Inhibition of Phosphatidylcholine Synthesis in Mammary Tissue by 2-Chloroethyltrimethylammonium Chloride

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The incorporation of choline into phosphatidylcholine in mammary tissue from lactating cows is impeded by 2-chloroethyltrimethylammonium chloride at concentrations ranging from 0.3 to 9 mM. This compound is a non-competitive inhibitor of choline kinase (K_m 0.25 mM), having K_i 17 mM. It is suggested that 2-chloroethyltrimethylammonium chloride may inhibit choline entry into the tissue.

2-Chloroethyltrimethylammonium chloride, commonly called chlorocholine chloride (CCC), is a potent plant growth regulant that produces varied responses in a wide variety of plant species (Stoddart, 1966; Anonymous, 1966; Wittwer, 1970). In higher plants 2-chloroethyltrimethylammonium chloride stimulates choline kinase, but in yeast it inhibits this enzyme (Tanaka & Tolbert, 1966).

Despite the structural similarity of 2-chloroethyltrimethylammonium chloride to choline, little information is available on its possible inhibitory effects on phospholipid synthesis, especially in animal tissues. Experimental studies, including both acute and chronic administration of 2-chloroethyltrimethylammonium chloride to rats, revealed that toxicity symptoms are elicited only at high concentrations (Anonymous, 1966; Lampeter, 1970). Lampeter (1970), however, reported that sheep showed toxicity symptoms at much lower concentrations than did rats.

Since 2-chloroethyltrimethylammonium chloride may be present in feedstuffs and because it can enter milk via the mammary gland (Lampeter, 1970), we investigated the effect of the compound on mammary phospholipid synthesis *in vitro*. Mammary tissue from lactating animals was used because of the relatively rapid rate of phosphatidylcholine synthesis that is apparently necessary for milk-fat globule formation and secretion (Kinsella, 1968, 1973) and because this tissue could conceivably be exposed to 2-chloroethyltrimethylammonium chloride from feeds (Lampeter, 1970; Wittwer, 1970).

Materials and methods

Mammary-tissue cells from lactating Holstein cows were prepared and dispersed by the procedure of Ebner *et al.* (1961). The cells (approx. 5 mg of protein) were incubated in 6 ml of medium containing $8.3 \mu\text{M}$ -[1,2- ^{14}C]choline ($10 \mu\text{Ci}/\mu\text{mol}$; New England

Nuclear Corp., Boston, Mass., U.S.A.) at 37°C . After specific intervals the lipids were extracted and the phospholipids were fractionated as detailed previously (Kinsella, 1968). Radioactivity was monitored in a Packard liquid-scintillation spectrophotometer. The choline kinase activity was assayed in the high-speed supernatant ($44000g \times 1\text{ h}$; type 21 head, Beckman 12-65 preparative ultracentrifuge) from lactating bovine mammary tissue. Control incubation mixtures contained saturating concentrations of ATP and MgCl_2 (6.7 mM each), various amounts of [$\text{Me-}^{14}\text{C}$]choline chloride ($7.6 \mu\text{Ci}/\mu\text{mol}$; Amersham-Searle, Chicago, Ill., U.S.A.) in 50 mM-potassium phosphate buffer, pH 7.8, and 2.5 mg of protein in a final volume of $375 \mu\text{l}$. Initial velocities were obtained from 10 min incubations at 37°C . Reactions were stopped with $50 \mu\text{l}$ of 1.0 M-trichloroacetic acid. With the same assay and incubation conditions, the inhibitory effect of 5.3 mM-2-chloroethyltrimethylammonium chloride was determined. Substrate and products were separated by paper chromatography (Plagemann, 1969) and the radioactivity in the excised spots was determined by liquid-scintillation spectrometry. Protein was determined by the method of Lowry *et al.* (1951), with crystalline bovine serum albumin as standard. Each assay was done in duplicate and the results were fitted to a straight line by a computerized least-squares method.

Results

Preliminary studies revealed that 2-chloroethyltrimethylammonium chloride caused inhibition of choline incorporation into mammary-cell phospholipids. Since the preponderance (90%) of the choline metabolized is consistently incorporated into phosphatidylcholine by mammary cells (Kinsella, 1973), the effect of 2-chloroethyltrimethylammonium chloride on phosphatidylcholine synthesis was emphasized in all experiments.

Fig. 1. Time-course study showing the inhibition of $[1,2-^{14}\text{C}]$ choline incorporation into phosphatidylcholine by 2-chloroethyltrimethylammonium chloride in mammary tissue from lactating cows

Experimental details are indicated in the text. Curve A, control; curve B, in the presence of 3.0 mM 2-chloroethyltrimethylammonium chloride.

The synthesis of phosphatidylcholine in dispersed bovine mammary cells was progressively inhibited by increasing amounts of 2-chloroethyltrimethylammonium chloride in the incubation medium ranging from 0.3 to 9 mM. Thus the amount of labelled choline incorporated into the cells exposed to 0 M-, 0.3 M-, 1.5 M-, 3.0 M- and 9.0 mM 2-chloroethyltrimethylammonium chloride decreased from 250 000 to 130 000, 100 000, 40 000 and 6000 c.p.m. respectively. Whereas radioactive choline was progressively incorporated into cellular phospholipids in the controls, the presence of 3.0 mM 2-chloroethyltrimethylammonium chloride caused almost complete inhibition after 3 h (Fig. 1).

Because this inhibitory effect of 2-chloroethyltrimethylammonium chloride might have been exercised on the enzyme choline kinase (ATP-choline phosphotransferase, EC 2.7.1.32) we determined the effect of this compound on this enzyme (Fig. 2). 2-Chloroethyltrimethylammonium chloride behaved as a non-competitive inhibitor of the choline kinase. The apparent K_i (17 mM) was large, being approximately two orders of magnitude greater than the apparent K_m for choline, i.e. 0.25 mM.

Fig. 2. Lineweaver-Burk plots of choline kinase activity in the absence ($\circ$; $r = 0.998$) and in the presence ($\bullet$; $r = 0.994$) of 5.3 mM 2-chloroethyltrimethylammonium chloride

Assay conditions were as stated in the text, and duplicate results of duplicate assays are presented. The apparent K_m for choline is 0.25 mM, the apparent K_i for 2-chloroethyltrimethylammonium chloride is 17 mM and the apparent V_{max} values are 43 and 33 nmol of phosphatidylcholine/h per mg of protein in the absence and in the presence of 2-chloroethyltrimethylammonium chloride respectively.

Discussion

Lampeter (1970) reported that cows excreted administered 2-chloroethyltrimethylammonium chloride mainly via the urine, although measurable quantities entered milk via the mammary gland. This worker did not quantify or assay the biochemical effects of the compound in mammary tissue. Our results indicate that 2-chloroethyltrimethylammonium chloride inhibits the synthesis of mammary phosphatidylcholine, but choline kinase may not be the site of inhibition by this compound in cell dispersions. The K_m obtained for mammary choline kinase (0.25 mM) compares favourably with that obtained with preparations from other tissues (McCaman, 1962; Tanaka *et al.*, 1966; McCaman *et al.*, 1971).

Inhibition by 2-chloroethyltrimethylammonium chloride of choline incorporation into phosphatidylcholine cannot be explained by its observed effects on isolated choline kinase. Its high K_i with the enzyme *in vitro* and the non-competitive inhibition indicates that the compound did not exert its effect directly on choline kinase in metabolizing tissue. The discrepancy between its effects on dispersed cells and on the isolated enzyme may indicate that 2-chloroethyltrimethylammonium chloride was impeding choline entry into the cells, that once in the cell it was converted into a product that became a potent inhibitor

of the choline kinase or that it is a general non-specific toxicant.

Plageman & Roth (1969) and Plageman (1971) reported that choline entry rate (choline permease) followed simple Michaelis-Menten kinetics (K_m $5\mu\text{M}$) and was only one-third the rate of phosphorylation (choline kinase). Choline entry was limiting at concentrations below $20\mu\text{M}$. Thus inhibition of the permease by 2-chloroethyltrimethylammonium chloride would be a plausible explanation for the present observation, since the choline concentration was $8.3\mu\text{M}$. 2-Chloroethyltrimethylammonium chloride, being lipophilic, may concentrate in the lipid-rich plasma membrane and therein behave like hemicholinium-3, a lipid-soluble choline analogue that is a potent competitive inhibitor of choline permease with a low K_i , $40\mu\text{M}$ (Diamond & Kennedy, 1969).

The non-competitive inhibition by 2-chloroethyltrimethylammonium chloride may be due to its alkylating property, i.e. the active site of choline kinase may be alkylated via nucleophilic substitution. The potent effect of 2-chloroethyltrimethylammonium chloride on mammary cells could also be due to a general effect on cell protein and enzyme synthesis (Plagemann, 1968; Knypl & Chylinska, 1972), and the interrelationship between choline metabolism and protein synthesis as reported by Mookerjee & Marai (1971) could possibly be involved.

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