the excess of ADP^{3-} was made large; rather, it decreased to a level near that of aqueous Zn^{2+}. For these reasons, it seems necessary to offer an alternate interpretation of line narrowing by excess ADP^{3-}. A reasonable possibility is that excess ADP^{3-} forms a new complex by reacting with the nucleotide base of $Zn(ADP)^-$ rather than with the bound Zn^{2+}. This type interaction could, by blocking a nucleotide base atom normally used for internal chelation of Zn^{2+}, lead to a less rigid configuration for the Zn^{2+} site and a corresponding decrease in τ. Intermolecular hydrogen bonding between nucleotide bases as well as stacking of the bases are reactions of this type, which are well substantiated for NDP concentrations $\geq$ those used in excess NDP experiments.

It has been noted that the Zn^{2+} environment in $Zn(CDP)^-$ (which, like that in $Zn(ADP)^-$, is characterized by a relatively high $\bar{\nu}$) appears to be more stable toward an interaction with a second CDP^{3-} molecule. It is tempting to attribute this to a stronger metal ion chelation to the nucleotide ring in the $Zn(CDP)^-$ complex. Certainly this would correlate well with the basicities of the adenosine and cytidine rings. An internal chelate involving the cytidine ring ($pk = 4.6$) would on this basis be more stable than one involving the adenine ring ($pk = 3.9$).

The changes which occur in the ^{35}Cl relaxation produced by $Zn(ADP)^-$ and $Zn(IDP)^-$ as the solution pH is raised beyond 6.0 are also indicative of the structure of the complexes. For $Zn(ADP)^-$, hydrolysis of the metal ion leads to very extensive removal of ^{35}Cl relaxation by bound Zn^{2+}. $Zn(ADP)^-$ produces a maximum ^{35}Cl line broadening near pH 6.5 but only one-third of this remains at pH 8.7. If the Zn^{2+} ion of $Zn(ADP)^-$ were chelated between two phosphate groups and the nucleotide base, then hydrolysis of the Zn^{2+} would most likely produce a coordinately saturated metal ion inaccessible to Cl^- ions. This would account for the observed behavior.

For $Zn(IDP)^-$ it has been noted that a pH-dependent change in the complex takes place over the pH region 6.0 to 7.0. The resulting complex produces ^{35}Cl relaxation to a considerably greater extent than does $Zn(IDP)^-$. This was particularly evident in solutions containing excess Zn^{2+} where ^{35}Cl relaxation in the ADP and IDP systems became nearly the same at pH 7.0. We tentatively suggest that hydrolysis of the Zn^{2+} ion in $Zn(IDP)^-$ leads to the formation of an internal chelate structure characterized by a longer τ_{rot}, essentially equal to that for $Zn(ADP)^-$. The apparent stability of the structure may be due to hydrogen bonding between the inosine OH and $>Zn(OH)^-$ moiety. Removal of a proton from the inosine ring ($pk \approx 9.0$) would seem unlikely in this pH region.

As a result of these studies, we conclude that internal chelation of Zn^{2+} occurs in $Zn(ADP)^-$ and $Zn(CDP)^-$, while the Zn^{2+} is bound only to the phosphate chain of $Zn(IDP)^-$. It appears that the latter complex may also attain a folded configuration as a result of metal ion hydrolysis. It also seems clear that conditions of pH and molar ratio of Zn^{2+}:NDP can readily influence the Zn^{2+} ion environment in these solutions. Results quite similar in nature have been obtained for Zn^{2+}–nucleotide triphosphate complexes and will be the subject of a later report.

Acknowledgment. This work was supported in part by the Bio-Medical Department of the Lawrence Radiation Laboratory, Livermore.

The Carbonic Anhydrase Catalyzed Hydrolysis of 2-Hydroxy-5-nitro-α-toluenesulfonic Acid Sultone

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Contribution from the Searle Chemistry Laboratory, University of Chicago, Chicago, Illinois 60637. *Received March 18, 1969*

Abstract: An ionizable group in bovine carbonic anhydrase (BCA) with a pK of 7.3 appears to be involved in the enzyme-catalyzed hydrolysis of 2-hydroxy-5-nitro-α-toluenesulfonic acid sultone (I). Similar observations have been reported previously for the pH–rate behavior of the BCA-catalyzed hydration of CO_2, hydration of carbonyl compounds, and hydrolysis of nitrophenyl esters of carboxylic acids. The BCA-catalyzed hydrolysis of I is subject to sulfonamide inhibition as are the other reactions mentioned above. Also, human carbonic anhydrases B and C have been demonstrated to be effective catalysts for the hydrolysis of I. On the basis of our observations taken in conjunction with those of other investigations we have proposed that a zinc bound hydroxide ion is the active catalytic species in carbonic anhydrase action and we have suggested a cyclic mechanism for the carbonic anhydrase catalyzed solvolysis of I, involving no net proton transfer to the solvent.

A previous communication from this laboratory presented preliminary information on the carbonic anhydrase catalyzed hydrolysis of a new sulfonate ester substrate, 2-hydroxy-5-nitro-α-toluenesulfonic acid sultone (I).[2] In the present article we report a full account of our findings with this compound which appears to be the most rapidly hydrolyzed ester substrate of carbonic anhydrase known. We have explored the esterase properties of bovine erythrocyte carbonic anhydrase (BCA) and human carbonic anhydrases B and C (HCAB and HCAC).

(1) Fellow of the Alfred P. Sloan Foundation.

(2) K.-W. Lo and E. T. Kaiser, *Chem. Commun.*, 834 (1966).

Experimental Section

Enzyme. Bovine erythrocyte carbonic anhydrase was obtained from Mann Research Laboratories, New York, and stored dry in a desiccator at +4° immediately upon receipt. The enzyme, in the form of a lyophilized powder, is a mixture of BCAA and BCAB (hereafter designated as BCA) and was used without further purification or fractionation. The following batches of BCA were used [lot (activity from data supplied by Mann)]: P 2631 (2500 units/mg), R 2662 (not specified), S 1020 (3400 units/mg), and T 1288 (not specified).

Stock solutions were prepared by dissolving suitable amounts of the enzyme in 0.09 *M* Tris–HCl buffer, pH 7.4 (0.09 *M* Tris–H_2SO_4 buffer, pH 7.6, was used in stock solutions employed during the later phases of this work), and dialyzed overnight at +1° against the same buffer. Insoluble materials were removed by centrifugation. Enzyme stock solutions were stored in 10- or 25-ml volumetric flasks at +4° and their activities were checked periodically with 2-hydroxy-5-nitro-α-toluenesulfonic acid sultone (I). It was found that stock solution showed practically no loss in activity for more than 1 year. Since there is no satisfactory way of titrating the active site of CA, the enzyme concentration was determined spectroscopically using an ϵ value at 280 mμ of 56,100 l. mol^{-1} cm^{-1},[3] based on a molecular weight of 30,000. For all the enzyme preparations, the ratio of the absorbance at 280 mμ (A_{280}) to that at 260 mμ (A_{260}) ranged from 1.58 to 1.70, which is in good agreement with the values of 1.60 and 1.70 reported by Lindskog[4] and Lieflander,[3] respectively. Enzyme stock solutions showed a faintly yellow color and a small absorption maximum at 410 mμ (about 0.06 OD unit for an enzyme concentration of 2.75×10^{-5} *M*). This is probably due to the residual hemoglobin in the enzyme preparation.

Human CA solutions were kindly supplied by Dr. P. L. Whitney of Professor J. T. Edsall's Laboratory, Harvard University. HCAB (about 50 mg/ml) in Tris–ammonium sulfate solution was dialyzed against 0.09 *M* Tris–HCl buffer (μ 0.09), pH 7.4, for 36 hr with three changes of dialysate. Since the sample of HCAC (about 25 mg/ml) in Tris–ammonium sulfate solution which we received was turbid, we dialyzed it as in the case of HCAB and then centrifuged it. As in the case of BCA, enzyme concentrations were determined by measuring the absorbance of solutions at 280 mμ, using ϵ_{280} = 49,000 l. mol^{-1} cm^{-1} and ϵ_{280} = 56,000 l. mol^{-1} cm^{-1} for HCAB and HCAC, respectively.[5] The ratio A_{280}/A_{260} was also calculated in each case and is shown in Table I.

Table I

Enzyme	Source	A_{280}/A_{260}
BCA	Mann	1.58–1.70
HCAB	Whitney	1.78
HCAC	Whitney	1.61

Materials. The deionized water used was obtained by passing distilled water through a Continental demineralizer.

Acetonitrile was Eastman spectrograde or Baker analyzed reagent grade and was refluxed and distilled from P_2O_5 (bp 80.5°).

Reagent grade acetone was fractionally distilled and a center cut boiling at 55.5° was used.

Acetazolamide was obtained from K & K Laboratories and was used directly.

2-Amino-2-hydroxymethyl-1,3-propanediol (Tris) obtained from Matheson Coleman and Bell was recrystallized from MeOH, mp 170–171°. Ultra-pure Tris from Mann Research Laboratories was also used.

2-Amino-2-methyl-1,3-propanediol (Ammediol) obtained from Matheson Coleman and Bell was recrystallized from ethyl acetate, mp 112–114°.

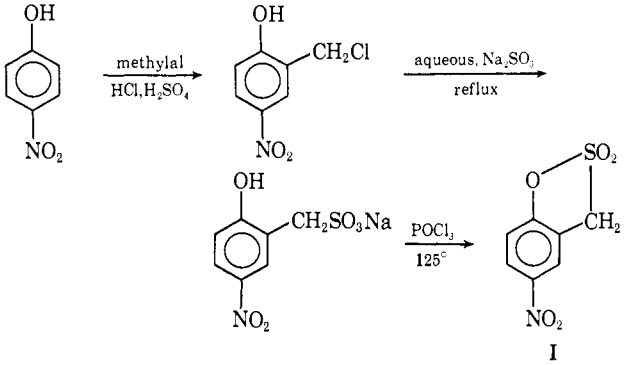
Pyridine–SO_3 complex was obtained from Aldrich Chemical Co.

Syntheses.[6] **2-Hydroxy-5-nitro-α-toluenesulfonic acid sultone (I).**[7] 2-Hydroxy-α-toluenesulfonic acid sultone[8] (2.4 g, 14.1 mmol) was dissolved in 20 ml of concentrated H_2SO_4 and the resultant solution was cooled in an ice bath; 1.07 ml (1.52 g, 16.92 mmol) of 70.3% HNO_3 was added dropwise with stirring over a period of 15 min. The solution became pale yellow and was allowed to stand in the ice bath for 10 min. Small ice cubes were slowly added until no further precipitate was obtained. The pale yellow precipitate was filtered through a sintered glass funnel, washed with a small amount of ice-cold water, and dried by suction. Recrystallization from ethanol gave very pale yellow crystals, mp 148.5–149.5° (lit.[9] 148°). The yield was 2.85 g (94%).

Anal. Calcd for $C_7H_5NO_5S$: C, 39.15; H, 2.32; N, 6.52; S, 14.88. Found: C, 39.22; H, 2.27; N, 6.45; S, 14.61.

Spectral data were as follows: nmr (τ values, in acetone-d_6) singlet 5.00 (2 H), doublet 2.60 (1 H), and doublet 1.65 (2 H) (TMS was used as an internal standard); ir (KBr pellet) 3.27, 3.36, 8.23, 8.48, 8.58, 12.44, 12.64, 13.39, and 13.99 μ; uv (acetonitrile) peak at λ 276 mμ (ϵ 9.85×10^3 l. mol^{-1} cm^{-1}). I has also been synthesized from *p*-nitrophenol, albeit in a very low yield, according to Scheme I. 2-Hydroxy-5-nitrobenzyl chloride (5.61 g, 30 mmol) prepared from *p*-nitrophenol and methylal according to the method of Buchler,

Scheme I



I

et al.,[10] was mixed with 11.34 g (90 mmol) of anhydrous sodium sulfite and 100 ml of deionized water. The mixture was heated under reflux for 3 hr and the color of the solution changed to yellow. Water was removed from the solution under reduced pressure and the residue was dried under vacuum. The crude sodium salt was treated with 100 ml of phosphorus oxychloride and refluxed at a temperature of 125° for 30 hr. $POCl_3$ was carefully removed by distillation under reduced pressure, and the residue was ground and added to ice-cold 1 *N* H_2SO_4. The resultant mixture was allowed to stand at ice temperature for 4 hr and filtered. The light brown precipitate was washed thoroughly with ice-cold water and dried by suction. Three recrystallizations from ethanol gave a faintly yellow compound, mp 148°, yield, 0.3 g. This compound has identical ir and uv spectra to I prepared by direct nitration. Also there was no depression on mixture melting point and the two preparations have the same rate constant for their alkaline hydrolysis.

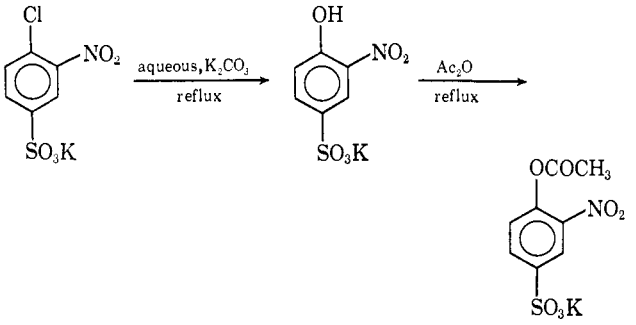
Potassium 4-Acetoxy-3-nitrobenzenesulfonate. Potassium carbonate (30 g, 217 mmol) in 350 ml of deionized water was added to 35 g (127 mmol) of potassium 4-chloro-3-nitrobenzenesulfonate (Eastman) in a 1-l., round-bottomed flask equipped with a reflux condenser and an efficient magnetic stirrer. The chloronitrobenzenesulfonate was not soluble and floated on the surface. The mixture was refluxed for 4 days giving a cherry red solution which was allowed to cool and filtered. The filtrate was carefully acidified with concentrated HCl until no further precipitate of the nitrophenol was obtained. This was filtered and dried, yielding 30 g (92%) of potassium 4-hydroxy-3-nitrobenzenesulfonate.

Potassium 4-hydroxy-3-nitrobenzenesulfonate (5.1 g, 20 mmol) was treated with 50 ml of acetic anhydride in a 100-ml, round-bottomed flask fitted with a reflux condenser and $CaCl_2$ tube. The mixture was refluxed for 18 hr, cooled, filtered, washed thoroughly with dry benzene, and dried, giving 5.8 g (97% yield) of white solid which was recrystallized from 1:1 (v/v) EtOH–CH_3CN.

(3) M. Lieflander, *Z. Physiol. Chem.*, **335**, 125 (1964).
(4) S. Lindskog, *Biochim. Biophys. Acta*, **39**, 218 (1960).
(5) J. M. Fine, G. A. Boffa, M. Charrel, G. Laurent, and Y. Derrien, *Nature*, 200, 371 (1963).
(6) Melting points are uncorrected and were obtained on a Thomas-Hoover "Uni-melt" capillary melting point apparatus. Analyses were performed by Micro-Tech Laboratories, Inc., Skokie, Ill. Nmr, ir, uv, and mass spectra were taken on Varian A-60, Perkin-Elmer Infracord, Cary 14 or Cary 15, and MS 9 instruments, respectively. Only the principal and characteristic peaks are included in the ir listings.
(7) This compound can also be called 5-nitro-3H-1,2-benzoxathiole 2,2-dioxide according to "The Ring Index," A. M. Patterson and L. T. Capell, Reinhold Publishing Corp., New York, N. Y., 1940.
(8) O. R. Zaborsky and E. T. Kaiser, *J. Amer. Chem. Soc.*, **88**, 3084 (1966).
(9) W. Marckwald and H. H. Frahne, *Ber.*, **31**, 1854 (1898).
(10) C. A. Buchler, F. K. Kirchner, and G. F. Deebel, *Org. Syn.*, 20, 59 (1940).

Anal. Calcd for $C_6H_6NO_7SK$: C, 32.10; H, 2.02; N, 4.68; S, 10.71. Found: C, 32.23; H, 2.19; N, 4.66; S, 10.54.

Infrared analysis (KBr pellet) showed peaks at: 5.66, 7.99, 8.09, 9.29, 9.58, 11.23, 11.74, and 13.24 μ.



4-Chloro-3-nitrobenzenesulfonamide. Potassium 4-chloro-3-nitrobenzenesulfonate (6 g, 21.76 mmol) (Eastman) was mixed thoroughly with 12 g (57.62 mmol) of PCl_5 in a 100-ml, round-bottomed flask fitted with a reflux condenser and drying tube. The mixture was heated in an oil bath for 30 min at 150°. After cooling to room temperature, 40 ml of dry benzene was added and the mixture was heated on a steam bath with stirring of the solid mass. This was allowed to cool and filtered on a dry fiber glass filter paper. The precipitate was washed with a little benzene. The combined washings and filtrate were again washed with three 25-ml portions of ice-cold water and slowly poured into 30 ml of ice-cold concentrated ammonia solution with stirring. A pale yellow precipitate was formed. This was filtered,[11] washed with water, and recrystallized in 50% (v/v) ethanol giving pale yellow shiny crystals, mp 175–176°. The yield was 3 g (59.5%).

Anal. Calcd for $C_6H_5N_2O_4ClS$: C, 31.11; H, 2.17. Found: C, 30.83; H, 2.39.

Spectral analysis gave the following results: ir (KBr pellet) 2.96, 3.22, 8.50, 8.65, 9.00, 9.47, 10.91, 11.30, 11.93, 12.84, and 13.21 μ; uv (0.09 *M* Tris–H_2SO_4 buffer, pH 7.6) peaks at λ 262.5 mμ (ϵ 6.78 × 10^4 l. mol^{-1} cm^{-1}), 224 mμ (ϵ 3.59 × 10^4 l. mol^{-1} cm^{-1}), and trough at λ 233.5 mμ (ϵ 3.16 × 10^4 l. mol^{-1} cm^{-1}).

4-Hydroxy-3-nitrobenzenesulfonamide. Potassium 4-acetoxy-3-nitrobenzenesulfonate (4 g, 14.5 mmol) was mixed thoroughly with 10 g (48 mmol) of PCl_5 in a 100-ml, round-bottomed flask fitted with a reflux condenser and drying tube. The mixture was heated in an oil bath at 150° for 30 min and allowed to cool to room temperature. Dry benzene (30 ml) was added and the mixture was heated on a steam bath with stirring to give a reddish brown solution and a white precipitate which was removed by filtration. The filtrate was washed with three 30-ml portions of ice-cold water. After drying the resultant solution with anhydrous sodium sulfate, the benzene was removed by evaporation to give a pale yellow solid (the sulfonyl chloride), mp 85–86°.

The sulfonyl chloride (2 g) in 15 ml of benzene was slowly poured into 10 ml of ice-cold concentrated ammonia solution. The benzene layer was washed with water and dried over anhydrous sodium sulfate, leaving a brownish oil on evaporation, which solidified on prolonged standing. The aqueous layer was adjusted to pH 7 (Hydrion paper) with concentrated HCl. The sulfonamide was formed as a bright red precipitate on standing. This was filtered and recrystallized from acetone–hexane to yield pale yellow crystals, mp 203–204° dec.

Anal. Calcd for $C_6H_6N_2O_5S$: C, 33.03; H, 2.77. Found: C, 33.56; H, 2.87.

Spectral analysis gave the following results: ir (KBr pellet) 2.96, 3.03, 7.48, 8.65, 9.00, 10.09, and 11.61 μ; uv (0.09 *M* Tris–H_2SO_4 buffer, pH 7.6) peak at λ 395 mμ (ϵ 4.17 × 10^3 l. mol^{-1} cm^{-1}) and trough at λ 313 mμ (ϵ 6.40 × 10^2 l. mol^{-1} cm^{-1}).

α-Toluenesulfonyl Fluoride. α-Toluenesulfonyl chloride (19.06 g, 100 mmol) (Eastman) and 20.4 g (500 mmol) of anhydrous sodium fluoride (Fisher) were suspended in 20 ml of DMF and stirred for 4 hr at 110°. After cooling, 80 ml of water was added and the solution was repeatedly extracted with ether. The ether layer was dried with anhydrous sodium sulfate and evaporated to dryness leaving a brown residue. This was taken up in chloroform and precipitated by petroleum ether (bp 30–60°) when a cream-colored precipitate was obtained. Recrystallization of this precipitate with benzene–cyclohexane gave colorless needles, mp 90–92°. The yield was 7.0 g (40%). The mass spectrum showed the absence of the sulfonyl chloride; ir (KBr pellet) 3.33, 3.39, 7.12, 8.21, 8.39, 12.36, 12.83, 13.17, and 14.32 μ.

pH Measurements. The pH values of buffer solutions employed in dialyses and spectrophotometric kinetic runs were measured on a Beckman Research pH meter or a Radiometer Model PHM 4 instrument which were standardized against fresh standard buffers (Fisher Certified) shortly before use. Two types of combination glass–calomel electrodes were used, namely Thomas No. 4858-L15 and Radiometer GK2021C. The difference in pH of the buffered reaction mixtures in the spectrophotometer cuvettes before and after enzymatic reaction is usually less than 0.05 pH unit. All runs with pH differences greater than this were discarded.

Kinetic Measurements. Most of the kinetic runs were performed on either a Cary 14 recording spectrophotometer provided with a 0.0–0.2 slide wire or a Beckman DU spectrophotometer equipped with a Gilford Model 220 optical density convertor and a Honeywell recorder. Unless otherwise stated, the cell compartments and the cell jacket of the Cary 14 were thermostated at 25.00 ± 0.10°. At least two half-lives were followed for each run and the infinity absorbance was not taken before seven half-lives had elapsed. About 20 points were read off from the absorbance trace at suitable time intervals and subjected to manual plotting.

In a typical run, 3.0 ml of an appropriate buffer was introduced to a 1-cm optical path quartz cuvette, capacity 4 ml. A small aliquot of enzyme stock solution (usually 10 μl) was then added[12] and the cuvette placed in the thermostated cell compartment for at least 15 min.[13] The initial absorbance (the base line) was set to zero with the "balance" knob of the spectrophotometer. The reaction was initiated by adding a convenient amount of the substrate stock solution to the cuvette with a microliter pipet and the contents stirred well with the pipet or a glass rod flattened at one end. The time elapsed (20 to 30 sec) between the introduction of the substrate solution and the activation of the master switch of the spectrophotometer was noted on an electric timer or a stop watch.

For some of the faster reactions, kinetic measurements were made on a Durrum Gibson stopped-flow instrument. The enzyme in a suitable buffer was introduced into one of the two syringes and the substrate in deionized water in the other. Five minutes was allowed for temperature equilibrium and the run was performed within 30 min after making up the solutions.

Studies with Pyridine–SO_3 Complex. To 1.00 ml of 0.11 m*M* BCA stock solution in 10 m*M* Tris–HCl buffer, pH 8.70 (μ 0.09), was added 50 μl of 30 m*M* pyridine–SO_3 solution in 90 m*M* Tris–HCl buffer, pH 7.42 (μ 0.09). The resultant solution was placed in a refrigerator at +4° and a 25-μl aliquot was assayed with I after 40 hr. A control was run where pyridine–SO_3 was replaced by 90 m*M* Tris–HCl buffer, pH 7.42.

Studies with 2,4-Dinitrofluorobenzene (DFB). BCA (100 μl, 78.6 μ*M*) in 100 m*M* Tris–H_2SO_4 buffer, pH 7.84 (μ 0.5), was diluted to 9.9 ml with the same buffer. DFB (100 μl, 97.4 m*M*) in acetone was added and the solution was incubated at 25 ± 0.2°. Aliquots (100 μl) were withdrawn at suitable intervals and assayed with I. A control was also run on enzyme incubated in the absence of the inhibitor.

Studies with α-Toluenesulfonyl Fluoride (TSF). Ten microliters of 10.11 μ*M* BCA in 90 m*M* Tris–HCl buffer (μ 0.09), pH 7.50, was added to 2.89 ml of the same buffer in a stoppered uv cell with a capacity of 4 ml. To this solution was added 90 μl of 30 m*M* α-toluenesulfonyl fluoride in acetonitrile. The mixture was incubated at 25.0° for 19 hr and then assayed by adding 10 μl of 3 m*M* I in acetonitrile. A control was also run on enzyme incubated in the absence of the inhibitor.

Results

Kinetic Studies with 2-Hydroxy-5-nitro-α-toluenesulfonic Acid Sultone. Even at the highest concentrations of 2-hydroxy-5-nitro-α-toluenesulfonic acid sul-

(11) The benzene layer of the filtrate was separated and washed with water until the washings were neutral to Hydrion paper. After drying over anhydrous sodium sulfate, the benzene was evaporated leaving a brown oil, the ir spectrum of which was quite different from the solid sulfonamide and was not further characterized.

(12) The inhibitor is also added at this stage for inhibition studies.

(13) No reference or blanking was necessary for most of the runs, as the reactions were usually followed at wavelengths far away from the region where the enzyme absorbs strongly. Also, the enzyme concentrations used were too low to contribute significantly to the total absorbance.

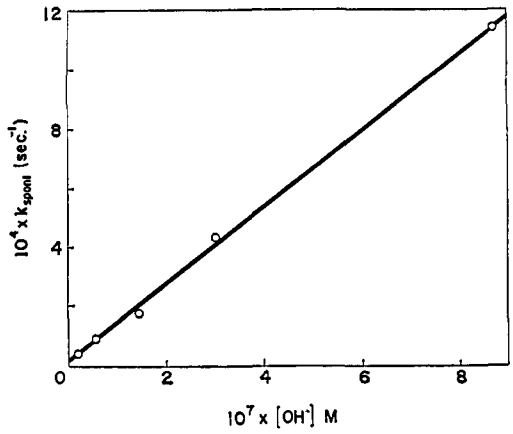


Figure 1. Variation of k_{spont} for the hydrolysis of 2-hydroxy-5-nitro-α-toluenesulfonic acid sultone with hydroxide ion concentration at 25.0 ± 0.2°. Reaction medium: 10 mMTris-HCl or Tris-HOAc buffer (μ 0.09), containing 3.3% (v/v) acetonitrile. k_{OH} = slope of line = 1.31×10^3 l. mol^{-1} sec^{-1}.

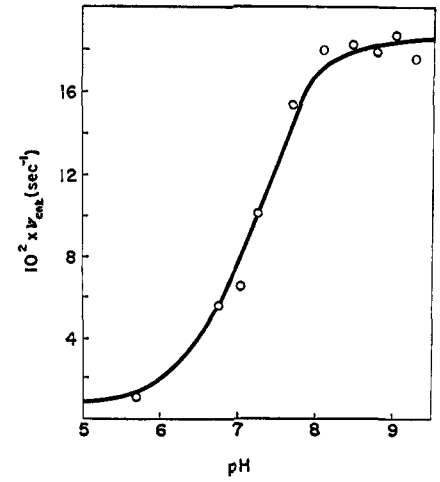


Figure 2. Variation of v_{enz} with pH for the hydrolysis of 2-hydroxy-5-nitro-α-toluenesulfonic acid sultone catalyzed by bovine carbonic anhydrase at 25°. Reaction medium: 50 mM buffer (μ 0.25, maintained with Na_2SO_4) containing 0.5% (v/v) acetone. E_0 = 0.3932 μM. S_0 = 25 μM. Curve is a theoretical sigmoid with a pK of 7.28.

tone employed in our work with the carbonic anhydrases pseudo-first-order kinetics were observed under the usual substrate-in-excess conditions. Since we found that the product of the hydrolysis reactions, 2-hydroxy-5-nitro-α-toluenesulfonic acid, does not act as an inhibitor, our kinetic results imply that the Michaelis constants, K_M, for the reactions investigated are much greater than the highest substrate concentrations used. Accordingly, it was not possible to obtain the catalytic rate constants, k_{cat}, or values of K_M from our data. The rate constant, k_{enz}, is thus used to give an indication of the efficiency of catalysis by carbonic anhydrase with k_{enz} defined by eq 1 where k_{obsd} is the observed pseudo-first-order rate constant in the presence of enzyme and k_{spont} is the rate constant for the nonenzymatic hydrolysis of the substrate under comparable conditions. Particularly in the neutral pH range, the value of k_{spont}

$$k_{enz} = \frac{k_{obsd} - k_{spont}}{E_0} \quad (1)$$

is quite appreciable relative to k_{obsd}. A study of the dependence of k_{spont} on [OH$^-$] reveals a linear relationship (see Figure 1) with a second-order rate constant, k_{OH}, of 1.3×10^3 M^{-1} sec^{-1} at 25.0°. The value of k_{enz} measured at pH 7.55 for bovine carbonic anhydrase (BCA) was found to be independent of the substrate concentration for enzyme concentrations from 3.37×10^{-8} to 3.93×10^{-7} M and substrate concentrations up to 2.3×10^{-4} M.

The pH–v_{enz} ($v_{enz} = k_{obsd} - k_{spont}$) profile (Figure 2) (from data shown in Table II) of the BCA-catalyzed hydrolysis of I at 25.0° is sigmoidal with a pK of 7.28. Three different buffers (phosphate, Tris, and Ammediol) were used and a smooth transition was observed on going from one buffer to another.[14,15]

Human carbonic anhydrases B and C also are effective catalysts for the hydrolysis of 2-hydroxy-5-nitro-α-toluenesulfonic acid sultone (I). Table III illustrates the variation of k_{enz} with enzyme species. It should be noted that here, as in the case of the carbonic anhydrase catalyzed hydration of CO_2,[16] HCAC is a better catalyst than HCAB. BCA has about the same activity as HCAC toward I.

Table II. Variation of Kinetic Parameters with pH for the Hydrolysis of 2-Hydroxy-5-nitro-α-toluenesulfonic Acid Sultone Catalyzed by Bovine Carbonic Anhydrase at 25.0[a]

pH	Buffer[b]	$10^2 k_{obsd}$, sec^{-1}	$10^2 k_{spont}$, sec^{-1}	$10^2 v_{enz}$, sec^{-1}
5.72_1	P	1.07	0.059	1.01
6.83_2	P	5.65	0.158	5.49
7.08_4	T	6.46	0.040	6.42
7.32_2	T	10.02	0.083	9.94
7.72_2	T	15.44	0.148	15.29
8.10_5	A	18.20	0.191	18.01
8.41_0	A	18.90	0.546	18.35
8.79_5	A	19.13	1.176	17.95
9.03_9	A	21.15	2.414	18.74
9.42_0	A	23.22	5.773	17.45

[a] Reaction medium: 0.05 M buffer (P, T, or A), μ = 0.25 (maintained with Na_2SO_4), containing 0.5% acetone: E_0 = 0.393 μM, S_0 = 25.0 μM, $v_{enz} = k_{obsd} - k_{spont}$. Reactions were followed at λ 350 mμ for k_{obsd}, and at λ 320 or 410 mμ for k_{spont}. [b] P = phosphate; T = Tris; A = ammediol.

Table III. Comparison of k_{enz} Values Obtained with Different Enzyme Species for the CA-Catalyzed Hydrolysis of I at 25.0 ± 0.2°[a]

Enzyme	Source	E_0, μM	$10^{-4} \times k_{enz}$, l. mol^{-1} sec^{-1}
HCAB	Whitney	0.312	3.64
HCAC	Whitney	0.147_2	23.1
BCA	Mann	0.096_8	28.3

[a] Reaction medium: 90 mM Tris-HCl buffer (μ 0.09), pH 7.42 to 7.47, containing 10% (v/v) acetonitrile. S_0 = 50 μM, k_{spont} = 9.45×10^{-4} sec^{-1}.

The BCA-catalyzed hydrolysis of I can be inhibited by the sulfonamide, acetazolamide, which is a potent inhibitor for the enzymatic hydration of carbon diox-

(14) At the same pH, buffer concentrations, and ionic strength phosphate is a much better catalyst than Tris and Ammediol in the nonenzymatic hydrolysis of I.

(15) Above pH 8 the scatter in the values of v_{enz} was somewhat worse than at lower pH values because the correction which had to be made for the nonenzymatic hydrolysis of I was larger.

(16) B. H. Gibbons and J. T. Edsall, *J. Biol. Chem.*, 239, 2539 (1964).

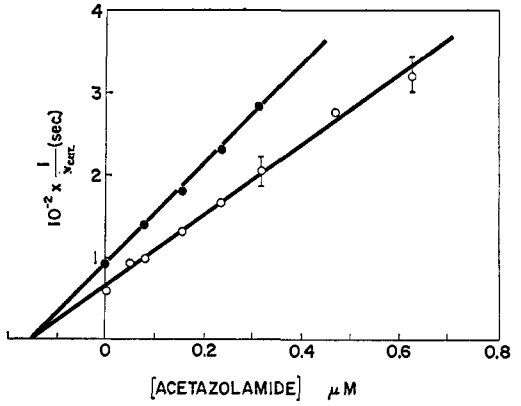
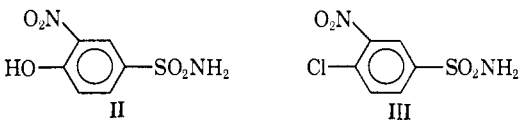


Figure 3. Dixon plot of the acetazolamide inhibition of the BCA-catalyzed hydrolysis of 2-hydroxy-5-nitro-α-toluenesulfonic acid sultone. Reaction medium: 10 m*M* Tris-HCl buffer (μ 0.09), pH 7.50, containing 3% (v/v) acetonitrile. $E_0 = 0.273\ \mu M$. (○) $S_0 = 50\ \mu M$; (●) $S_0 = 75\ \mu M$. Temperature = 25.0 ± 0.2°.

ide.[17] A Dixon[18] plot of data obtained with this inhibitor at two different substrate concentrations is given in Figure 3, and from this plot a value of 1.45×10^{-7} *M* was calculated for the noncompetitive inhibition constant, K_I.[2,19] The effects of two new carbonic anhydrase inhibitors, 4-hydroxy-3-nitrobenzenesulfonamide (II) and 4-chloro-3-nitrobenzenesulfonamide (III), on the BCA-catalyzed hydrolysis of I were also examined. Noncompetitive inhibition was established to be the



mode of action of II, and the same kind of inhibition was assumed for III. The K_I values[20] obtained from Dixon plots are 7.38×10^{-7} *M* and 1.15×10^{-8} *M* for II and III, respectively. The observation that III is a more powerful inhibitor of the action of BCA in catalyzing the hydrolysis of I probably reflects the electronic effect of the chloro substituent.[21] It is known that the magnitude of the inhibition of carbonic anhydrase catalyzed carbon dioxide hydration by a series of structurally related sulfonamides increases with the ease of ionization of the $-SO_2NH_2$ group, suggesting that the ionization of this group is essential for binding with the enzyme.[22,23]

Kinetic Studies with Potassium 4-Acetoxy-3-nitrobenzenesulfonate. Since the solubility limit of the nitrosultone I is on the order of 1×10^{-3} *M* and since at the highest substrate concentrations we employed, we were not able to detect any saturation of the enzyme we decided to explore the possibility that we could saturate the enzyme if we used a more soluble substrate. A substrate which seemed to be worth testing therefore was potassium 4-acetoxy-3-nitrobenzenesulfonate (IV). Compound IV has a solubility in water of about 0.085 *M* at 25° and no organic solvent such as acetonitrile or acetone has to be added to bring it into solution.[24] We found that even at a substrate concentration of 0.071 *M* pseudo-first-order kinetics were observed for more than two half-lives of the hydrolysis of IV as catalyzed by BCA (under conditions of $S_0 >> E_0$). Thus, the K_M for the reaction of IV with BCA must be substantially greater than 0.1 *M*.

In Table IV values of k_{enz} obtained for different forms of CA are shown. It can be seen that HCAB and HCAC have about the same catalytic effect on the hydrolysis of the *o*-nitrophenyl ester IV, and they are somewhat more effective catalysts toward this substrate under the conditions used than BCA is. Verpoorte, *et al.*,[25] have reported that the activities of HCAB and HCAC toward *o*-nitrophenyl acetate differ much less than their relative activities toward the hydrolysis of *p*-nitrophenyl acetate. We briefly investigated the inhibition of the BCA-catalyzed hydrolysis of IV by acetazolamide, and we found a K_I value of approximately 6×10^{-6} *M*.[26]

Table IV. Variation of k_{enz} with Species for the CA-Catalyzed Hydrolysis of IV at 25.0 ± 0.2° [a]

Enzyme	Source	E_0, μ*M*	k_{enz}, l. mol^{-1} sec^{-1}
HCAB	Whitney	8.30	153
HCAC	Whitney	5.92	149
BCA	Mann	6.71	83.7

[a] Solutions of IV in deionized water were freshly prepared shortly before use.

Inactivation Studies

In order to obtain some information about the nature of the groups responsible for the esterase action of CA, several compounds known to react with certain functional groups in proteins were used in inactivation studies. Three compounds, pyridine–SO_3 complex, α-toluenesulfonyl fluoride, and 2,4-dinitrofluorobenzene, were found to inactivate BCA slowly (see Table V). The pyridine–SO_3 complex has been used to sulfonate the 3′-nitrogen of histidine and phenolic OH of tyrosine in insulin[27] and since it has been reported[28] that chemical modification of the only "available" tyrosine in BCAB does not affect the activity of the enzyme, the inactivation of BCA by pyridine–SO_3 complex which we have observed may be due to the modification of at least one histidine in the enzyme.[29] Although α-tol-

(17) R. P. Davis, *J. Amer. Chem. Soc.*, 81, 5674 (1959).
(18) M. Dixon, *Biochem. J.*, 55, 170 (1953).
(19) Pocker and Stone have shown that $K_I = 2 \times 10^{-7}$ *M* for the noncompetitive inhibition by acetazolamide of the BCA-catalyzed hydrolysis of *p*-nitrophenyl acetate in 10% (v/v) acetonitrile in a Tris buffer of ionic strength 0.09 at pH 8.45 and 25.0°. See: Y. Pocker and J. T. Stone, *Biochemistry*, 7, 2936 (1968).
(20) These K_I values were determined for BCA solutions in 0.05 *M* Tris–H_2SO_4 buffer (ionic strength 0.25), pH 7.8, containing 0.47% (v/v) acetone.
(21) The Hammett σ_p values are +0.23 and −0.37 for –Cl and –OH, respectively [D. H. McDaniel and H. C. Brown, *J. Org. Chem.*, 23, 420 (1958)].
(22) W. H. Miller, A. M. Dessert, and R. O. Roblin, Jr., *J. Amer. Chem. Soc.*, 72, 4893 (1950).
(23) The sulfonamide II possesses a chromophoric group which absorbs in the visible region of the spectrum and should therefore provide a useful probe for the study of the environment of the sulfonamide binding site in the enzyme. Studies along these lines and toward the determination of the kinetics of sulfonamide binding are under way in our laboratory.
(24) Solutions of IV in deionized water were prepared shortly before use.
(25) J. A. Verpoorte, S. Mehta, and J. T. Edsall, *J. Biol. Chem.*, 242, 4221 (1967).
(26) The reaction medium consisted of 0.09 *M* Tris-HCl buffer (ionic strength 0.09), pH 7.34, containing 10% (v/v) of acetonitrile.
(27) L. A. Ae. Sluyterman and J. M. Kwestroo-van den Bosch, *Biochim. Biophys. Acta*, 38, 102 (1960).
(28) Z. Nilsson and S. Lindskog, *European J. Biochem.*, 2, 309 (1967).

Table V. Results of Inactivation Studies on Bovine Carbonic Anhydrase[a]

	TSF	PS[b]	DFB
Concn, m*M*	0.90	1.43	0.974
E_0, μM	0.0337	105	0.078_6
Incubation time, hr	19	40	75
Incubation temp, °C	25	4	25
Incubation medium	90 m*M* Tris-HCl buffer (μ 0.09) pH 7.50, 3% CH_3CN	10 m*M* Tris-HCl buffer (μ 0.09) pH 8.70	100 m*M* Tris-SO_4^{2-} buffer (μ 0.5), pH 7.84, 1% acetone
S_0, μM	10	10	23.7
Assay medium	90 m*M* Tris-HCl buffer (μ 0.09) pH 7.50, 3% CH_3CN	90 m*M* Tris-HOAc buffer (μ 0.09), pH 7.1, 10% CH_3CN	100 m*M* Tris-SO_4^{2-} buffer (μ 0.5), pH 7.84, 0.5% acetone
% inactivation	30	21	31

[a] TSF = α-toluenesulfonyl fluoride, PS = pyridine-SO_3 complex, DFB = 2,4-dinitrofluorobenzene. [b] In the studies of inhibition by TSF and DFB, I was the substrate used to assay the % inactivation of enzymatic activity. However, in the case of PS another new substrate, 8-hydroxy-5-nitronaphthalenesulfonic acid sultone, was employed.

uenesulfonyl fluoride reacts stoichiometrically with α-chymotrypsin,[30] sulfonating the active site serine residue (Ser-195), the inactivation of BCA by this reagent which we have found is likely to be due to the modification of a histidine residue near the active site in view of the observation that sulfonyl chlorides are substrates of CA.[31] Whitney, *et al.*,[29] explained the inactivation of HCAB by dinitrofluorobenzene which they observed as being due to the modification of one or possibly two histidines in the enzyme. There is, however, one difference between the inactivation of BCA or HCAB by 2,4-dinitrofluorobenzene. That is, whereas the inactivation process is fast in the case of HCAB,[29] only a very slow inactivation was found by us with BCA (see Figure 4). The reason for this difference in the behavior of BCA and HCAB is not known at the present time. Although no dialysis was performed on the BCA solutions incubated with α-toluenesulfonyl fluoride and 2,4-dinitrofluorobenzene prior to their rate assay, the inactivations we observed using these modifiers cannot be due to inhibition by fluoride ion formed from hydrolyses of the reagents because the concentrations of the modifiers used are well below the K_I value (0.4 *M*) reported for F.[25]

Conclusions

Our studies on the pH-v_{enz} profile for the BCA-catalyzed hydrolysis of the cyclic sulfonate ester I at 25° show that an ionizable group in the enzyme with a pK of 7.28 is important in the catalytic action observed. This value is in good agreement with the observation that in the case of the BCA-catalyzed hydration of CO_2 the function v_{max}[32] has a sigmoidal pH dependence due to the ionization of a group on the enzyme with a pK of 6.9. Similar pH-rate profiles have been found for the action of BCA in catalyzing the hydration of acetaldehyde[33] and the hydrolysis of nitrophenyl esters of carboxylic acids.[34]

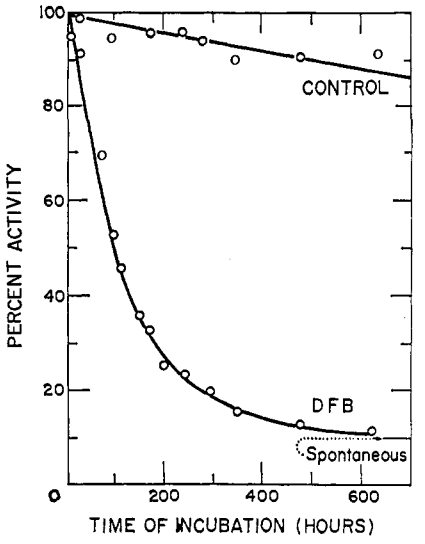


Figure 4. The inactivation of BCA by 2,4-dinitrofluorobenzene (DFB). See Table V for incubation medium and assay medium.

We have shown that another respect in which the catalytic action of BCA in the hydrolysis of the sulfonate ester I resembles its action in the other catalytic reactions previously investigated is that it is subject to strong inhibition by the addition of sulfonamides. Furthermore, our investigation has demonstrated that human carbonic anhydrases B and C are effective catalysts in the hydrolysis of I as they are for the hydration of CO_2[16] and the hydrolysis of nitrophenyl esters of carboxylic acids.[25]

Finally, we have found that several compounds which probably react with histidine residues in BCA inactivate the enzyme slowly. As already mentioned, evidence has been presented in the literature[29] that reagents specific for histidyl residues cause the inactivation of HCAB.

(29) Evidence has been presented by Whitney, *et al.*, that reagents specific for histidyl residues (one or two being modified) which may be bound to Zn(II) in HCAB can cause the inactivation of the enzyme [P. L. Whitney, P. O. Nyman, and B. G. Malmstrom, *J. Biol. Chem.*, 242, 4214 (1967)].

(30) A. M. Gold and D. E. Fahrney, *Biochemistry*, 3, 784 (1964), and A. M. Gold, *ibid.*, 4, 897 (1965).

(31) P. L. Whitney, G. Folsch, P. O. Nyman, and B. G. Malmstrom, *J. Biol. Chem.*, 212, 4206 (1967).

(32) J. C. Kernohan, *Biochim. Biophys. Acta*, 81, 346 (1964). In the BCA-catalyzed hydration of CO_2 values of v_{max} can be obtained. This stands in contrast to our findings with the esters I and IV with which we could not detect any saturation of BCA.

(33) Y. Pocker and J. E. Meany, *Biochemistry*, 4, 2535 (1965).

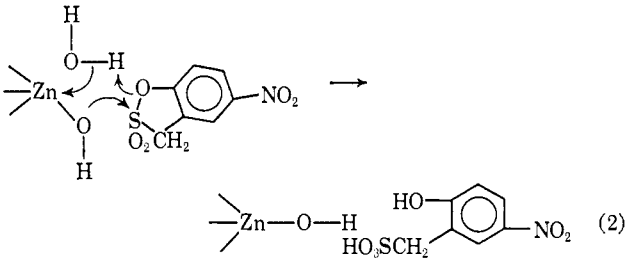
(34) Y. Pocker and J. T. Stone, *ibid.*, 6, 668 (1967).

From the foregoing summary of our findings it seems clear that the mechanism of the carbonic anhydrase catalyzed hydrolysis of I must be very closely related to the mechanisms for the action of the enzyme on other types of substrates. A fundamental problem then in postulating a mechanism for the action of carbonic anhydrase on the sulfonate ester I is to decide what group on the enzyme with a pK of approximately 7 is responsible for the observed catalytic properties.

The suggestion has been made that a zinc-bound water molecule or hydroxide ion is directly transferred to carbon dioxide in the carbonic anhydrase catalyzed hydration of this substrate.[35] Two different types of mechanisms for the action of carbonic anhydrase on various substrates have evolved from this suggestion. On the one hand it has been postulated that the attack of the zinc-bound water on carbonic anhydrase substrates is assisted by a histidine residue with a pK of about 7 in the enzyme which acts in its basic form.[36] Alternatively, recent infrared studies on the action of carbonic anhydrase have been interpreted in terms of a mechanism which involves a zinc-bound hydroxide ion as the active species.[37,38] According to this hypothesis, then, the ionization of the zinc-bound water in carbonic anhydrase occurs with a pK of $\sim$7.

On the basis of presently available evidence we cannot make a final judgment as to the relative merits of the alternative mechanisms. Certainly, at least in the case of HCAB[29] chemical modification of histidine residues can cause inhibition of enzymatic activity. However, we feel that there is no really compelling reason to invoke the direct participation of a histidine residue in the catalytic mechanism of carbonic anhydrase action. An aspect of the mechanisms postulating the involvement of a histidine residue as a general base which is particularly unattractive to us is that a protonated imidazolium species is formed in the step in which the zinc-bound water attacks the substrate. For the enzyme to regain its catalytic activity this imidazolium ion must lose its proton to the solvent. However, we know that proton transfer from the imidazolium ion to water occurs with a rate constant of approximately 10^3 sec^{-1},[39,40] a value which is below the maximum turnover number which has been found for carbonic anhydrase (turnover number $>10^4$ sec^{-1}).[41]

Indeed, it is because of the large magnitude of this maximum turnover number that we postulate the scheme shown below in eq 2 for the action of carbonic anhydrase in catalyzing the hydrolysis of I. In this scheme a zinc hydroxide complex is considered to be the catalytically active species. However, the cyclic reaction pathway of eq 2 avoids the difficulty of postulating a net proton transfer from the enzyme to the solvent, a reaction which may be too slow to be consistent with the high catalytic efficiency of carbonic anhydrase.[42]



(2)

Acknowledgment. The support of the National Institute of General Medical Sciences is gratefully acknowledged.

(35) R. P. Davis, *Enzymes*, 5, 545 (1961).
(36) Y. Pocker and D. R. Storm, *Biochemistry*, 7, 1202 (1968).
(37) M. E. Riepe and J. H. Wang, *J. Amer. Chem. Soc.*, **89**, 4229 (1967).
(38) M. E. Riepe and J. H. Wang, *J. Biol. Chem.*, 243, 2779 (1968).
(39) I. Amdur and G. G. Hammes, "Chemical Kinetics: Principles and Selected Topics," McGraw-Hill Book Co. Inc., New York, N. Y., 1966, p 185.
(40) M. Eigen, G. G. Hammes, and K. Kustin, *J. Amer. Chem. Soc.*, 82, 3482 (1960).
(41) H. DeVoe and G. B. Kistiakowsky, *ibid.*, 83, 274 (1961).
(42) The authors acknowledge the great value of their discussions with various participants at the First Conference on the Chemistry and Mechanism of Enzyme Action held in New Orleans in Dec 1968 in their formulation of the mechanism of eq 2. Obviously, mechanisms equivalent to that shown in eq 2 can be written for the carbonic anhydrase catalyzed hydration of carbon dioxide and the hydrolysis of nitrophenyl esters of carboxylic acids.

Communications to the Editor

Terpenoids. LXIV.[1] Chemical Studies of Marine Invertebrates. V.[2] The Isolation of Three New Holothurinogenins and Their Chemical Correlation with Lanosterol[3]

Sir:

Hydrolysis of the toxic glycosides[4] of various species of sea cucumbers (family *Holothurioideae*) has led to the isolation[5-8] of several sapogenins, which have been assumed to possess a lanostane skeleton (see, for instance, structure IV for griseogenin[6]) on the basis of extensive spectral and circumstantial chemical evidence.[5-8] We should now like to report[9] the isolation

(1) For part LXIII see T. Nakano, M. Hasegawa, T. Fukumaru, L. J. Durham, H. Budzikiewicz, and C. Djerassi, *J. Org. Chem.*, in press.
(2) For part IV see W. Rudiger, W. Klose, B. Tursch, N. Houvenaghel-Crevecoeur, and H. Budzikiewicz, *Ann.*, 713, 209 (1968).
(3) The work at Stanford University was supported by Grant GM 06840 from the National Institutes of Health, while the investigations in Brussels were aided by the Centre Belge d'Océanographie and the I.R.S.I.A.
(4) For leading references see J. D. Chanley, R. Ledeen, J. Wax, R. F. Nigrelli, and H. Sobotka, *J. Am. Chem. Soc.*, **81**, 5180 (1959); S. Shimada, *Science*, **163**, 1462 (1969).
(5) J. D. Chanley, T. Mezzetti, and H. Sobotka, *Tetrahedron*, 22, 1857 (1966).
(6) B. Tursch, I. S. de Souza Guimaraẽs, B. Gilbert, R. T. Aplin, A. M. Duffield, and C. Djerassi, *ibid.*, 23, 761 (1967).
(7) J. D. Chanley and C. Rossi, *ibid.*, 25, 1897 (1969).
(8) J. D. Chanley and C. Rossi, *ibid.*, 25, 1911 (1969).