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PROTECTIVE EFFECTS OF SEMICARBAZIDE AND *p*-AMINOBENZOIC ACID AGAINST OZONE TOXICITY

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Abstract—Ozone-induced crosslinking of spectrin was counteracted by semicarbazide and *p*-aminobenzoic acid. This could be ascribed to the action of these compounds as ozone scavengers. Inhibition of hexokinase and acetylcholinesterase by ozone was also impeded by these drugs. In the case of semicarbazide the effect appeared to be complex. When the drug was present during exposures of the enzymes to ozone, inhibition was counteracted. Ozonolysis of semicarbazide yielded a product, however, that was itself inhibitory with respect to hexokinase and acetylcholinesterase activity. This dual effect was even more pronounced in ozone-induced K^+ leakage from red blood cells. *p*-Aminobenzoic acid inhibited this K^+ leakage very strongly. With semicarbazide an initial inhibition was observed, followed by a subsequent augmentation of K^+ leakage. In control experiments it could be shown that the increase leakage was caused by an ozonolysis product of semicarbazide.

Deleterious cellular effects of ozone, the major oxidant of photochemical air pollution, have been documented in several different systems. For instance, ozone effects on structure and function of red blood cells [1–4], membrane-bound enzymes [5–7] and lung tissue [8–12] have been described in some detail, whereas a possible role of ozone in the process of tissue ageing has been suggested [13].

Although in model systems ozone-induced lipid peroxidation [6, 7, 14], oxidation of aminoacids and proteins [15–19] and covalent protein crosslinking [20] have been described, the pathochemical background of ozone toxicity *in vivo* is not yet clear. Further, it still has to be established whether ambient levels of ozone produce significant toxicity in man. Despite these uncertainties, several suggestions for treatment or prevention of ozone toxicity appeared in recent literature.

For instance, Goldstein *et al.* [1] demonstrated protection by *p*-aminobenzoic acid against ozone toxicity and suggested that these experimental results warranted exploration of the drug as possible protective agent in populations exposed to significant photochemical air pollution. Further, Kesner *et al.* [7] suggested the possibility of treating persons with semicarbazide in order to protect them against the consequences of ozone inhalation. This suggestion was based on the observation that semicarbazide counteracted inhibition of erythrocyte membrane $(Na^+ + K^+)$-ATPase by ozonised phospholipids.

In a recent study we demonstrated the action of semicarbazide as an ozone scavenger [21]. This action was complicated, however, by the fact that ozonolysis of semicarbazide yielded a product, causing inhibition of glyceraldehyde-3-phosphate dehydrogenase. For this reason a more detailed study of the effects of both semicarbazide and *p*-aminobenzoic acid seemed appropriate. The effects of both agents on ozone-induced crosslinking of spectrin, inhibition of acetylcholinesterase and hexokinase and red cell membrane permeability were studied. The results are presented in this communication.

MATERIALS AND METHODS

Ozone was generated in an oxygen flow of 4 ml/min with a Supelco Micro-Ozonizer at a rate of 2.5 μmoles/min. Ozone production was measured by titration with KI–$Na_2S_2O_3$ at pH 7.0. Treatment of solutions or suspensions with ozone was carried out by bubbling the gas into 4 ml samples.

All reagents were analytical grade, and used without further purification. ^{14}C-Labeled compounds were obtained from ICN Pharmaceuticals (Irvine). Hexokinase from yeast was obtained from Boehringer. Heparinized blood was centrifuged and the erythrocytes were washed three times in isotonic phosphate buffered saline pH 7.4.

Hemoglobin-free ghosts were prepared by gradual osomotic lysis as described by Weed *et al.* [22]. Spectrine was extracted from ghosts according to Bennett and Branton [23].

Protein concentrations were determined by the method of Lowry *et al.* [24] with bovine serum albumin as a standard.

K^+ determinations were carried out with a flame photometer. As reference for 100% intracellular K^+ concentrations an erythrocyte sample was hemolysed in water.

Sodium dodecylsulphate polyacrylamide gel electrophoresis was performed as described by Fairbanks *et al.* [25].

Acetylcholinesterase activity was measured according to Ellman *et al.* [26] and hexokinase activity according to Bergmeyer *et al.* [27].

Analysis of ^{14}C-labeled semicarbazide and *p*-aminobenzoic acid during ozone treatment was done via thin layer chromatography on silica gel, with the solvent system *n*-butanol/acetic acid/water (4:1:1, v/v) and subsequent autoradiography.

RESULTS

During exposure of a spectrin solution to ozone an increasing amount of the protein disappeared

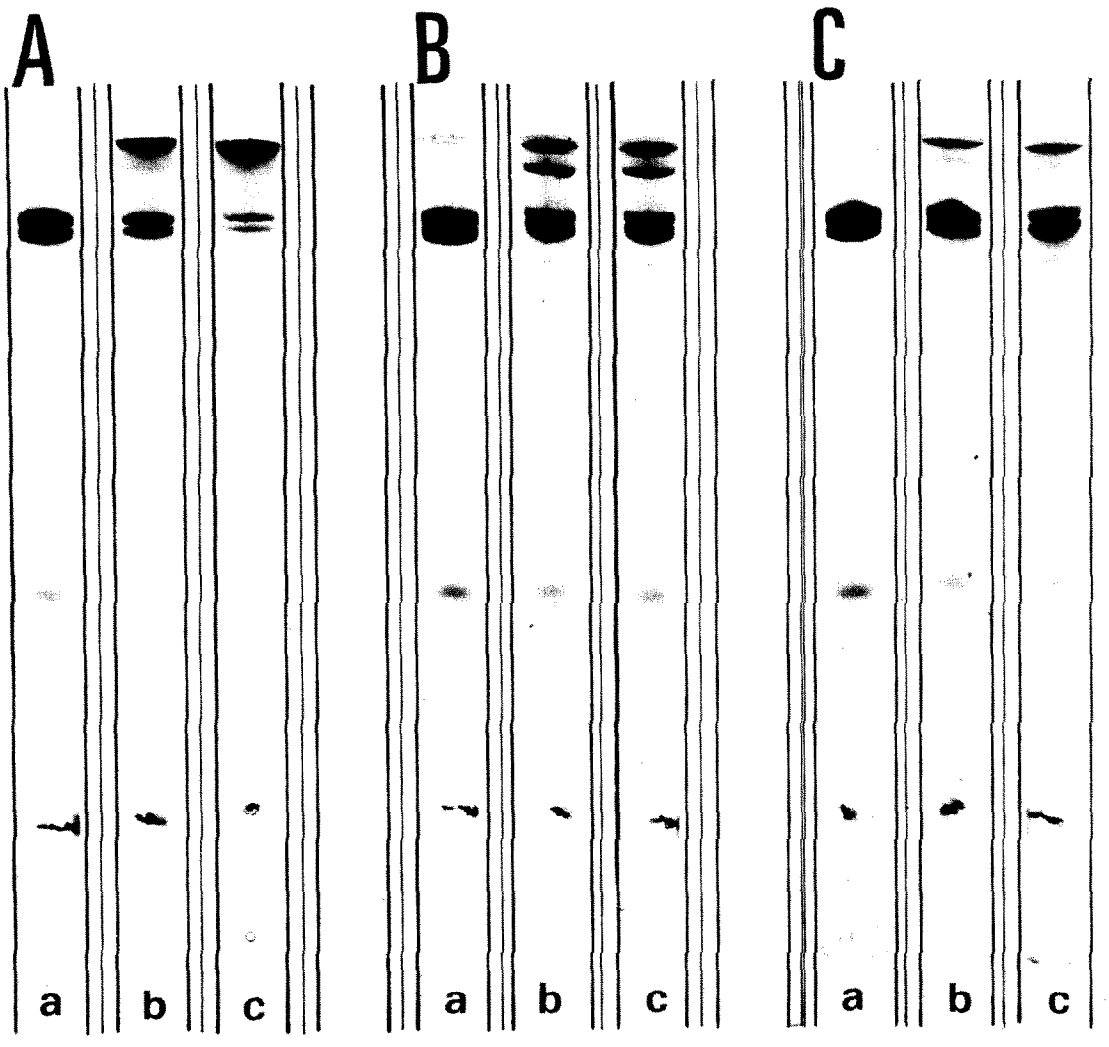

Fig. 1. Effect of semicarbazide and *p*-aminobenzoic acid on ozone-induced crosslinking of spectrin. 3 ml spectrin solution (1.0 mg protein/ml) was treated with ozone at pH 7.4 during 0 (a), 1(b) and 2(c) min. A: in the absence of drugs, B: in the presence of 40 mM *p*-aminobenzoic acid; C: in the presence of 40 mM semicarbazide.

from its normal position in the electrophoretogram and was recovered as high molecular weight aggregates (Fig. 1). This crosslinking is not caused by disulfide bridges, as reduction with dithiotreitol was carried out prior to electrophoresis. No crosslinking is observed during exposure to oxygen. Semicarbazide and *p*-aminobenzoic acid protected against ozone-induced crosslinking (Fig. 1).

Both semicarbazide and *p*-aminobenzoic acid react readily with ozone, as shown in Fig. 2. Solutions of these drugs, extensively treated with ozone, had no visible effect on spectrin and did no longer protect the protein against ozone-induced crosslinking (not shown).

Red cell membrane-bound acetylcholinesterase is inhibited by ozone. Again this inhibition is counteracted by the ozone scavengers semicarbazide and *p*-aminobenzoic acid (Fig. 3A). When an ozone-treated solution of *p*-aminobenzoic acid is added to the ghost suspension, the enzyme activity is not influenced. Ozone-exposed semicarbazide, however, caused a strong inhibition of enzyme activity (Fig. 3B). Apparently ozonolysis of semicarbazide yields a product that is inhibitory with respect to acetylcholinesterase activity, whereas ozonolysis of *p*-aminobenzoic acid does not generate toxic products.

As shown in Fig. 4A and B similar results were obtained with the solubilized enzyme hexokinase.

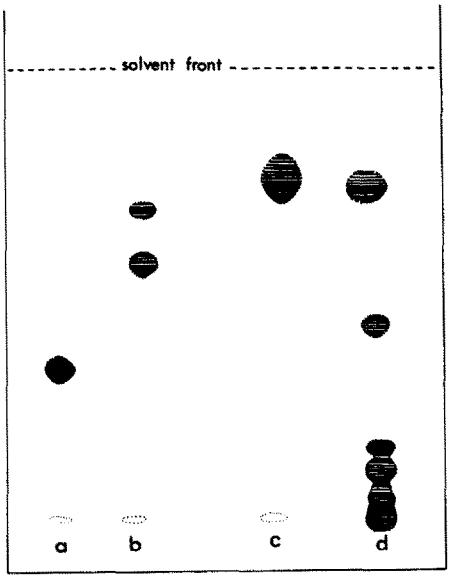

Fig. 2. Autoradiography of silicagel thin-layer chromatograms of semicarbazide (a), ozone-treated semicarbazide (b), *p*-aminobenzoic acid (c) and ozone-treated *p*-aminobenzoic acid (d).

Both scavengers protect against inactivation by ozone, but again ozonolysis of semicarbazide yielded a product that inhibited the enzyme.

K^+ leakage due to ozone exposure is shown in Fig. 5A. This K^+ leakage was strongly impeded by *p*-aminobenzoic acid. With semicarbazide an initial inhibition of K^+ leakage was also observed. After longer exposure times to ozone, however, K^+ leakage in the presence of semicarbazide exceeded K^+ leakage in the absence of the drug (Fig. 5A), suggesting again the generation of a toxic ozonolysis product. Whereas ozone-treated *p*-aminobenzoic acid had no effect on the passive permeability of the membrane to cations, significant K^+ leakage was provoked by ozone-treated semicarbazide (Fig. 5B).

DISCUSSION

It is still a matter of dispute whether ambient ozone levels, even in highly industrialized areas, produce significant toxicity in man. On the other hand the potential health hazard of ozone has been demonstrated in many experimental designs. In this context a search for drugs, usable for treatment or prevention of ozone toxocity is rational. Based on

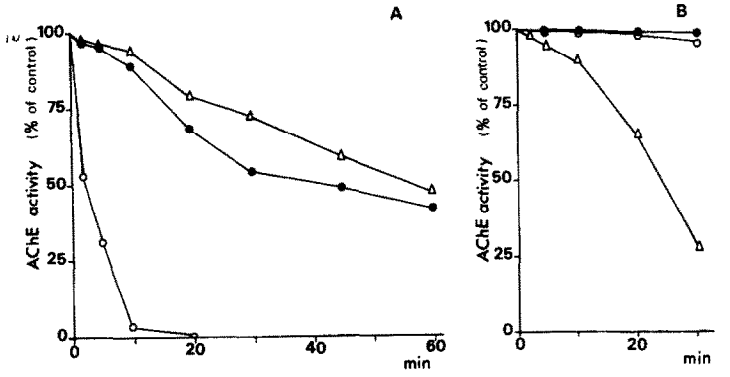

Fig. 3. Effect of semicarbazide and *p*-aminobenzoic acid on ozone-induced inactivation of erythrocyte membrane acetylcholinesterase. (A) 4 ml 25% ghost suspension treated with ozone at pH 7.4 in the absence of drugs (○), in the presence of 50 mM semicarbazide (△) or in the presence of 50 mM *p*-aminobenzoic acid (●). Prior to enzyme assay 100 μl samples were incubated with 300 μl 10 mM Na-phosphate (pH 7.4) for 15 min at 37°. (B) Treatment of 4 ml 10 mM Na-phosphate (○), 50 mM semicarbazide (△) or 50 mM *p*-aminobenzoic acid (●) with ozone. Thirty minutes after ozone treatment 100 μl samples were incubated for 15 min at 37° with 33 μl packed ghosts and subsequently assayed for enzyme activity.

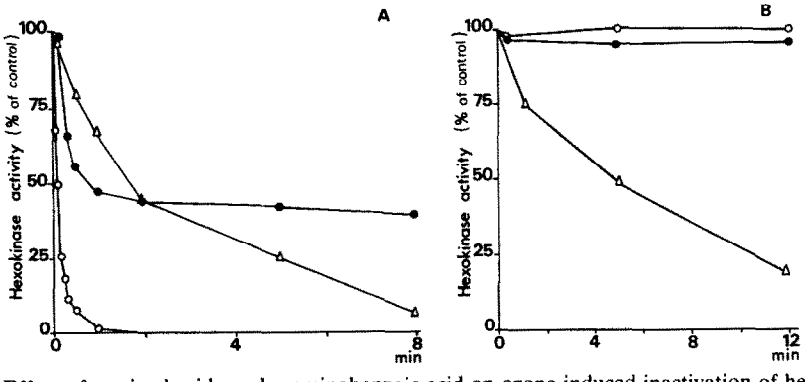

Fig. 4. Effect of semicarbazide and *p*-aminobenzoic acid on ozone-induced inactivation of hexokinase (0.25 mg/ml, pH 7.4). Symbols and experimental details as in Fig. 3.

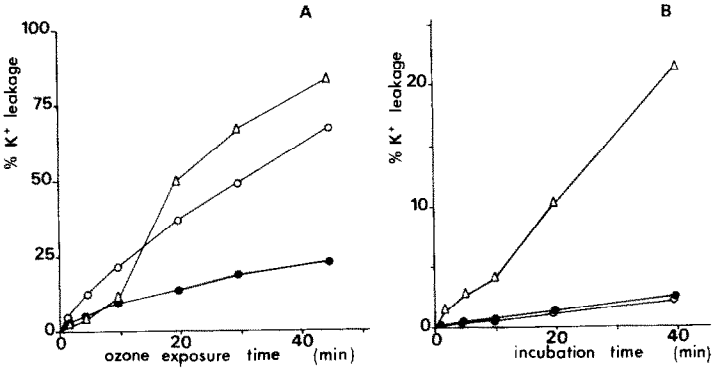

Fig. 5. Effects of semicarbazide and *p*-aminobenzoic acid on ozone-induced K^+ leakage from erythrocytes. (A) 5 ml 16.7% suspensions treated with ozone in the absence of drugs (○), in the presence of 40 mM semicarbazide (△) or in the presence of 40 mM *p*-aminobenzoic acid (●). (B) 4 ml of isotonic NaCl (○), 50 mM semicarbazide in isotonic NaCl (△) or 50 mM *p*-aminobenzoic acid in isotonic NaCl (●) were treated with ozone during 20 min and further incubated at 37° during 30 min. After mixing with red blood cell suspensions (final concentrations: 16.7% cells, 37.5 mM drug) K^+ leakage was monitored. In control experiments with O_2 replacing ozone, K^+ leakage never exceeded 3.5%/hr.

the observed impediment of ozone-induced inhibition of (Na^+ + K^+) ATPase, Kesner *et al.* [7] suggested in a recent paper the possible therapeutical use of semicarbazide.

As shown in the results section, semicarbazide is a potent ozone scavenger, like *p*-aminobenzoic acid. This effect can be explained by the fact that both drugs react readily with ozone, as shown in Fig. 2. In equimolar concentrations semicarbazide was slightly more effective than *p*-aminobenzoic acid in counteracting ozone-induced spectrin crosslinking and inhibition of acetylcholinesterase activity.

A serious problem, however, is the generation of toxic ozonolysis products from semicarbazide. Ozonolysed semicarbazide did not produce spectrin crosslinking, but it caused inhibition of hexokinase, acetylcholinesterase and, as shown in a preceding paper [21], glyceraldehyde-3-phosphate dehydrogenase. During exposure of these enzymes to ozone in the presence of semicarbazide this effect was obscured by the activity of the drug as ozone scavenger. This notion is supported by the results shown in Fig. 4A. After short exposure times to ozone hexokinase activity was better protected by semicarbazide then by *p*-aminobenzoic acid. After longer exposure times the protective effects were reversed, apparently by the accumulation of a toxic ozonolysis product of semicarbazide. The detrimental effects of this product are quite clear, when ozonolysed semicarbazide is added to the enzyme preparation (Figs. 3B and 4B).

Even more pronounced were the effects on ozone-induced K^+ leakage from red blood cells. Initially semicarbazide caused inhibition of ozone-induced K^+ leakage, apparently by its activity as ozone scavenger. During prolonged exposure to ozone, however, semicarbazide clearly potentiated the ozone effect, via the generation of a highly toxic ozonolysis product (Fig. 5).

These observations cast serious doubts on the possible use of semicarbazide against ozone toxocity. On the other hand, ozonolysis of *p*-aminobenzoic acid did not generate products that showed detrimental effects. Although the described studies are too limited to allow generalised conclusions, they indicate that *p*-aminobenzoic acid seems to be preferable to semicarbazide and related compounds for application to ozone-exposed biological systems.

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