Inactivation of superoxide dismutase by several thiocarbamic acid derivatives[1]

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Summary. 4 thiocarbamic acid derivatives inhibited superoxide dismutase (SOD) activity in vitro. Dimethyldithiocarbamate also inhibited tissue SOD in mice in vivo. These data extend previously published results on the inhibitory action of diethyldithiocarbamate on SOD activity.

It was shown previously[2] that diethyldithiocarbamate (DEDC) inhibited the copper-zinc form of superoxide dismutase (SOD). Inhibition was observed after incubation of DEDC with either solutions of purified bovine SOD or with mouse tissue homogenates (viz., brain, liver) that contained endogenous SOD. Inhibition was also observed in vivo in erythrocytes, liver and brain after injection of DEDC into mice. An inhibitory action of DEDC on SOD isolated from spinach leaves has also been reported[3].

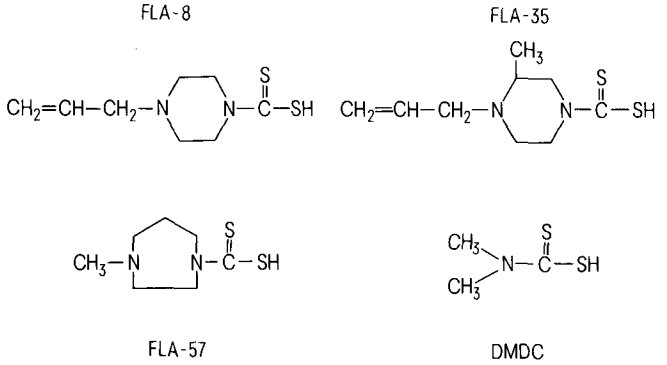
In the present study, 4 other thiocarbamic acid derivatives were studied and found to inhibit purified copper-zinc SOD in vitro. The structures of these compounds are shown in the figure. Dimethyldithiocarbamate (DMDC) strongly inhibited tissue SOD when it was administered in vivo to mice. The other compounds (FLA-8, FLA-35, FLA-57) proved lethal at dose levels where inhibition of SOD may have occurred and were not further studied in vivo. All of the active thiocarbamic acid derivatives are known copper chelating agents and known inhibitors both in vitro and in vivo of dopamine beta-hydroxylase, a copper enzyme[4].

Materials and methods. The sources of the compounds used were as follows: DMDC (Aldrich), FLA-57 (Regis), and FLA-8 and FLA-35 (Dr L. Florvall, Astra Lakemedel AB, Sodertalje, Sweden). For in vitro studies, stock solutions of DMDC and FLA-35 were prepared in distilled water at 0.1 M. FLA-57 and FLA-8 were dissolved in 0.2-0.3 ml 0.1 N NaOH and brought to 0.1 M with an equal volume of 0.1 N HCl and with distilled water. The thiocarbamic acids were then diluted to 0.01 M in solutions containing 0.5 mg/ml of purified bovine erythrocyte SOD (Truett Labs) in H_2O or in 0.05 M potassium phosphate buffer, pH 6.5, containing 10^{-4} M EDTA. After 2 h of incubation at 37 °C, 10µl aliquots were removed and assayed for SOD activity. Control samples were incubated without the thiocarbamic acids or without the SOD. SOD activity was measured spectrophotometrically in the above buffer by a method based on the inhibitory action of the enzyme on the rate of autoxidation of 6-hydroxydopamine[2,5]. All results were verified with the cytochrome c reduction method[6] and, in several experiments, results were further confirmed with assay methods based on the autoxidation of pyrogallol[7] or the reduction of nitroblue tetrazolium[8]. For these latter methods, some changes in experimental protocol were necessary to prevent interference from the thiocarbamic acids, as described earlier for DEDC[2]. In vivo experiments were performed with male Swiss-Webster mice (20-30 g). For details see the table.

Results. Each of the thiocarbamic acid derivatives produced essentially a complete (greater than 95%) inhibition of the activity of SOD in vitro. Inhibition was accompanied by the appearance of a colored (yellow to orange) enzyme-inhibitor complex. As had been observed earlier with DEDC[2], the loss in enzymatic activity could not be reversed by dialysis against 500 vol. of distilled water at 4 °C for 16 h, but after dialysis the original activity was restored by incubation with 5×10^{-4} M $CuSO_4$ for 1 h at 37 °C.

In other experiments, a number of other copper-chelating agents were tested but were found to be without inhibitory action on purified bovine SOD in vitro. These agents included methimazole, penicillamine, bathocuproine, diethyldithiophosphate, propylthiouracil, 1-phenyl-3-(2-thiazolyl)-thiourea, pyridyldiphenyltriazine and cuprizone. The last 4 agents were not soluble in water and therefore experiments with these compounds were conducted in dimethylformamide as solvent. Control studies showed that dimethylformamide did not affect the activity of SOD and that DEDC caused complete inactivation in this solvent.

When DMDC was injected into mice in doses of 375 and 750 mg/kg, a dose-dependent loss of SOD activity was observed in liver (table). Brain and blood SOD also were inhibited at the higher dose (table); these tissues were not analyzed at the lower dose. Results with whole blood reflect mainly the SOD activity of erythrocytes. The in vivo action of DMDC on SOD in liver and brain appeared more effective than that previously reported for DEDC. Therefore, a direct comparison of DMDC and DEDC at dose



The structures of 4-allyl-1-piperazinedithiocarboxylic acid (FLA-8), 4-allyl-3-methyl-1-piperazinedithiocarboxylic acid (FLA-35), 4-methyl-1-homopiperazinedithiocarboxylic acid (FLA-57) and dimethyldithiocarbamate (DMDC).

The in vivo effect of dimethyldithiocarbamate (DMDC) on superoxide dismutase activity of mouse tissues

Tissue	DMDC (mg/kg)	SOD (µg/g)	Loss (%)
Brain	0	91± 6 (11)	-
	750	43± 7 (12)	53
Liver	0	694±85 (15)	-
	375	441±35 (7)	36
	750	113±62 (17)	84
Blood	0	73± 7 (6)	-
	750	11± 7 (6)	85

DMDC at 375 mg/kg or 750 mg/kg was administered i.p. in 0.9% (w/v) saline. Control mice received saline alone. Superoxide dismutase activity was measured 3 h later in 1:10 lysates of erythrocytes prepared in distilled water, or in brain or liver homogenates prepared in 9 vol. cold buffer followed by centrifugation for 10 min at 700× g. Data are the mean ±SD for the number of mice shown in parentheses. Brain and liver were assayed for SOD at pH 6.5 while blood was assayed at pH 7.4[2].

levels of 750 mg/kg was performed. Results confirmed the initial impression: the loss in enzyme activity in liver caused by DEDC was 52±13%, while that for DMDC was 86±5% (mean±SD, for 6 mice in each group; $p < 0.05$).

Discussion. DMDC, FLA-8, FLA-35 and FLA-57, as well as DEDC[2], can be used to reversibly inhibit the activity of the copper-zinc form of SOD in studies performed in vitro. Inhibited enzyme preparations can be dialyzed to provide inactive enzyme free of excess inhibitor. Reversal of inhibition can be achieved with $CuSO_4$. For in vivo studies, DMDC presents an alternate to the use of DEDC to inhibit tissue SOD.

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2 R.E. Heikkila, F.S. Cabbat and G. Cohen, J. biol. Chem. *251*, 2182 (1976).
3 K. Asada, M. Takahashi and M. Nagate, Agr. biol. Chem. *38*, 471 (1974).
4 L. Florvall and H. Corrodi, Acta pharm. suecica *7*, 7 (1970).
5 R.E. Heikkila and F.S. Cabbat, Analyt. Biochem. *75*, 356 (1976).
6 J. McCord and I. Fridovich, J. biol. Chem. *244*, 6049 (1969).
7 S. Marklund and G. Marklund, Eur. J. Biochem. *47*, 469 (1974).
8 C. Beauchamp and I. Fridovich, Analyt. Biochem. *44*, 276 (1971).

Serum triglycerides and post-heparin lipolytic activity in guinea-pigs with latent vitamin C deficiency

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Summary. Guinea-pigs with latent vitamin C deficiency have a raised serum triglyceride concentration and significantly reduced post-heparin lipolytic activity in blood plasma.

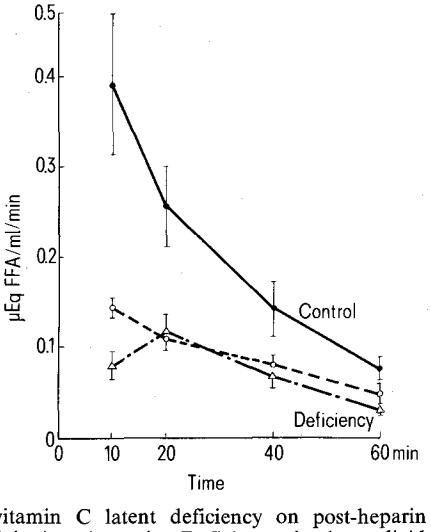
Microsomal 7α-hydroxylation of cholesterol, a rate-limiting reaction in the transformation of cholesterol to bile acids, slows down in the liver of guinea-pigs with latent ascorbic acid deficiency[1,2]. In consequence, cholesterol catabolism also slows down, hypercholesterolemia develops, cholesterol accumulates in certain tissues and, in long-term experiments, pathological changes may occur in the vascular system[3-5]. In recent years, evidence has accumulated showing that latent vitamin C deficiency also affects plasma triglyceride metabolism[6-10]. This study is a contribution to knowledge of the mechanism of the development of hypertriglyceridemia in guinea-pigs with chronic latent vitamin C deficiency.

Material and methods. We used growing male guinea-pigs fed ad libitum on a scorbutogenic diet[11] containing 15% w/w fat, the main source of which in this diet is butter and dried milk.

Experiment I. Guinea-pigs with an initial weight of 350 g were put on 3 very different doses of ascorbic acid. In the 1st group, latent vitamin C deficiency was induced by our routine method: for 2 weeks the animals were given a vitamin C-free diet and then received a maintaining dose of 0.5 mg ascorbic acid/animal/day. The 2nd group was given the same diet plus 0.05% w/w crystalline ascorbic acid and the 3rd group the same diet plus 0.5% w/w ascorbic acid. The last 2 diets were freshly prepared twice a week and were kept in the cold in sealed plastic vessels. 3 other groups of guinea-pigs had the same graded ascorbic acid intake, but their diet contained 0.2% w/w cholesterol dissolved in butter. The experiment lasted 17 weeks. The guinea-pigs were then deprived of food; 18 h later they were killed under mild ether anesthesia and the triglyceride (Boehringer UV test) and vitamin C concentration[12] were determined in their serum.

Experiment II. Guinea-pigs with an initial weight of 370 g were given the basic scorbutogenic diet without added cholesterol. In the 1st group, latent vitamin C deficiency was induced in the same way as in experiment I; for the 2nd group, 0.5% w/w ascorbic acid was added to the diet. The experiment lasted 9 weeks. The animals were then deprived of food for 20 h and the total lipolytic activity of post-heparin plasma was determined in blood samples taken from the jugular vein of thiopental-anesthetized animals 10, 20, 40 and 60 min after the i.v. administration of heparin (Spofa) in a dose of 5 U/100 g b. wt. Lipolytic activity was determined by quantification of the fatty acids[13] released during the incubation of 0.2 ml plasma in medium containing intralipid (Vitrum) as substrate[14].

Results and discussion. Experiment I. The weight curves in all the groups were similar, and at the end of the experiment the animals b. wt was 600-650 g. Table 1 shows that the serum vitamin C level rose together with the ascorbic acid intake, while the triglyceride concentration fell. There is a moderately close negative linear correlation between the serum vitamin C and triglyceride level ($r_{xy} = -0.548$, $p < 0.002$): in the presence of high vitamin C levels, the triglyceride level is low and vice versa. Several authors have described an incidence of hypertriglyceridemia in the presence of vitamin C deficiency[6,7,9]. The hypotriglyceridemic effect of large doses of ascorbic acid has also been described, in guinea-pigs[7,10,15], rabbits[16], golden hamsters[9], monkeys[17,18] and man[9,16]. It is interesting to note that the addition of cholesterol to the diet of guinea-pigs given small doses of ascorbic acid caused a



Effect of vitamin C latent deficiency on post-heparin plasma lipolytic activity in guinea-pigs. Deficient animals are divided into 2 subgroups: 1: Maximum activity was reached within the same period of time as in control, that is, 10 min after heparin administration; 2: maximum activity was reached even 20 min after heparin administration, ±SEM is given by verticals at each value.