenized with 25 strokes in a glass homogenizer containing allyl-cyanide with 12.5 mg/ml of sodium tetraphylboron [5] to extract choline metabolites. After centrifugation, the organic phases were transferred to new tubes containing 2 ml of 1 M HCl. After being shaken for 15 sec, the tubes were centrifuged and the organic phases discarded. The aqueous phases were washed with 4 × 5 ml of ether and then freeze-dried. The recovery of ACh by this extraction procedure was 97–102%.

Chromatography

The freeze-dried pellets were dissolved in 75% ethanol and chromatographed using the following solvent systems.

Paper chromatography (*Whatman No. 1 paper*). Butanol–ethanol–acetic acid–water (8:2:1:3), ascending and descending (solvent system); propanol–1 M ammonium formate–water (39:18:18), descending (solvent system 2); propanol–1 M sodium acetate buffer (pH 5)–water (7:1:2), ascending (solvent system 3).

Thin layer chromatography (*glass plates covered*

with cellulose, DC-Fertigplatten Cellulose®). Butanol–ethanol–conc. hydrochloric acid–water (16:4:1:5).

The following reference substances were run in parallel with the samples: ACh (15 μg), choline (10 μg) and 2-methylthiamine (15 μg). The reference spots were stained with Dragendorff's reagent [6]. In some experiments where [^{3}H]choline had been used for labelling. [^{14}C]choline or [^{35}S]thiamine was added in order to localize the choline or thiamine spots. The identity of the ACh-spot was further confirmed by AChE treatment and subsequent chromatography. After the chromatograms had been developed for about 16 hr, they were dried, cut or scraped into 0.5 cm pieces and put into Packard plastic vials with 2,5 ml of 0.9% NaCl. After they had been shaken for 2 hr, 10 ml of Instagel scintillation fluid was added (Packard Instrument Co.) and the radioactivity was measured in a liquid scintillation counter.

2-Methylthiamine was synthetized by reacting 2,4-dimethyl-5-hydroxyethylthiazole with 2-methyl-4-amino-5-pyrimidylmethyl bromide hydrobromide as described by Andersag and Westphal [7]. The thiazole derivative was synthetized according to Lindberg *et al.* [8]. Chemicals for the thiazole synthesis were kindly supplied by Astra Co., Södertälje, Sweden. The pyrimidyl compound was a gift from Merck Co., Darmstadt, West Germany. Other chemicals were obtained from usual commercial sources and were of analytical grade or of the purest grade commercially available. Choline chloride, (N-(C^3H_3)$_3$) (sp. act. 1 Ci/m-mole); choline chloride, (N-$^{14}CH_3$)$_3$) (sp. act. 50 mCi/m-mole); [^{35}S]thiamine (sp. act. 1 mCi/m-mole) and betaine chloride, (N-$^{14}CH_3$)$_3$) (sp. act. 1 mCi/m-mole) were obtained from NEN Chemicals, Germany. Adenosyl-L-(S-$^{14}Ch_3$) methionine (sp. act. 25 mCi/m-mole) was obtained from the Radiochemical centre, Amersham, England. The glass-plates for thin layer chromatography were obtained from Merck Co. The radiochemical purity of [^{3}H]choline, [^{14}C]choline and [^{35}S]thiamine was controlled by paper chromatography (system 2) and was more than 99%.

RESULTS

Chemical identification of compound A

When extracts of ventral roots ($n > 15$) injected with 20 μCi of [^{3}H]choline were chromatographed with solvent system 1 or 2, three main peaks of radioactivity appeared, two with R_f-values corresponding to those of ACh and choline and a third due to an unknown compound A (Fig. 1). Similar results were obtained when 5 μCi of [^{14}C]choline was used as precursor ($n = 3$). The R_f-value of compound A was found to correspond to that of thiamine. When solvent system 3 was used for chromatography, however, all the radioactivity moved with the solvent front ($n = 3$). When the radioactive substance with an R_f-value of 0.25 (corresponding to the thiamine reference) was eluted from the paper developed by solvent system 1 and rechromatographed on paper developed by solvent system 3, the radioactivity moved as 2-methylthiamine with an R_f-value (0.65) slightly higher than that for thiamine (0.60)($n = 2$). A reference spot containing ACh and 2-methylthiamine which was developed by system 3 also moved with the solvent front whereas 2-methylthiamine alone had an R_f-value of 0.65 ($n > 10$). Thus compound A and 2-methylthiamine behaved identically in system 3. It was impossible to separate compound A and thiamine completely by paper chromatography, but compound A was clearly separated from thiamine on thin layer chromatography; compound A showed the chromatographic properties of 2-methylthiamine (Fig. 2).

Compound A was also found when extracts from ventral roots injected with 5 μCi of [^{35}S]thiamine were investigated using thin layer chromatography (Fig. 2)($n = 5$). Thus both choline and thiamine may serve as precursors for compound A. Choline, ACh and compound A constituted 6–14% of the total radioactivity in ventral roots. In dorsal roots the levels of both ACh and compound A were lower (Fig. 1). The content of compound A in the ventral roots was about 2–100 pmol (7–35 ng) per 0.1 g of ventral roots, if calculations are carried out using the known specific activities of the isotopes and if compound A is assumed to have the same specific radioactivity as the added precursor [^{3}H]choline. Control experiments ($n = 6$), where [^{3}H]choline or [^{3}H]ACh was added to the ventral roots immediately before the homogenization with allylcyanide, showed only choline or ACh respectively in the chromatograms. Thus compound A is not an artefact of the procedure used.

Cat dorsal roots

In chromatograms from extracts of cat dorsal roots *in vivo*, the peaks corresponding to both compound A and ACh were much smaller (Fig. 1b) than in those from extracts of ventral roots, despite the fact that the dorsal roots contained large amounts of free choline ($n = 5$).

Choline is metabolized along three pathways, namely to metabolites in the intermediary metabolism (by donating methyl groups after oxidation to betaine [9]), to ACh and to phospholipids after oxidation to betaine [9]), to ACh and to phospholipids.

Compound A and intermediary metabolism of choline

The possibility that compound A is identical with one of the known choline metabolites in the intermediary metabolism was investigated by dissolving each of the following substances (100 μg) in 1.0 ml of 0.9% NaCl, extracting it and chromatographing the extract: ethanolamine, betaine-aldehyde, betaine

Table 1.

Chromatography, R_f-values	Compound A	2-methylthiamine
Butanol–ethanol–HAc–H_2O	0.25–0.28	0.26–0.28 ($n = 10$)
Propanol–1 M sodium acetate buffer (pH 5)–water	0.46–0.51	0.44–0.50 ($n = 4$)
Propanol–1 M ammonium fornate–water	0.64–0.67	0.64–0.68 ($n = 6$)
Butanol–ethanol–conc. hydrochloric acid–water	0.21–0.23	0.20–0.23 ($n = 10$)

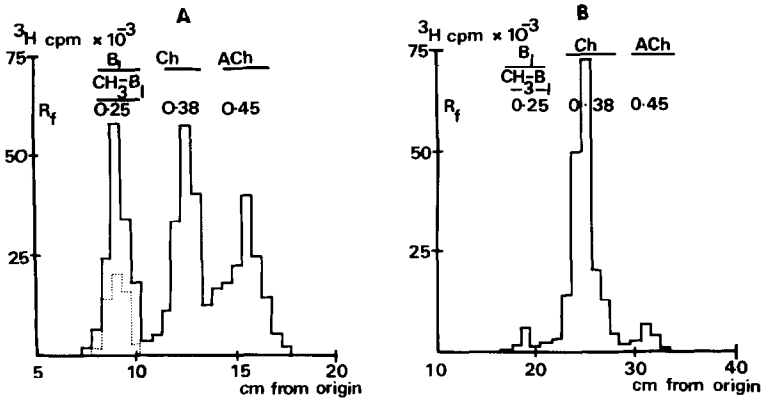

Fig. 1. Paper chromatogram of extracts from (A) ventral roots labelled with [^{3}H]choline. Solvent system: *n*-butanol–ethanol–acetic acid–water (8:2:1:3), ascending. Three main peaks are visible corresponding to compound A, choline and ACh. The broken line indicates the [^{35}S]thiamine peak. (B) Dorsal roots labelled with [^{3}H]choline. Solvent system: the same but descending. Three peaks are visible. Compared with A, the peak corresponding to choline dominates and the peaks corresponding to ACh and compound A are both much smaller. All chromatograms with significant peaks had the same appearance as those shown in the Figs.

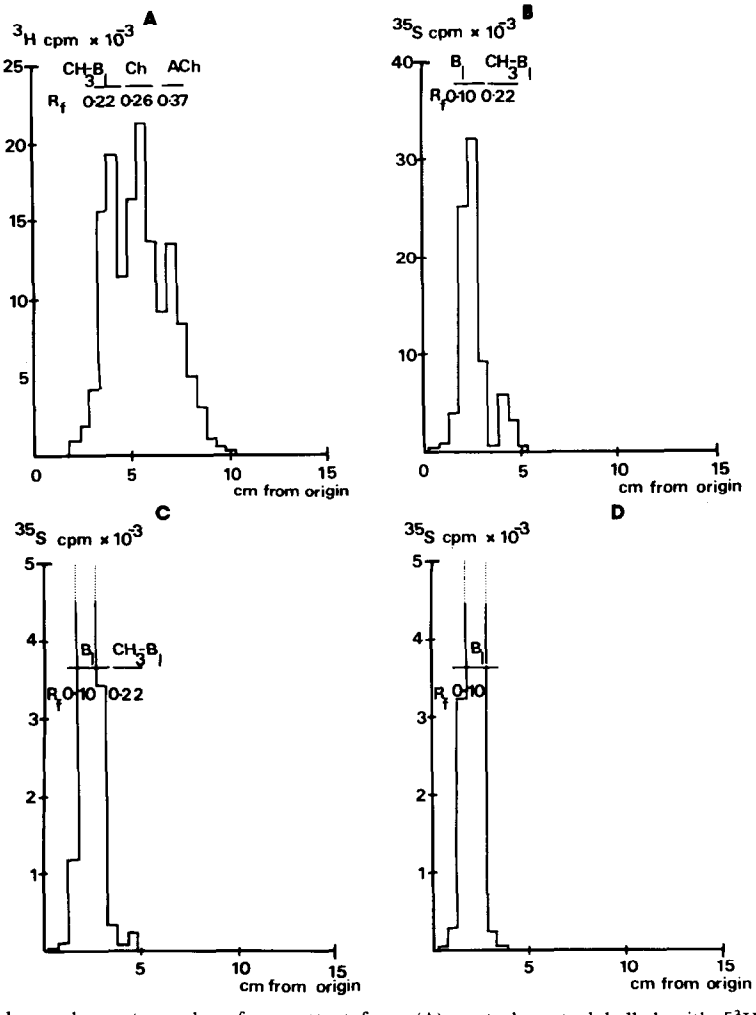

Fig. 2. Thin layer chromatography of an extract from (A) ventral roots labelled with [^{3}H]choline. Solvent system: *n*-butanol–ethanol–conc. HCl–water (8:2:0.5:2.5). Three peaks corresponding to compound A, choline and ACh are visible. (B) Ventral roots labelled with [^{35}S]thiamine. Two peaks are seen, one corresponding to thiamine and the other to compound A. (C) The *in vitro* incubation of radioactive thiamine and cold ACh at pH 9. The main peak corresponds to thiamine (B_1) and the small peak to compound A. (D) The *in vitro* incubation at pH 3. No peak corresponding to compound A can be seen.

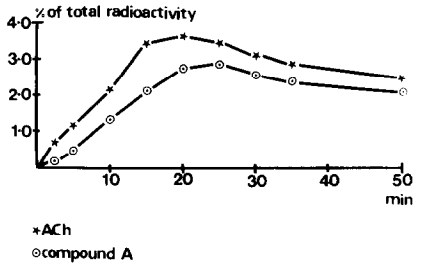

Fig. 3. Content of compound A and ACh at different time intervals after injections of radioactive choline (1 μCi) into different ventral roots. It was difficult to get the same amount of radioactive choline into the different roots, the radioactivity corresponding to ACh and compound A was expressed as a percentage of the total radioactivity.

chloride, *N*,*N*-dimethylglycine, *N*-hydroxy-sarcosine, *N*-hydroxy-methylglycine, methionine, purine, thymine, creatine and creatinine. Each substance was investigated 5 times. Compound A was not identical with any of the intermediary choline metabolites investigated.

Injections with 5 μCi of adenosyl-L-(*S*-$^{14}CH_3$)methionine or 5 μCi of [^{14}C]betaine hydrochloride which are formed from choline before the release of methyl groups to the methyl pool of the intermediary metabolism gave considerable radioactivity in the ventral roots. Less than 150 cpm of [14]choline chloride could be found when the ventral roots were extracted and chromatographed ($n = 6$). No compound A was detectable in the chromatograms.

Compound A and ACh

In order to study the possible correlation between the formation of compound A and ACh, 1 μCi of [^{3}H]choline was injected directly into the ventral roots. Extractions with subsequent chromatography were carried out after 2.5, 5, 10, 15, 20, 25, 30, 35 and 50 min ($n = 3 \times 9$). The levels of both ACh and compound A increased similarly with time (Fig. 3).

Compound A and lipid-soluble metabolites

Lipid-soluble choline metabolites were studied after injections of [^{3}H]choline into the ventral roots. The metabolites were extracted by homogenization in 3 × 1 ml of chloroform-methanol (2:1) after 2.5, 5, 10, 15, 20, 25, 30, 35, 50 and 270 min ($n = 3 \times 10$). The amount of lipid-soluble choline (phospholipids) continued to increase for at least 3 hr whereas the amount of compound A reached a maximum after 20–30 min.

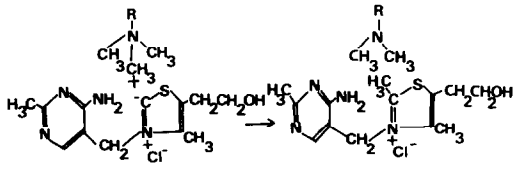

Fig. 4. A suggested reaction model for the formation of 2-methylthiamine. The thiamine molecule can form an anion at the C2-carbon of the thiazole [7]. Since the main positive charge of the ammonium ion of ACh is distributed on the methyl groups [9], an electrostatic attraction will result between the thiamine anion and one of the methyl groups of the ammonium ion. A demythylation of the quarternary ammonium will then be possible with the thiamine anion acting as the methyl acceptor. After demethylation, the electrostatic polarity and thus also the ionic attraction will be lost.

Formation of compound A in rat brain, liver, kidney and spleen

The formation of compound A in various tissues in the rat was studied by injecting [^{3}H]choline directly into brain, liver, kidney and spleen using a microsyringe (5 μCi in 2 μl of 0.9% NaCl into each organ). Three rats were used. Compound A was found in the brain but not in the liver, kidney or spleen. The brain was the only organ of those investigated in which detectable formation of radioactive ACh took place. The chromatograms ($n = 9$) from extracts of liver, kidney and spleen contained only traces of free choline (150–200 cpm), suggesting rapid incorporation into phospholipids. Less compound A was found in rat brain than in cat ventral roots: the amount of compound A in the rat brain corresponded to 4.1–6.3% of the ACh formed (23,000–39,000 cpm of [^{3}H]ACh) as calculated from the radioactivity ($n = 3$).

In vitro *incubations*

With tissue. No radioactivity corresponding to compound A was formed when homogenates of ventral roots were extracted and chromatographed according to the procedure described. When chopped tissue was used for incubation, a small peak (850–1400 cpm) corresponding to compound A appeared after incubation with [^{3}H]choline. Blank incubations in which the tissue had been kept at 70° for 30 min prior to incubation gave only labelled choline in the chromatograms. Labelled adenosyl-L-(*S*-$^{14}CH_3$)methionine gave only trace amounts of choline in the chromatograms.

Without tissue. Trace amounts of radioactive material which behaved like compound A were formed at pH 9.0, while nothing was formed at pH 7.9 or pH 3.0 when [^{3}H]ACh and thiamine were incubated *in vitro*.

DISCUSSION

Chemical identification of compound A

Evidence is presented that nerve tissue, especially ventral roots, can convert choline to a compound A with several characteristics in common with 2-methylthiamine. Both compounds behave identically with the ion pair extraction used. Compound A has the chromatographic properties of 2-methylthiamine when chromatographed in various systems.

The formation of compound A occurs from two precursors, namely choline and thiamine– supporting the hypothesis that compound A is identical with 2-methylthiaimine.

Compound A and choline metabolism

The formation of compound A required the presence of intact or chopped tissue, and was not detected to any measurable extent in cell-free extracts or in buffer solutions (pH 7.9). Compound A could not be recovered from heated tissue, which suggests that one

or several enzymes may be involved in its formation. It was not formed from [^{14}C]betaine chloride or adenosyl-L-(*S*-$^{14}CH_3$)methionine *in vivo* or *in vitro*. The time course of the synthesis of compound A following a single injection into cat ventral roots was similar to that for ACh (Fig. 3), but distinct from the synthesis of labelled lipid-soluble choline (phospholipids), which continued to increase for more than 3 hr. No compound A was found in tissues with non-detectable amounts of *radioactive* ACh—such as liver, kidney and spleen.

The levels of both ACh and compound A were lower in dorsal roots than in ventral roots even though the dorsal roots contained much free choline. In ventral roots there was a parallel increase of ACh and compound A. These results suggests that compound A may be formed from ACh.

Demethylation and nicotinic receptor activity

A demethylation reaction with ACh as the methyl donator and the unmethylated compound A (thiamine) as the methyl acceptor is shown in Fig. 4. Demethylation of ACh may be of importance in the interaction between nicotinic receptors and ACh for the following reasons. The trimethyl ammonium group in ACh is essential for its intrinsic activity while the ester group contributes to the affinity [10]. The main positive charge of the trimethyl ammonium ion is distributed on the methyl groups surrounding the nitrogen, which is almost neutral [11]. Thus the three methyl groups form a sphere of positive electrical charge that is capable of interacting with the anionic centre of the nicotinic cholinergic receptor. An interaction of thiamine with nicotinic receptors is more probable than with muscarinic receptors since thiamine has a selective affinity to structures mediating the nicotinic response [12]. The charge distribution may facilitate an electrostatical interaction between a methyl group of the ammonium ion and the negatively polarized receptor, leading to a demethylation of ACh (see Fig. 4 for a proposed reaction model). The protonized "demethylated ACh" (dimethylaminoethylacetate) has 200 times less activity than ACh on the frog rectus abdominis muscle [13].

The effect of the ACh molecule is thus diminished 200-fold by the demethylation. Free energy of about 8500 cal is released by the demethylation of the trimethylammonium ion of betaine [14]. The methyl groups of the dimethylammonium ion have first to be oxidized before they can be split off from the molecule. Thus the different effects of trimethyl alkyl and dimethylalkyl ammonium ions on the nicotinic receptor can be explained by their different abilities to be demethylated and to release energy. The intrinsic activity of ACh would be due to demethylation which in turn releases energy.

Could the demethylation of ACh (including the intermolecular forces resulting in demethylation) explain its effect on striated muscle? This question may be partly answered by comparing the chemical structure of substances that have an effect with those that have not. If the *N*-alkyl substituents are increased in size and the binding characteristics changed from ionic to non-ionic, a decline in the cholinomimetic action and an increase in the cholinolytic activity appear [15]. In this way, a direct electrostatic contact between the positive ammonium ion and the receptor anion is sterically hindered, thus preventing a dealkylation according to the reaction model suggested. Depolarizing blocking agents of the neuromuscular junction contain trimethylalkylammonium groups and thus allow a direct electrostatic interaction between a methyl group and the receptor anion.

The present model for interaction of ACh on the nicotinic receptor which involves methylation of thiamine is hypothetical. More information is needed about the conditions determining the extent of compound A formation and about interactions between thiamine and nicotinic receptors. A chemical characterization of compound A is also desirable.

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