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THE EFFECTS OF INHIBITING CHOLINE DEHYDROGENASE ON CHOLINE METABOLISM IN MICE

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Abstract—3,3-Dimethylbutanol (Dimbunol), a competitive inhibitor of choline dehydrogenase (CDH), and ethylcholine mustard aziridinium (ECMA), an effective irreversible inhibitor of both CDH and choline transport, were investigated for their effects upon the uptake and metabolism of [^{3}H]choline in mice.

Thirty minutes after Dimbunol administration (i.p. 0.5 mmoles/kg) a reduction in the rate of choline oxidation was accompanied by an inhibition of choline phosphorylation in the kidney. Choline had accumulated to 5-fold the control level. After ECMA (i.v. 4 μmoles/kg), kidney choline was elevated 18-fold and both oxidation and phosphorylation rates were severely inhibited.

In the liver Dimbunol inhibited oxidation and phosphorylation of choline and generated a 2-fold rise in tissue choline. Ethylcholine mustard aziridinium inhibited both oxidation and phosphorylation in the liver to the same extent as in the kidney but produced only a 3-fold elevation of choline. Dimbunol failed to elevate serum choline 30 min after administration and brain choline and acetylcholine levels were also unchanged. Serum choline was doubled by ECMA. These studies suggest that both transport across the renal tubules and oxidation may be important in choline regulation, that high levels of choline may accumulate in the liver and kidney which are not available for acetylcholine synthesis but that longer term studies on the effects of Dimbunol might reveal useful ways of facilitating sustained elevation of serum choline in precursor therapy.

The use of precursor therapy in disorders associated with a deficiency in cholinergic function has been widely but inconclusively investigated (for review see [1]). One explanation for the inconsistent results obtained from such studies may arise from the lack of knowledge regarding the metabolic events which follow the entry of choline into the bloodstream. The success of precursor therapy probably relies on the persistence of elevated serum-precursor levels but the question of how choline levels are regulated has received comparatively little attention.

In this capacity a number of studies have pointed to the potentially important role played by the FAD-linked mitochondrial enzyme, choline dehydrogenase (EC 1.1.99.1). Zeisel and Wurtman [2] have demonstrated a relationship, in young rats, between developmental changes in choline levels and choline dehydrogenase activity. Haubrich *et al.* [3] have suggested an explanation for the action of dimethylaminoethanol (Deanol) in raising tissue-choline levels, by demonstrating an inhibition of choline metabolism in the liver and kidney of Deanol-treated mice. Choline dehydrogenase has been shown to be responsible for the production of large quantities of betaine (e.g. [4]) the metabolic value of which is questionable [5].

In the present investigation the regulation of choline metabolism by intervention with compounds designed to interfere with choline dehydrogenase activity has been studied in the hope that such a strategy might lead ultimately to the possibility of pharmacologically enhancing precursor therapy through the inhibition of choline catabolism.

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MATERIALS AND METHODS

[^{14}C-Methyl]choline chloride (58 mCi/mmole), [^{3}H-methyl]choline chloride (78 Ci mmole), [^{14}C-1] acetylcoenzyme A (3 mCi mmole) and [^{32}P]ATP (3.3 Ci/mmole) were all from Amersham Radiochemicals, U.K. 3,3-Dimethylbutanol (Dimbunol) was from Aldrich Chemical Co., Gillingham U.K. Ethylcholine mustard aziridinium (ECMA) was obtained in the form of its precursor, *N*-2-acetoxyethyl-2-chlorethylethanamine from Research Biochemicals Incorporated, Wayland, MA 91778, U.S.A., and made up by cyclization in distilled water, followed by deacetylation in NaOH and neutralization with HCl, as previously described [6].

Treatment of animals. Female CF/1 mice (20–25 g) were used in all experiments and allowed free access to food and drink before and after treatment. 3,3-Dimethylbutanol (75 mM solution adjusted to isoosmolarity with NaCl) was i.p. injected (0.48 mmoles/kg). Ethylcholine mustard aziridinium was administered i.v. (tail vein, 4 μmoles/kg, 6.5 ml/kg). [^{3}H-Methyl]choline chloride was also given i.v. (250 mCi/kg) 30 min after administration of the inhibitor. Animals were killed either by decapitation or microwave. Blood was drained from the carcasses into chilled test tubes, allowed to clot and then centrifuged (at 10,000 *g* for 10 sec) to give serum. Organs were excized into liquid nitrogen within 30 sec of death then stored at −80° for about a week.

Analysis of ^{3}H-*products*. It was decided, on the basis of previous studies (e.g. [7]) and for the sake of economy, to analyse for choline, betaine, phosphorylcholine and phosphatidylcholine and not to measure (in peripheral tissue) acetylcholine, betaine aldehyde and CDP-choline since these are probably minor or transitory products.

Water-soluble metabolites were analyzed using a method derived from the findings of Jellinek *et al.* [8] relating to the precipitation of choline and betaine with ammonium reineckate (ammonium tetrathiocyanodiaminochromate). It was demonstrated that betaine precipitates out with reineckate only when protonated while choline precipitation is pH independent. For the purpose of this analysis it was further shown that phosphorylcholine does not precipitate out with reineckate in the pH range 0.8 (10% TCA) to 14. Furthermore, precipitation of choline and betaine only happens when the solubility product (e.g. 10^{-7} M for [^{3}H]choline) is exceeded. Thus it was possible to reinforce the selectiveness of precipitation by the addition of suitable carriers at the appropriate stages of analysis. The inclusion of [^{14}C-methyl]choline standard enabled the calculation of supernatant contamination with choline.

Whole frozen tissue was homogenized (using a Polytron) in ice-cold distilled water (10 vol.) and aliquots for analysis rapidly deproteinated with 10% TCA. A sample (100 μl) of the supernatant was neutralized (5 N NaOH) and counted in 4 ml PPO-POPOP toluene scintillant in order to estimate total labelled water-soluble metabolites (contamination of this supernatant with ^{3}H-lipids was checked for by chloroform:methanol extraction and found to be negligible). Another aliquot (0.5 ml) was transferred to a separate tube containing 3 N NaOH (0.2 ml) and referred to as the alkali sample. To 0.5 ml of the acid sample (TCA supernatant) were added carriers to a final concentration of 10 mM betaine, 10 mM [^{14}C-methyl]choline (5000 dpm/ml). To the alkali sample [^{14}C-methyl]choline alone was added to give the same final concentration. Then 1.5 vol. of 2% ammonium reineckate was added and all tubes centrifuged at 13,000 *g* for 3 min. Aliquots of supernatant were taken for scintillation counting. By calculating the loss of radioactivity on precipitation in alkaline conditions, choline may be estimated. The further loss of radioactivity on precipitation at acid pH is due to betaine. The remaining activity is chiefly phosphorylcholine (confirmed by TLC using an 8:2:1:3 butanol:ethanol:acetic acid: water solvent and cellulose plates). Efficiencies of scintillation counting were calculated by the construction of quenching curves or by recounting vials after addition of known quantities of radioactivity in small volumes of methanol. The yield of radioactivity in pellets and supernatants were occasionally compared against totals and found to be 104 ± 5% (S.E.M., N = 5).

Lipids were extracted from homogenates by the method of Bligh and Dyer [9]. The chloroform layer was taken, dried by evaporation, chromatographed on a silica plate using 17:7:1 choloroform: methanol:water and developed with iodine vapour. More than 99% of radioactivity was found at R_F 0.24 corresponding to phosphatidylcholine while less than 1% was found at R_F 0.14 (spingomyelin) and 0.47 (phosphatidylethanolamine).

Choline and acetylcholine determinations. Endogenous choline levels were determined in unextracted tissue by a radioenzymatic assay based on the method of Haubrich *et al.* [10]. Whole frozen tissue was homogenized in cold 15% 1M formic acid/85% acetone (10 vol.) and centrifuged at 13,000 *g* for 5 min. Supernatants were washed with ether and freeze dried. Samples were then redissolved in a volume of 100 mM Tris buffer (pH 9.0 at 25°) to give an expected choline concentration of 1–10 nmoles/20 μl. Aliquots of 20 μl were taken and preincubated for 10 min at 37° with 9 mm $MgSO_4$, 12.5 mM NaCl, 12.5 mM NaF, 0.1 mM EDTA, 12 mM Tris base, 3 mM [γ-^{32}P]ATP (10^{-3} μCi/ml) in a volume of 130 μl (pH 9.0). The reaction was started by the addition of 0.01 units of choline kinase in 10 μl and incubation was for 90 min. At the end of this time the reaction was terminated by the addition of 1 ml of chilled Tris buffer (10 mM, pH 9.5) containing 10 mM $MgSO_4$ and 0.1 mg/ml phosphorylcholine. A 1-ml aliquot was taken and run through an ion-exchange column consisting of 1 ml of Dowex-1 (Cl^- form) in a pasteur pipette with a glass-bead in the neck. The sample was washed through with 2.5 ml of Tris/Mg^{2+} buffer (as above) and the eluate collected in plastic inserts, then counted directly for ^{32}P using Cerenkov radiation.

Brain choline and acetylcholine measurements were carried out by the methods of Goldberg and McCaman [11]. Animals (5 per group) were sacrificed by microwave and brains were homogenized in formic acid/acetone and washed with ether as above. Dried samples were analysed for choline with the following modifications. Incubation was for 2 hr and was at pH 7.0. The incubation mixture contained 0.2 units of choline kinase and a small quantity of eserine (0.1 mg/ml). The concentration of cold ATP was reduced to 0.3 mM. For acetylcholine measurement, samples were first incubated with cold ATP as above, then the [γ^{32}P]ATP was added and quickly followed by 10 μl of 10 mM Tris buffer (pH 9.0) containing 0.01 units of acetycholinesterase. Samples were incubated for a further 2 hr, then treated as before. Choline and acetylcholine standards were included in both assays to assess interference. Assays were performed in triplicate and standard errors were generally less than 3% of the mean.

Preparation of enzyme. Choline dehydrogenase was freshly solubilized from acetone-dried powder of rat-liver mitochondria using Na cholate [protein: detergent 1:1 (w/w)]. Detergent was removed by dialysis.

RESULTS

The data presented in Table 1 suggest that Dimbunol is an inhibitor of Na cholate-solubilized choline dehydrogenase. In other studies (not reported here) inhibition was demonstrated to be competitive with a K_i of 0.9 mM. Using the same assay system, dimethylaminoethanol had a K_i of 1.8 mM and the K_m for choline was 9 mM. It also appears from Table 1 that Dimbunol is neither a substrate for, nor an inhibitor of, choline kinase in the range of con-

Table 1. Effects of Dimbunol on choline metabolism and transport, *in vitro*

Enzyme/Transport system	Concentration of Dimbunol (mM)	Rate (per min/mg)
Choline dehydrogenase (rat liver mitochondrial)	0	19.0 ± 1.0 nmole (N = 8)
	5	3.0 ± 0.1 nmole (N = 4)
	10	2.0 ± 0.1 nmole (N = 4)
Choline kinase (from yeast)	0	4.9 ± 0.3 μmole (N = 2)
	5	5.1 ± 0.4 μmole (N = 2)
Choline acetyltransferase (from rat-brain acetone-dried powder)	0	335 ± 45 pmole (N = 2)
	0.15	410 ± 25 pmole (N = 2)
	1.5	460 ± 20 pmole (N = 2)
	15	410 ± 50 pmole (N = 2)
Choline transport (guinea-pig synaptosomes)	0	32 ± 3 pmole (N = 2)
	1.0	25 ± 5 pmole (N = 2)

Choline dehydrogenase (EC 1.1.99.1) and choline kinase (EC 2.7.1.32) were assayed as previously described [6]. Choline acetyltransferase (EC. 2.3.1.6) was measured according to Fonnum [12]. Synaptosomes were prepared from guinea pigs by a modification of the method of Gray and Whittaker [13]. Uptake was measured as follows. Synaptosomes, 100 mg equivalent, were pre-equilibrated for 10 min, washed and resuspended in 0.5 ml normal medium with 0.5 μCi/ml [^{14}C]choline (8.9 μM). After 4 min incubation they were sedimented and washed twice by resuspension in 1.0 ml of normal medium and the [^{14}C]choline which had been taken up was measured. To estimate the carrier-mediated component, it was incubated with 100 μM hemicholinium and the flux was reduced to 3.35 ± 0.30 pmol/min/mg. Errors are S.E.M. or, where N = 2, the range.

centrations tested. Dimbunol does not appear to inhibit choline acetyltransferase, although there remains a faint possibility that Dimbunol is itself acetylated by the enzyme and extracted with the [1-^{14}C]acetylcholine. Nor does Dimbunol significantly inhibit choline transport into synaptosomes. These results prompted the administration of Dimbunol to mice in order to study its effect on *in vivo* choline metabolism. Ethylcholine mustard aziridinium, a very powerful irreversible inhibitor of both choline dehydrogenase [6] and choline transport, was also administered in selected experiments as a comparison. Since tissue choline levels were to be measured using a radioenzymatic assay it was important to check that Dimbunol did not itself interfere with the assay. Figure 1 illustrates the perturbation of the standard curve introduced by the inclusion of 750 nmoles of Dimbunol in the reaction mixture and the effect of various concentrations of Dimbunol on the radioactivity recovered in the eluant obtained from a standard sample containing 2.5 nmoles of choline. It is reasonable to conclude that unless very high quantities (10 times the amount of choline) of Dimbunol have accumulated in the sample tissues, the effect of the compound on measurements of choline levels will not be significant.

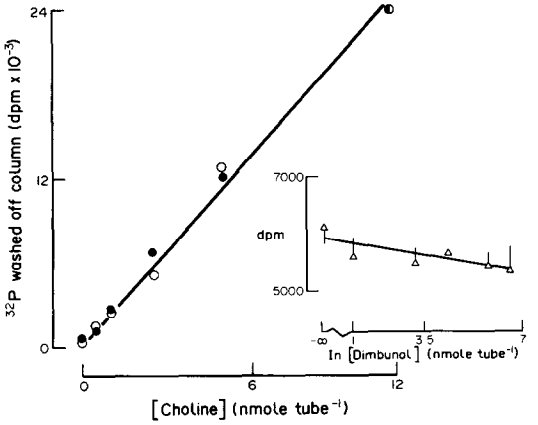

Fig. 1. The effect of Dimbunol on the assay employed for determination of choline concentration. Standard solutions of choline chloride were assayed in the presence and absence of Dimbunol using the modified procedure of Haubrich *et al.* [10] described in the text. —○—, no Dimbunol present: —●—, +750 nmoles of Dimbunol/tube. Inset: —△—, −2.5 nmoles of choline/tube + various quantities of Dimbunol. All points represent the mean of triplicate measurements. Error bars are the range.

Figure 2 summarizes the overall pattern of [^{3}H]-choline uptake from the blood and its subsequent distribution. Radioactivity is rapidly removed from serum with a half-life of 5–10 sec. It subsequently appears mainly in the liver and kidney which also contain most of the choline dehydrogenase activity.

The kidneys are initially the most active tissue in uptake, incorporating nearly 10% of the injected ^{3}H within 30 sec while the liver takes up label more slowly, accumulating 25% of the label over 10 min. Thirty minutes after Dimbunol treatment, the half-life of label in serum is extended 2–3 fold. It is notable that this effect is not a manifestation of slower rates of uptake in the kidney and liver which, as is apparent from Fig. 2b, are not significantly affected by Dimbunol.

From Figures 3a and b it would appear that, in control animals, choline is metabolized extremely rapidly once it has entered the tissue. Within 60 sec an approx. total of 12% of the original [^{3}H]choline is present in the liver and kidneys as [^{3}H]betaine

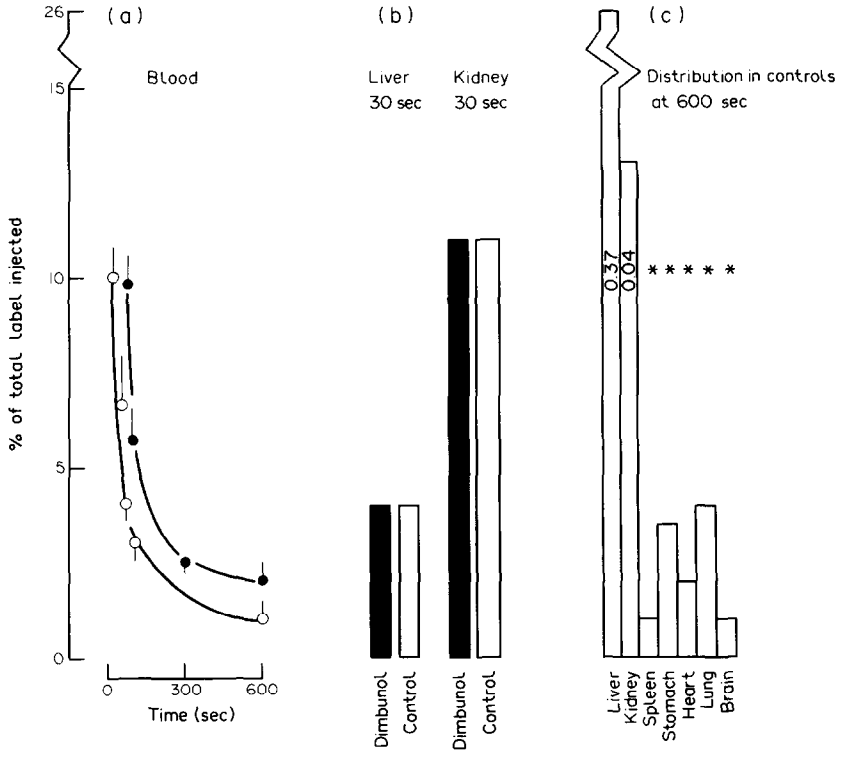

Fig. 2. The distribution, in mouse, of radioactivity following administration of [^{3}H]choline and the effect of prior i.p. injection of 0.5 nmoles/kg Dimbunol on the half-life in blood and uptake by kidney and liver, of the label. (a) ^{3}H-Label in blood: —○—, untreated animals; —●—, animals treated with Dimbunol (S.E.M., N = 4). (b) ^{3}H-Label in liver and kidney 30 sec post-injection of radioactivity: black bars, animals treated with Dimbunol; white bars, untreated animals (average of 4 animals/group) (c) Distribution of ^{3}H-label in control animals at 600 sec post-injection of radioactivity. The number in parentheses represents the choline dehydrogenase activity expressed as units/organ (* indicates < 0.01).

compared to the 3% present as [^{3}H]phosphorylcholine. In Dimbunol-treated animals, these totals are reduced to 6 and 1.5%, respectively.

Table 2 summarizes the effect of both ECMA and Dimbunol on tissue levels of choline and on the rate of oxidation and phosphorylation of choline. Ethylcholine mustard azirdinium, which is a much stronger *in vivo* inhibitor of both enzymes, is more effective in raising choline levels than Dimbunol. Both compounds cause a greater elevation in kidney than in liver choline. It is interesting that this is not a reflection of a lowering of choline oxidation or

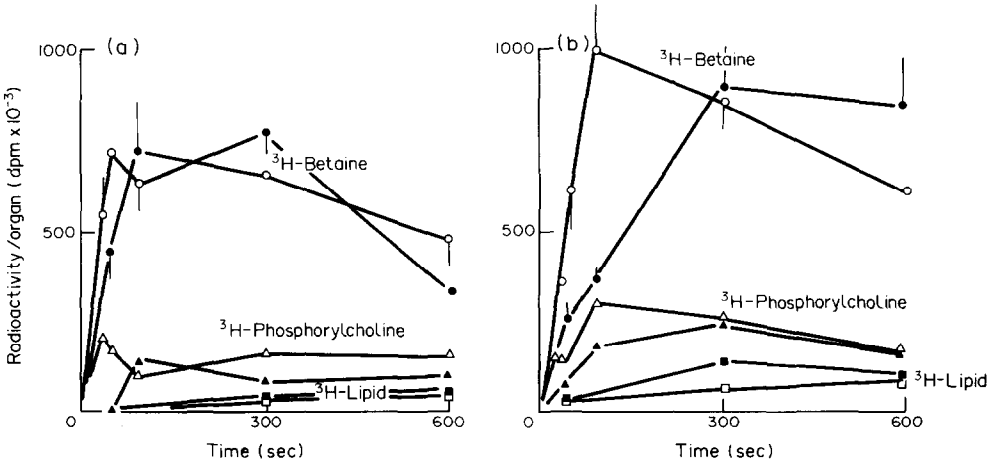

Fig. 3. The effect of Dimbunol on the metabolism of [^{3}H]choline in liver (a) and kidney (b) of mouse. —○—, betaine; —△—, phosphorylcholine; —□—, lipids. Closed symbols are Dimbunol-treated animals; open symbols are controls. Error bars are the S.E.M. for N = 4.

Table 2. Choline concentrations and turnover in the tissues of treated and untreated mice

Tissue	Treatment	Choline concentration (nmole/g or per ml)	Rate (nmole/min/g) of: Phosphorylation	Oxidation
Kidney	Control	40 ± 10	142 ± 30	648 ± 90
	Dimbunol	260 ± 20*	50 ± 10*	410 ± 40*
	ECMA	740 ± 190*	>5*	50 ± 10*
Liver	Control	120 ± 15	196 ± 25	632 ± 90
	Dimbunol	213 ± 25*	105 ± 25	380 ± 50*
	ECMA	275 ± 60*	6 ± 5*	56 ± 19*
Plasma	Control	15 ± 5	nc	nc
	Dimbunol	22 ± 2	nc	nc
	ECMA	40 ± 2	nc	nc

Animals were treated in the way described in the Methods section. The rates were approximated from the following simplified formula:

[^{3}H-Product (dpm/organ at time x) × Endogenous choline (nmol/g)]/[^{3}H-choline (dpm/organ at time x) × x (min)].
nc, not calculated.

* $P < 0.05$ (Student's t-test compared with controls).

phosphorylation rates measured 30 min after administration since these appear to be inhibited to a roughly similar extent in both tissues. Serum choline is significantly higher after ECMA treatment but not after Dimbunol—in neither case does the magnitude of elevation compare to that seen in the kidneys. Table 3 gives choline and acetylcholine concentrations in the brain of control and Dimbunol-treated animals sacrified by microwave radiation 30 min after treatment. There is no significant difference between the two groups.

DISCUSSION

Dimbunol is a better inhibitor of choline dehydrogenase than Deanol and *in vitro* appears not to affect other enzymes of choline metabolism or transport. In contrast to Deanol, it is extremely unlikely that Dimbunol could be metabolized to choline [15]. Hence the finding that Dimbunol administration (0.5 mmoles/kg) generates elevated choline levels in kidney and liver (though not in serum) supports the argument that the inhibition of choline dehydrogenase constitutes a feasible strategy for achieving raised tissue choline concentrations.

However, there are a number of points which obscure the exact mechanism of action of these compounds. Anomalously both ECMA and Dimbunol appear to inhibit choline phosphorylation *in vivo*.

Table 3. Effects of Dimbunol treatment on brain choline and acetylcholine concentrations

Treatment	Choline (nmole/g)	Acetycholine (nmole/g)
Control	63 ± 13	11.0 ± 2.6
Dimbunol	53 ± 6	11.4 ± 0.8

Animals were treated as described in the Methods section.
Errors are S.E.M.; N = 5.

At 30 min post-injection Dimbunol does not inhibit oxidation and phosphorylation in the kidney significantly more than in the liver. Yet kidney choline levels are elevated far more than choline levels in liver. It could be that the effects of Dimbunol are transitory and have allowed an accumulation of choline which persists after Dimbunol has dispersed and enzymatic rates are returning to normal. The fact that oxidation and phosphorylation rates are both much lower 30 min after treatment with ECMA (which inhibits irreversibly) would be consistent with this explanation.

Similarly metabolism is equally inhibited by ECMA in both liver and kidney but the elevation of choline in the kidney is 5-fold higher than in liver. It is conceivable that this is a result of another effect of ECMA, the inhibition of choline transport across the renal tubules. It might be the case that the transport of choline is at least as important an element in renal choline metabolism as is oxidation and therefore kidney choline concentrations are more susceptible to ECMA than hepatic choline levels.

It is also notable that the elevation of serum choline is relatively small compared with that of kidney and liver choline and it is hardly surprising that there is no significant increase in brain choline and acetylcholine levels after Dimbunol treatment. This result suggests that in the short-term at least, the kidney and liver can accumulate large quantities of free choline which are probably not immediately available for acetylcholine synthesis.

It is worth drawing attention to the fact that the dose of ECMA administered in this study (4 μmoles/kg i.v.) does not differ markedly from the intracerebroventricular doses used in mice by Fisher *et al.* [15] in their attempts to employ ECMA as a selective neurotoxin for cholinergic neurons. It is quite conceivable that ECMA crosses the blood–brain barrier and, if this is the case, one would expect over a period of days, large accumulations of choline in peripheral tissue. The implication of the presence

in the periphery of copious quantities of precursor should certainly be considered if ECMA is to be used as an agent for inducing animal models of Alzheimer's disease [16].

Intervention with Dimbunol and ECMA represents useful methods for unraveling the complexities of choline regulation. The long-term effects on serum choline of chronic Dimbunol treatment and the possibility of potentiating the effect of a high choline diet with Dimbunol would seem to be fruitful avenues for future studies.

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