Carbonic Anhydrase Inhibition

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Carbonic anhydrase (carbonate hydro-lyase, EC 4.2.1.1) is involved to some extent in a number of processes, among them the exchange of carbon dioxide in blood (MELDRUM and ROUGHTON, 1934), the regulation of CO_2 levels in photosynthesis by land plants and algae (POINCELOT, 1972; GRAHAM et al., 1971), and the deposition of $CaCO_3$ in shells of mollusks (WILBUR and JODREY, 1955) and eggshells (HASELTINE et al., 1974). It is thought that inhibition of carbonic anhydrase activity in certain organisms leads to inhibition of photosynthesis and to eggshell thinning, the latter being the subject of a good deal of interest (HEALD et al., 1968; PEAKALL, 1970; POCKER et al., 1971, 1971a). This enzyme is important in a number of different areas and it was of interest to test the inhibitory effectiveness of a number of different chemicals on its activity. We have found that the hydrolysis of p-nitrophenyl acetate by carbonic anhydrase is inactivated by certain heavy metal salts, that certain other heavy metal compounds and organomercurials inhibit carbonic anhydrase activity non-competitively, and that certain chlorinated insecticides, soluble in dioxane-water systems, are mild non-competitive inhibitors.

EXPERIMENTAL

Bovine carbonic anhydrase[1] was obtained from Sigma Chemical Co. and used without further purification. Concentrations of CA solutions were determined spectrophotometrically at 280 nm ($\epsilon = 5.40 \times 10^4$ l mole^{-1} cm^{-1} - POCKER and MEANY, 1967). The substrate used in the activity tests was p-nitrophenyl acetate, obtained from Eastman Organic Chemicals and purified by recrystallization from ethyl ether. Stock solutions of NPA were prepared in dioxane for stability. All experiments were performed in pH 7.50 phosphate buffer at 25°, a constant ionic strength of 0.01, and a final dioxane concentration of 0.33% v/v, except in the case of the pesticides, for which a final dioxane concentration of 5% was used to ensure solubility. Steady-state kinetic conditions were used for all activity tests. Typical concentrations were [CA] = 10^{-7} M and [NPA] = 5.5×10^{-1} M. In a typical experiment, 10µl of NPA

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[1] Abbreviations used: CA, bovine carbonic anhydrase; NPA, p-nitrophenyl acetate; dieldrin, 1, 2, 3, 4, 10, 10-hexachloro-6, 7-epoxy-1, 4, 4a, 5, 6, 7, 8, 8a-octahydro-endo, exo-1, 4:5, 8-dimethylnonaphthalene; DDT, 1, 1-bis(p-chlorophenyl)-2, 2, 2-trichloroethane; 2, 4, 5-T, 2, 4, 5-trichlorophenoxyacetic acid; chlordane, 1, 2, 4, 5, 6, 7, 8, 8-octachloro-3a, 4, 7, 7a-tetrahydro-4, 7-methanoindan; parathion, O, O-diethyl-O-(p-nitrophenyl) phosphorothioate.

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solution was added to 3.0 ml enzyme solution (with or without added inhibitor) in a cell in the spectrophotometer (Cary 14), the contents were mixed quickly, and the reaction was monitored from about 6 seconds after mixing. Initial velocities were obtained from the initial slopes of absorbance - time curves using a difference extinction coefficient for the product p-nitrophenol at 400 nm of 1.84×10^4 1 $mole^{-1}$ cm^{-1} (KEZDY and BENDER, 1962). At least three kinetic determinations were made for each set of conditions, and the standard deviation in the rates and derived parameters was usually about 10%. The rate data were treated using standard techniques of steady-state enzyme kinetics (LAIDLER and BUNTING, 1973). Corrections were made for NPA hydrolysis in the absence of enzyme.

The compounds tested for their effect on carbonic anhydrase were dioxane, mercuric chloride, p-chloromercuribenzoic acid, cadmium chloride, lead nitrate, silver nitrate, methylmercuric chloride, phenylmercuric chloride, dieldrin, DDT, 2, 4, 5-trichlorophenoxyacetic acid, chlordane, and parathion.

RESULTS AND DISCUSSION

The linear variation of the rate of hydrolysis of NPA with dioxane concentration is shown in Fig. 1.

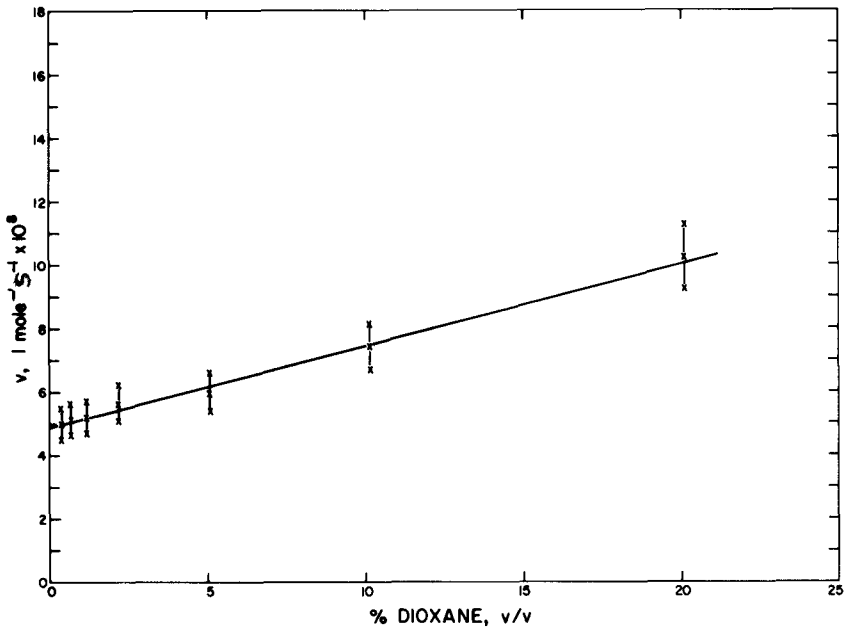

Figure 1. The effect of dioxane concentration on the rate of hydrolysis of p-nitrophenyl acetate by carbonic anhydrase. [CA] = 10^{-7} M, [NPA] = 5.5×10^{-1} M, pH 7.50.

The results of all experiments performed in 5% dioxane solution to ensure solubility of the insecticides have been corrected to 0.33% v/v dioxane.

Mercuric chloride, p-chloromercuribenzoic acid, and cadmium chloride are powerful inactivators of carbonic anhydrase. Complete abolition of activity occurs at a ratio of inhibitor concentration to enzyme concentration of 1:1, presumably due to reaction with a sulfhydryl group in the enzyme which is essential for activity. Methylmercuric chloride, phenylmercuric chloride, lead nitrate, and silver nitrate apparently inhibited the reaction non-competitively, as seen from plots of 1/v vs. 1/[NPA] for different inhibitor concentrations. The rates were corrected for the inhibitory effects of nitrate and chloride ions observed previously (POCKER and STONE, 1967) and the values for the dissociation constants which denote the concentration of inhibitor necessary for 50% inhibition of the rate of reaction are shown in Table 1.

TABLE 1

Inhibition constants for the cationic inhibition of the hydrolysis of p-nitrophenyl acetate by carbonic anhydrase

Compound	Ki[a], M
methylmercuric chloride	2.3×10^{-6}
phenylmercuric chloride	9.4×10^{-6}
lead nitrate	3.0×10^{-4}
silver nitrate	2.6×10^{-4}

[a]Ki is the amount of inhibitor necessary to produce 50% inhibition of the rate of the reaction at pH 7.5.

There may be some error in the determination of Ki values for the two organomercurials because at concentrations greater than 10^{-6} M they contribute significantly to the hydrolysis of NPA themselves. Methylmercuric chloride and phenylmercuric chloride are 30-100 times more potent inhibitors of carbonic anhydrase esterase action than are lead nitrate and silver nitrate, again presumably because of a more specific interaction with some group on the enzyme such as a sulfhydryl group.

Dieldrin, DDT, and chlordane (but not parathion) were all shown to be fairly weak non-competitive inhibitors of the reaction in 5% dioxane solution (2, 4, 5-T is a stronger inhibitor). The chlorinated hydrocarbons were tested over the range 10^{-8} - 10^{-5} M. At concentrations greater than 10^{-5} M the dieldrin, DDT, and chlordane precipitated from the 5% dioxane solution. The extent of inhibition at the upper limit of solubility was small (10%) but determined not to be an experimental artifact. Table 2 presents the results of the inhibition of NPA hydrolysis by the pesticides.

TABLE 2

Inhibition constants for the pesticide inhibition of the hydrolysis of p-nitrophenylacetate by carbonic anhydrase

Compound	Ki[a], M	Concentration range, M
dieldrin	10^{-4}	10^{-8} - 10^{-5}
DDT	10^{-4}	10^{-8} - 10^{-5}
chlordane	10^{-4}	10^{-8} - 10^{-5}
2, 4, 5-T	4×10^{-6}	10^{-8} - 2×10^{-3}
parathion	no inhibition	10^{-8} - 10^{-4}

[a]Ki is the amount of inhibitor necessary to produce 50% inhibition of the rate of the reaction at pH 7.5.

The values of Ki for the first three insecticides listed in Table 2 are subject to some error because of the restricted concentration range over which they could be employed; the values indicate that they are about 25 times less effective as inhibitors than is 2, 4, 5-trichlorophenoxyacetic acid.

The inhibition by dieldrin and DDT in 5% dioxane solutions reported here is somewhat at odds with results reported previously by POCKER et al. (1971) which indicated that these two compounds are not inhibitory at concentrations of 10^{-5} M in 34% dimethylformamide solution; we feel that the observed effect is small but real. In consideration of in vivo effects in which there is no solubilizing agent for the insecticides, however, it is probable, as pointed out by POCKER et al. (1971), that insecticides present in excess of their solubility limit form precipitates which occlude enzyme from solution and furnish a physical explanation of the supposed inhibition. The lack of inhibition by parathion indicates that carbonic anhydrase activity is not dependent on a serine residue at the active site, as are such enzymes as chymotrypsin and trypsin.

Although bovine carbonic anhydrase was used in this study because of its ready commercial availability, note should be made that certain reactions and patterns of inhibitory behaviour may not be the same for all species. For example, it has been demonstrated that carbonic anhydrases isolated from green leaves of spinach (BRADFIELD, 1951) and the elderberry bush (DAY and FRANKLIN, 1946) are not affected by sulfanilamide, a potent inhibitor of bovine and human carbonic anhydrase. Thus caution should be exercised in comparing the effects of potential environmental pollutants on the enzymic systems of different species.

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