Kinetics of Inhibition by Acetazolamide and Sulfanilamide of Bicarbonate Dehydration Catalyzed by Bovine Carbonic Anhydrase.* (32025)

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(Introduced by T. H. Maren)
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The sulfonamide carbonic anhydrase inhibitors, acetazolamide and sulfanilamide, have been shown to obey the kinetics of non-competitive inhibition of the hydration of carbon dioxide(1,2). Two other carbonic anhydrase-catalyzed reactions of water with carbonyl compounds, the hydration of aldehydes and the hydrolysis of aromatic esters, appear to be inhibited by acetazolamide according to non-competitive kinetics(3,4). The present report concerns experiments on the inhibition of the dehydration of bicarbonate, using a partially purified carbonic anhydrase preparation from bovine erythrocytes.

Materials and methods. The enzyme was prepared from bovine erythrocytes according to Lindskog(5). The DEAE cellulose eluate fractions containing the major peak of carbonic anhydrase activity were pooled and lyophilized. The preparation contained 530 units per mg, as measured by the changing-pH method(6), when the concentration of peptone in the assay reaction chamber was 0.01%.

The apparatus is shown diagrammatically in Fig. 1. Nitrogen (prepurified grade) was passed through a cooling coil (A) and into the bottom chamber of a Toribara ultrafiltration tube (B) (Will no. 13000). The upper chamber of this tube, which served as the reaction cell, was separated from the lower by a sintered glass plate. It was fitted with a rubber stopper through which was inserted a 17-gauge trochar needle equipped with a stopcock, for addition of reactants, and a gas delivery tube, through which evolved carbon dioxide was swept and led to the lower chamber of a second Toribara tube (C). The upper part of this tube, which was cut down for the purpose, contained the CO_2-absorbing fluid, into which dipped electrodes (Radiometer models G202A and K4312) connected to a Radiometer model TTT-1 pH meter (D), which, with the Radiometer model SBR-2 titrigraph (E), was set for pH-stat operation at pH 8. The absorbing fluid consisted of 10 ml of a 1:2000 dilution of laked dog blood, titrated to pH 8. As the pH fell below this value, due to acid formation, (accelerated by the carbonic anhydrase of the blood) from the incoming carbon dioxide, the relay and motorized micrometer (F) fed a dilute, standardized sodium hydroxide solution from a syringe microburet into the collecting cell. The rate of addition of alkali was plotted on the titrigraph, and was constant for the first 30 seconds of reaction. Components A, B, and C of the apparatus were kept at 5°C in a refrigerated water bath, as were the storage bottles of all components of the reaction and collection systems.

The reaction mixtures had total volumes of 10 ml. To 8.5 ml of 0.05 M phosphate buffer were added, as required, 0.2 unit of the carbonic anhydrase preparation in 0.5 ml of 0.05% peptone and inhibitor in 0.5 ml water. Enzyme and inhibitor were allowed to eqilibrate for 3-5 min. Water was added q. s. 9.5 ml, and the reaction was initiated by the addition of substrate (0.5 ml of a freshly-prepared solution of sodium bicarbonate in CO_2-free water) from a syringe through the needle. The final pH in each case was 7.0; the proper pH of the phosphate buffer required with each substrate concentration in order to attain this final pH, was determined in preliminary experiments.

Results. When enzyme was omitted from the reaction cell (the crude carbonic anhydrase was always a part of the collection system), initial rate of reaction was directly

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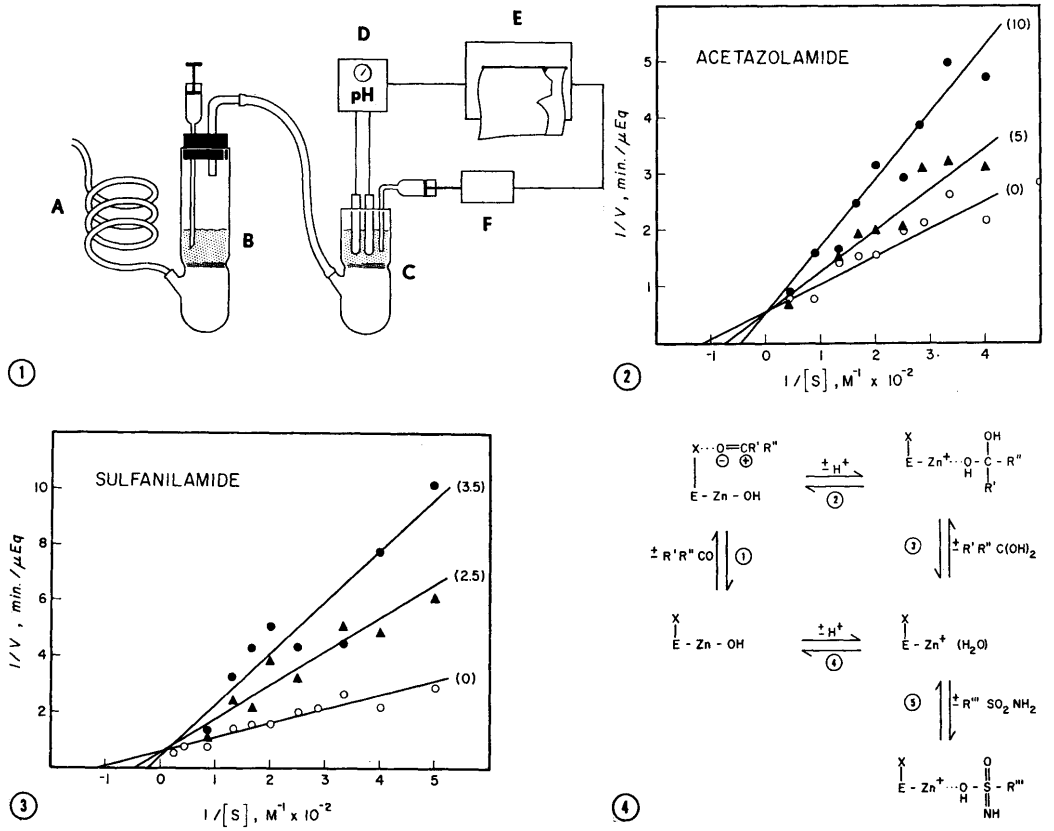

FIG. 1. Diagram (not drawn to scale) of the apparatus. Letters refer to description in text.
FIG. 2. Bicarbonate dehydration in presence of acetazolamide at molar concentrations shown in parentheses x 10^{-8}.
FIG. 3. Bicarbonate dehydration in presence of sulfanilamide at molar concentrations shown in parentheses x 10^{-5}.
FIG. 4. Hypothetical reaction scheme.

proportional to substrate concentration. It may therefore be concluded that the titration rates measured in these experiments were proportional to the rate of liberation of carbon dioxide in the reaction chamber, over the range of bicarbonate concentration used. A plot of these data was subsequently used to determine the rate of the corresponding uncatalyzed reaction to be subtracted from the measured overall rates in the presence of enzyme, in order to calculate the rates of the enzyme-catalyzed reactions.

Means of the reciprocals of substrate concentrations and of titration rates were plotted, and Lineweaver-Burk-Dixon(7) plots constructed from all points by the method of least squares. Figs. 2 and 3 show that both acetazolamide and sulfanilamide obey, under these conditions, the kinetics of competitive inhibition of the carbonic anhydrase-catalyzed production of carbon dioxide from bicarbonate. K_I, calculated from the constants of the least-square lines, is 8.1 and 9.1 (avg 8.6) $\times$ 10^{-8} M for acetazolamide, and 1.0 and 2.5 (avg 1.8) $\times$ 10^{-5} M for sulfanilamide. These values are appreciably higher than those found by various investigators for the inhibition of both dehydration and hydration reactions catalyzed by a variety of non-bovine carbonic anhydrase preparations of varying degrees of purification. They are, however, very similar to the I_{50} values (acetazolamide, 7.2 and 7.9 $\times$ 10^{-8} M; sulfanilamide, 1.3 and 1.7 $\times$ 10^{-5} M) reported from two different laboratories for inhibition of carbon dioxide hydration catalyzed by semi-

purified bovine erythrocyte enzyme preparations(8,9).

The apparent value of K_M derived from these data is 9.0 mM. This agrees well with the value of 9.6 mM determined for bovine erythrocyte carbonic anhydrase by DeVoe and Kistiakowski(10).

Discussion. The kinetics of inhibition indicated here for the formation of carbon dioxide differ from those found previously for the reverse reaction(1,2). A number of possible explanations for this situation come to mind.

If the active sites for binding of the two substrates, the carbonyl compound and its hydrate, were different, and if inhibitor were bound to only one of these sites, then the reaction would be competitively inhibited in one direction and non-competitively in the other. Recent evidence indicates that carbonic anhydrase exists in two forms which differ by one unit of charge, the basic form being active in hydration and the acid form in dehydration(11,12). Sulfonamide inhibitors appear to react with the acid form(11). The ionizing site, which is not identical with the carbonyl-binding site(3,4,12), has been suggested to be a zinc-bound water molecule which loses a proton to become a zinc-bound hydroxyl(12). A series of equilibria consistent with these findings and hypotheses are shown in Fig. 4. Here, X represents the carbonyl-binding group; the mode of binding of the carbonyl compound shown is suggestive only. E stands for the remainder of the apoenzyme. The carbonyl substrates (CO_2, aldehydes, esters) are symbolized by R'R''CO, while R'R''C(OH)$_2$ represents the corresponding hydrated compounds. The latter, and the sulfonamide inhibitors, are shown in Fig. 4 in the protonated form; if these are anionic, reaction 2 would not involve a proton. This diagrammatic representation is consistent with the favored kinetic mechanism of DeVoe and Kistiakowski(10). In this scheme, neither sulfonamide inhibitors nor ionization at the hydroxy-metallic site would affect binding of the carbonyl substrate. Competitive binding between sulfonamides and bicarbonate has previously been suggested on the basis of structural resemblance (13,14), but not in a manner which would explain non-competitive inhibition of CO_2 hydration. The very low K_I values of sulfonamide inhibitors suggest that additional binding of these compounds at some locus on the protein molecule may occur. This might take place *via* the sulfonamido nitrogen atom; this would be consistent with the fact that sulfonamides substituted on this atom are inactive as inhibitors(15,16).

Alternatively, the observed non-competitive inhibition kinetics of the hydration reactions(1-4) might not be indicative of the true nature of the inhibition. If, under the conditions of the assays, the enzyme-inhibitor complex were relatively slowly dissociable, there might result a situation of "pseudoirreversibility" which would tend to mask the true kinetic picture. Such a situation was studied in a different context by Goldstein (17), and is the subject of a recent note by Kernohan(18).

Finally, it must be pointed out that the experiments on inhibition of CO_2 hydration were carried out with relatively crude preparations from human erythrocytes(1,2). In the present work, a partially purified enzyme from bovine erythrocytes was employed. Differences between the two types of enzyme preparation cannot be ruled out. However, non-competitive inhibition of other carbonyl hydration reactions(3,4) was observed with bovine erythrocyte carbonic anhydrase.

Dobry-Duclaux has recently reported(19) that Roussin salt, $K[Fe_4S_3(NO)_7]$, is a competitive inhibitor of bicarbonate dehydration, but an *un*competitive inhibitor of CO_2 hydration. She made use of a commercial enzyme preparation of bovine erythrocyte origin. It is not immediately apparent how such a situation, in which the enzyme-inhibitor complex is stabilized by the association of the carbonyl substrate with its binding site, may be reconciled with the existing body of knowledge concerning this enzyme and its inhibitors.

Summary. The kinetics of inhibition by acetazolamide and sulfanilamide of the formation of carbon dioxide from bicarbonate, catalyzed by partially purified carbonic anhydrase preparations from bovine erythro-

cytes, were those of competitive inhibition. This is contrasted with the non-competitive kinetics of sulfonamide inhibition of a number of carbonic anhydrase-mediated hydration reactions. K_I for acetazolamide was 8.6×10^{-8} M while that for sulfanilamide was 1.8×10^{-5} M; these values are similar to those reported for inhibition of CO_2 hydration in the presence of bovine erythrocyte enzyme.

Note added in proof. From other types of evidence, J. E. Coleman (personal communication) has written a reaction scheme similar to that presented here.

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^{35}S 2-Hydroxyethanesulfonic Acid (Isethionic Acid) in Urine of Human Subjects Given ^{35}S Taurine.* (32026)

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Koechlin(1) was the first to report the occurrence of isethionic acid in a biological system. He found it to be the major anion in the axone of the squid, *Loligo pealii.* Later Braun and Fromageot(2) found that the mold *Aspergillus niger* could grow in a medium containing taurine as the sole source of sulfur and that isethionic acid accumulated as a presumed intermediate in the degradation of taurine to sulfate. Welty, Read and Shaw (3) demonstrated isethionic acid in the dog heart.

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In studying the metabolism of ^{35}S taurine in Down's syndrome (mongolism, trisomy 21) and in normal subjects, Wainer, King, Goodman and Thomas(4) observed radioactivity in urine not attributable to taurine or sulfate. The present paper reports on studies indicating that isethionic acid is present in the urine of normal and mongoloid subjects as a catabolite of taurine.

Experimental procedures. Normal subjects were the present investigators and mongoloid subjects were institutionalized young adults whose clinical diagnoses of mongolism were confirmed by chromosomal analyses. For most studies, ^{35}S taurine obtained from Volk Radiochemical Co. was administered orally in doses of 50 μc to fasting subjects. Urine