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Effect of desmethylimipramine on acetylcholine uptake by slices of rat brain cortex

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INHIBITION of the neuronal membrane pump for amine uptake mechanism appears to be an outstanding feature of the action of tricyclic antidepressants. Imipraminics are known to interfere with the neuronal uptake of norepinephrine,[1–3] serotonin[4,5] and dopamine[6] in various areas of the brain. It has also been reported that imipramine-like compounds possess anti-cholinergic and anti-acetylcholine properties[7–10] and recent evidence [11,12] indicates that desmethylimipramine (DMI) is capable of altering the acetylcholine content in the striatum and the ratio of "free" and "bound" acetylcholine in the whole brain of rats. The aim of the present experiments was to investigate whether DMI, a potent inhibitor of norepinephrine and serotonin uptake, would also affect the accumulation of acetylcholine (ACh) by brain tissue. Although earlier investigations[13,14] failed to demonstrate a significant accumulation of ACh by brain slices, recent studies[15–18] have shown that, in the presence of a suitable inhibitor of cholinesterase, ACh is taken up by cerebral tissue *in vitro* and that this process can be modified by drugs.

Male Sprague–Dawley rats, 180–220 g, were killed by decapitation and their brains were rapidly removed. Brain cortex slices (about 0·5 mm thick) were cut from the cerebral hemospheres using a Stadie–Riggs slicer, weighed immediately as described by McIlwain and Rodnight[19] and suspended in oxygenated incubation medium of the following composition (mM): NaCl, 128·0; KCl, 5·0; $CaCl_2$, 2·7; $MgSO_4$, 1·3; NaH_2PO_4, 10·0 (brought to pH 7·0 with 1 N HCl); glucose, 10·0. All of the above procedures were performed in a cold room (0–4°). Slices (50–70 mg/vessel) were pre-incubated (at 37° under oxygen) with a cholinesterase inhibitor, Sarin (isopropyl methylphosphonofluoridate, 10^{-5} M) in a Dubnoff metabolic shaker for 15 min after which the labeled acetylcholine (C^{14}-ACh) was added to make up to the desired final concentration. The incubation was then continued under oxygen for predetermined periods of time (15–120 min). When the effect of drugs was studied on ACh uptake, the various drugs were added at the same time as ACh. The final volume of the incubation medium in each vessel was 2·5 ml. At the end of the incubation period, slices were quickly removed from the vessels and rinsed with 20 ml of fresh incubation medium containing a concentration of non-radioactive ACh equal to that of labeled ACh in the original medium. Slices were then lightly blotted and transferred to a counting vial containing 0·5 ml of N.C.S. Tissue Solubilizer (Amersham/Searle). After they were completely dissolved, 20 ml of scintillation liquid was added to the vials and the radioactivity measured in a Nuclear Chicago Mark I liquid scintillation counter. The scintillation liquid contained 4 g of Omnifluor (98% of 2,5-diphenyloxazole (PPO) and 2% of Bis-MSB; New England Nuclear Corp.) per litre of a 2:1 (v/v) mixture of scintillation grade

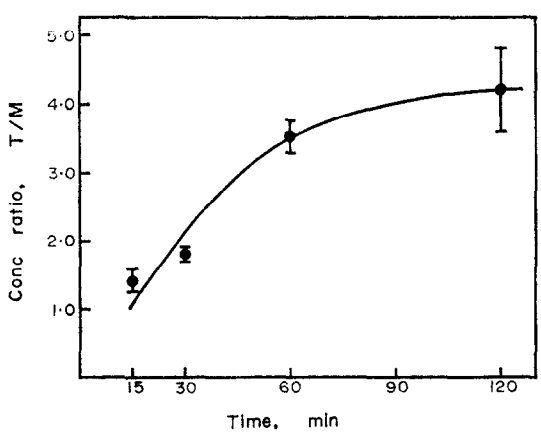

FIG. 1. Time course of C^{14}-ACh accumulation by cerebro-cortical slices of rats. Concentration of acetylcholine in the incubation medium was 10^{-5} M. Cholinesterase was inhibited by Sarin (10^{-5} M). Each point represents the mean ± S.E.M. of at least four determinations.

TABLE 1. UPTAKE OF C^{14}-ACh BY CORTICAL SLICES IN THE PRESENCE OF SARIN OR ESERINE AND IN THE ABSENCE OF AN INHIBITOR OF AChE*

Concn of labeled ACh in medium	AChE inhibitor	ACh concn ratios (T/M) corrected for passive diffusion
1×10^{-5} M	Sarin (10^{-5} M)	3·4 (2·6; 4·2)
1×10^{-5} M	Eserine (10^{-5} M)	1·1 (1·1; 1·1)
1×10^{-5} M	None	1·15 (1·1; 1·2)

* Incubation conditions: 1 hr at 37° in O_2.

toluene and Triton X-100. A 0·1-ml aliquot was added to 20 ml of scintillation liquid and used to measure the radioactivity of the suspending media at the end of the incubation period. Quench corrections were made by the channel ratio procedures. The counting efficiency for C^{14} ranged from 82 to 85 per cent.

Concentration ratios (tissue:medium) for C^{14}-ACh were calculated by dividing the amount of radioactivity (dis/min) in the tissue per g of initial wet weight by the amount of radioactivity in 1·0 ml of incubation medium. Tissue–medium (T/M) ratio of C^{14}-ACh at 0° incubation under nitrogen for 60 min was 0·84 ± 0·03 (N = 4). This value is close to the one (0·85) found at high external ACh concentrations under aerobic incubation at 37° and is believed to represent a passive diffusion[16,17]. The active component of ACh uptake was calculated by subtracting 0·84 from the experimentally found values. No attempt was made to identify the labeled ACh taken up by slices. However, indirect evidence does indicate that ACh was not broken down under our experimental conditions since Sarin (10^{-5} M) had already inhibited all measurable cholinesterase activity in slices after 15 min of pre-incubation. Radioactive acetyl-1-C^{14}-choline chloride (specific activity 13·7 mCi/m-mole) was obtained from Amersham/Searle Corp. and eserine sulphate from National Biochemicals Corp. Sarin was kindly supplied by Dr. Heggie from Defence Research Establishment, Ottawa, and desmethylimipramine (HCl) by Geigy Pharmaceuticals.

In order to study the effect of DMI on the uptake of ACh, we first established the characteristics of C^{14}-ACh accumulation in brain tissue under our experimental conditions. When slices of rat brain cortex were incubated in a medium containing radioactive ACh (10^{-5} M) and a cholinesterase inhibitor, Sarin (10^{-5} M), C^{14}-ACh was found to accumulate in the tissue against the concentration

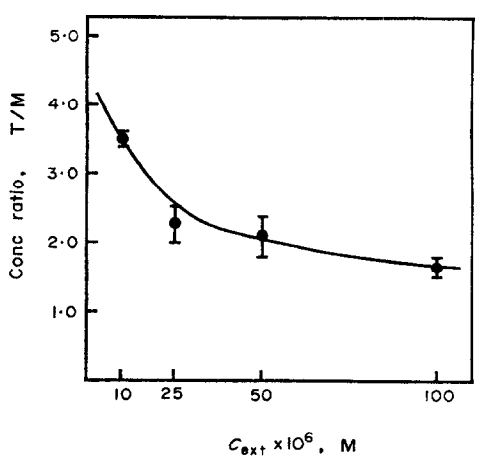

FIG. 2. Effect of variation in external acetylcholine concentration on the accumulation of radioactive C^{14}-ACh in slices of rat brain cortex incubated for 60 min under oxygen. C_{EXT}, concentration of labeled acetylcholine in incubation medium. Each point represents the mean ± S. E. M. of at least four determinations. Concentration of Sarin in incubation medium was 10^{-5} M.

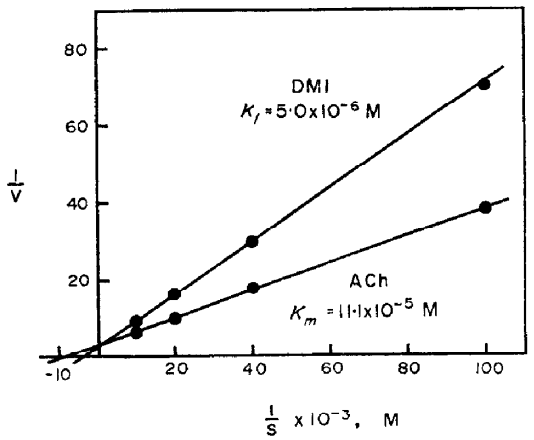

FIG. 3. Graphic analysis of the inhibition of C^{14}-ACh accumulation into brain cortex slices by desmethylimipramine (DMI). Plot of the reciprocals of external C^{14}-ACh concentration (S) and its accumulation (V) into cortical slices in the absence (ACh) and presence of desmethylimipramine, 10^{-5} M (DMI). Slices were preincubated in 10^{-5} M Sarin for 15 min, after which various concentrations of labeled acetylcholine and desmethylimipramine were added and the incubation was continued at 37° for 60 min. ACh accumulation (V) is expressed as micromoles per gram of tissue. Each point represents a mean of two to four independent determinations.

gradient. Time–course study of C^{14}-ACh accumulation presented in Fig. 1 showed a significant increase in T/M concentration ratio between 30 and 60 min of incubation with a value of 3·53 ± 0·23 (N = 9) being attained at the latter time interval. It appears that during the second hr, steady-state levels were reached, since the T/M ratio at 120 min did not increase markedly and the curve flattened. Table 1 shows the values for active uptake of C^{14}-ACh by cortical slices incubated for 60 min in the presence of Sarin or eserine and in the absence of a cholinesterase inhibitor. When eserine (10^{-5} M) was used instead of Sarin, the active uptake of C^{14}-ACh was considerably lower; T/M ratios were similar to those obtained in the absence of an inhibitor of cholinesterase. The effect of variation in external ACh concentration on the accumulation of C^{14}-ACh in cortical slices during 60 min of incubation is shown in Fig. 2. The highest T/M ratio was obtained at the lowest external ACh concentration and the ratios were decreased by increasing the amount of ACh in the medium. The kinetics of C^{14}-ACh accumulation were analyzed by Lineweaver–Burk plots.[20] Results presented in Fig. 3 demonstrate that when reciprocals of active ACh uptake (1/V) were plotted against the reciprocals of external ACh concentration (1/S), a linear relationship was obtained. The apparent Michaelis constant (K_m) given by the intercept on the ordinate is $11{\cdot}1 \times 10^{-5}$ M.

To study the effect of DMI on the accumulation of labeled ACh, cortical slices were incubated in the presence of Sarin with varying concentrations of C^{14}-ACh (10^{-5}–10^{-4} M) and with a fixed amount of DMI (10^{-5} M). The analysis of kinetic data (Fig. 3) indicated a competitive type of inhibition with a K_i value of $5{\cdot}0 \times 10^{-6}$ M. To further characterize the inhibitory effect of DMI on ACh uptake, experiments were carried out in which the accumulation of C^{14}-ACh by cortical slices was studied in the presence of varying concentrations of DMI (10^{-4}–10^{-6} M) but of a fixed amount of ACh (10^{-5} M) in the incubation medium. As shown in Fig. 4, the relationship between the log of the DMI concentration and the percentage of ACh uptake was linear. The ID_{50}-value for DMI derived from this plot was $3{\cdot}5 \times 10^{-6}$ M.

Our results confirm the recent observation[16–18,21] that brain cortex slices incubated in the presence of a suitable cholinesterase inhibitor take up ACh by an active process and demonstrate that the uptake of C^{14}-ACh by slices of rat brain cortex is competitively inhibited by DMI. The K_m-value for C^{14}-ACh uptake found in our experiments ($11{\cdot}1 \times 10^{-5}$ M) is similar to the one ($10{\cdot}8 \times 10^{-5}$ M) reported by Liang and Quastel[22] for rat brain cortex slices incubated in the presence of a different cholinesterase inhibitor, paraoxon. The observation that much smaller uptake of C^{14}-ACh occurred when eserine was used instead of Sarin as an inhibitor of cholinesterase is not surprising since eserine has been found to competitively inhibit the ACh uptake by cerebro-cortical slices of mice[16] and rats.[17] Moderate accumulation of radioactivity found in the absence of a cholinesterase inhibitor is presumably due to the influx of labeled acetate, since ACh under this condition is completely

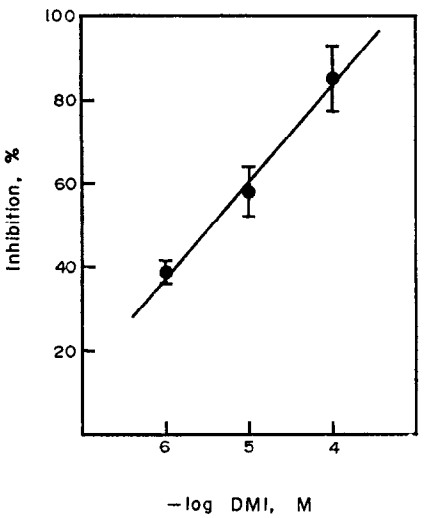

FIG. 4. Inhibition of the accumulation of C^{14}-ACh in brain cortex slices by desmethylimipramine (DMI). On the ordinate: per cent of inhibition taking the T/M concentration ratio of ACh in absence of the drug as 100 per cent. On the abscissa: negative log of DMI concentration (M) in the incubation medium. Each point represents the mean value ± S.E.M. of at least four independent determinations.

hydrolyzed.[23] Recently, a small accumulation of C^{14}-acetate by brain cortex slices in the presence of Sarin has been demonstrated by Heilbronn[21].

DMI, a tricyclic antidepressant compound with known anti-cholinergic properties, competitively inhibited the accumulation of C^{14}-ACh by rat cerebro-cortical slices. The K_i value for DMI ($0{\cdot}5 \times 10^{-5}$ M) is of the same order of magnitude as that reported by Liang and Quastel[22] for cocaine ($0{\cdot}33 \times 10^{-5}$ M), procaine ($0{\cdot}23 \times 10^{-5}$ M) or hemicholinium ($0{\cdot}5 \times 10^{-5}$ M), but considerably lower than that for atropine ($1{\cdot}8 \times 10^{-5}$ M). The inhibitory potency of DMI on ACh uptake by cortical slices, as expressed by ID_{50} ($3{\cdot}5 \times 10^{-6}$ M), is similar to that exhibited by this drug on the uptake of serotonin in hypothalamus and striatum ($2{\cdot}5$–$2{\cdot}8 \times 10^{-6}$ M and $8{\cdot}0$–$9{\cdot}0 \times 10^{-6}$ M, respectively), but is about 100 times less than its effect on the norepinephrine uptake in hypothalamus.[5] It appears that inhibition of the neuronal membrane pump for the uptake of putative neurotransmitters may indeed by a general feature of the action of imipramine-like antidepressant compounds.

The physiological significance of the uptake transport system for ACh at the brain cell membrane is, at present, unclear. The possibility exists, as suggested by Liang and Quastel,[17] that the reuptake of ACh might be a process additional to the action of cholinesterase in terminating the fate of the neurohormone. If such a system does operate *in vivo*, the inhibitory effect of DMI on the uptake of ACh observed in present experiments may be related to the reported ability of this drug to alter the levels of ACh in certain brain areas and may actually represent an additional aspect of the interference of imipramine-like compounds with central cholinergic mechanisms.

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Effect of ethoxyquin on the carbon tetrachloride-induced changes in rat hepatic microsomal enzymes

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Recent studies on the hepatotoxic effects of carbon tetrachloride (CCl_4) have related the toxic effects of CCl_4 to the activity of the hepatic cytochrome P-450 dependant-mixed function oxidases.[1,2] It is now believed that the toxic effects are mediated by an active metabolite of CCl_4.[3] In accordance with this view, pre-treatment of rats with phenobarbitone or DDT produces an increase in the rate of metabolism of CCl_4;[2–4] an increased destruction of cytochrome P-450 and drug metabolizing enzyme activity;[5–7] more extensive liver necrosis[8] and a reduction in the LD_{50} of the toxicant.[1–3] Further support for the central role of the drug metabolizing system in development of CCl_4 hepatotoxicity is provided by the findings that treatments that reduce the activity of the enzyme system (such as low-protein diets,[1,2] small doses of carbon disulphide[9] or inhibitors of drug metabolism such as SKF 525A[10] and disulphiram[11]) decrease CCl_4 hepatotoxicity.

In an earlier publication,[12] it was shown that 6-ethoxy-2,2,4-trimethyl-1-2-dihydroquinoline (ethoxyquin) given to rats 48 hr before CCl_4 protected them from a normally lethal dose of CCl_4. Ethoxyquin also prevented the CCl_4-induced necrosis and hepatic fat accumulation. Whilst ethoxyquin is a powerful antioxidant *in vitro*, the inability to detect a significant concentration of ethoxyquin in the liver at the time of dosage with CCl_4 suggested that the theory of antioxidant protection could not be invoked to explain the action of ethoxyquin. In fact, it was shown that ethoxyquin was an inducer of hepatic microsomal drug metabolizing enzymes and it was suggested that this could be the basis of its protective action against CCl_4-induced hepatotoxicity.[12] However, in contrast to the action of phenobarbitone, ethoxyquin presumably induces a metabolic pathway that leads to a reduction in the concentration of the postulated toxic metabolite.

Among the early effects of CCl_4 toxicity is the loss of drug metabolizing enzymes and glucose-6-phosphatase, perhaps due to the close proximity of these enzymes to the site of production of the postulated toxic metabolite. It therefore seemed useful to study the effects of ethoxyquin on the CCl_4-induced changes in these enzymes. In these studies, the rats were killed by cervical dislocation 24 hr after CCl_4 treatment, and their livers removed. Aminopyrine demethylase[13] and glucose-6-phosphatase activities[14] were measured as described previously. Hexobarbitone oxidase and aniline hydroxylase activities were determined by the methods of Gilbert and Golberg.[15] The amount of cytochrome P-450 was determined spectrophotometrically as described by Omura and Sato.[16] Protein concentrations were measured by the method of Lowry *et al.*[17] using bovine serum albumin as standard.