[CONTRIBUTION FROM THE CHEMOTHERAPY DIVISION, STAMFORD RESEARCH LABORATORIES, AMERICAN CYANAMID COMPANY]

Heterocyclic Sulfonamides as Carbonic Anhydrase Inhibitors[1]

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Since the original observations by Mann and Keilin,[2] other reports have appeared confirming the fact that only sulfonamides unsubstituted on the sulfonamide nitrogen are highly active as carbonic anhydrase inhibitors.[3] Our earlier interest in the specific inhibition of this enzyme system was re-awakened by the studies of Schwartz[4] with sulfanilamide in the control of edema associated with congestive heart failure. Toxicity and low activity appeared to limit the usefulness of sulfanilamide, and suggested the desirability of seeking more effective agents. Moreover, the controversial implication of carbonic anhydrase in the secretion of acid by the stomach indicated another interesting use for such inhibitors.[5]

The diuretic action of sulfanilamide is presumed to rest on the inhibition of renal carbonic anhydrase.[4] Since this enzyme catalyzes the equilibrium reaction, $CO_2 + H_2O \leftrightarrows H_2CO_3$, its inhibition decreases the rate of conversion of carbon dioxide to carbonic acid, and consequently the rate of production of hydrogen ions. The excretion of hydrogen ions has been proposed as the normal mechanism for the conservation of sodium.[6] Therefore, in order to maintain the ionic balance, blocking carbonic anhydrase might be expected to result in the excretion of sodium ions. Such an action should in turn lead to diuresis and to the relief of edema. Thus it is possible, as Schwartz has suggested, that carbonic anhydrase inhibitors offer a new approach to diuretic agents. Such substances may also be of interest for conditions in which the primary objective is the promotion of sodium excretion.

With the exception of thiophene-2-sulfonamide[7] and pyridine-3-sulfonamide,[2,3b] heterocyclic sulfonamides do not appear to have been investigated as carbonic anhydrase inhibitors. The synthesis of a series of these compounds is described in an accompanying paper.[8] As was pointed out there, our earlier studies with sulfanilamide derivatives[9] suggested that heterocyclic unsubstituted sulfonamides, in which the sulfur atom of the sulfonamide group is joined to a carbon atom of the heterocyclic ring, might be highly inhibitory to the carbonic anhydrase enzyme system.

For the purposes of this investigation a rapid approximate method for the determination of relative inhibitory activity was desired. Consequently, in a majority of the inhibition studies the colorimetric method of Philpot and Philpot,[10] which employs sodium bicarbonate as a buffer, was used. Some comparative observations were also made in a veronal buffer system suggested by Roughton and Booth[11] (see Experimental). The relative anticarbonic anhydrase activity of the heterocyclic sulfonamides at fifty per cent. inhibition is recorded in Tables I and II. These values, obtained by the Philpot method, indicate the inhibitory effectiveness with respect to sulfanilamide, which is assigned an arbitrary value of 1. Consequently, the larger the number, the greater the anti-enzymic activity. As indicated in the Experimental part, only differences greater than twofold in the fifty per cent. values are considered to be significant.

Approximately one hundred per cent. inhibitory activities were also determined for some of the more active compounds. These values are intended only as rough approximations, since sharp end-points were not obtainable with the colorimetric methods employed. The ratio of approximately one hundred per cent. to fifty per cent. inhibition is indicated wherever possible. Since it requires about twenty-five times as much sulfanilamide for more or less complete inhibition of the enzyme, the relative activity of some of the heterocyclic derivatives such as benzothiazole-2-sulfonamide, with a ratio of about 7, appears to be greater at approximately one hundred per cent.

In order to decide whether any relationship between acid dissociation constants and activity as enzyme inhibitors existed, the pK_a values for some of the heterocyclic sulfonamides were determined. Table II gives these values for the sulfur-containing heterocyclic derivatives, among which the most active inhibitors were found. An inspection of the pK_a data suggests that, within these particular ring systems, the activity increases with increasing acidity. Each ring system appears to constitute a separate series, since the correlation does not carry over from the thiazoles to the thiadiazoles. A number of other very approximate pK_a values were determined for the compounds in Table I. Because of the limited amounts of materials available in many cases, and the poor spectral shifts observed in the ultraviolet region, these results were not sufficiently re-

(1) Presented in part before the Division of Medicinal Chemistry, American Chemical Society, Philadelphia Meeting, April 10, 1950.

(2) Mann and Keilin, *Nature*, **146**, 164 (1940).

(3) (a) Locke, Main and Mellon, *Science*, **93**, 66 (1941); (b) Krebs, *Biochem. J.*, **43**, 525 (1948).

(4) Schwartz, *New Engl. J. Med.*, **240**, 173 (1949). We are greatly indebted to Dr. S. A. Levine and Dr. W. B. Schwartz for calling our attention to this work which led to the present investigation.

(5) Davenport, *Physiol. Rev.*, **26**, 560 (1946); Davies, *Biochem. J.*, **42**, 609 (1948).

(6) Pitts and Alexander, *Am. J. Physiol.*, **144**, 239 (1945).

(7) Davenport, *J. Biol. Chem.*, **158**, 567 (1945).

(8) Roblin and Clapp, THIS JOURNAL, **72**, 4890 (1950).

(9) Bell and Roblin, *ibid.*, **64**, 2905 (1942).

(10) Philpot and Philpot, *Biochem. J.*, **30**, 2191 (1936).

(11) Roughton and Booth, *ibid.*, **40**, 319 (1946).

TABLE I

RELATIVE CARBONIC ANHYDRASE INHIBITORY ACTIVITY OF HETEROCYCLIC SULFONAMIDES: NITROGEN HETEROCYCLES

Sulfonamide	Inhibitory activity[a] (Sulfanilamide ratio) 50%	~100%	Ratio ~100%/50%
Sulfanilamide	1	1	25
Imidazole-2-	5		
1-Methylimidazole-2-	20	3	150
1-Phenylimidazole-2-	2		
Benzimidazole-2-	14		
1,2,4-Triazole-3-	3		
Pyrido[2,1-c]s-triazole-3-	30	30	25
3-Hydroxy-4-phenyl-4,1,2-triazole-5-	30		
4-Phenyl-4,1,2-triazole-3,5-di-	18	5	75
1-Methyltetrazole-5-	14		
1-Phenyltetrazole-5-	190	650	10
Pyrimidine-2-	3		
4,6-Dimethylpyrimidine-2-	0.4		
5-Chloropyrimidine-2-	16		
Pyrazine-2-	18	40	15

[a] Values indicate approximate number of times as active as sulfanilamide on a molar basis—see Experimental.

TABLE II

RELATIVE CARBONIC ANHYDRASE INHIBITORY ACTIVITY AND pK_a VALUES FOR HETEROCYCLIC SULFONAMIDES: SULFUR AND NITROGEN HETEROCYCLES

Sulfonamide	Inhibitory activity (Sulfanilamide ratio)[a] 50%	~100%	Ratio ~100%/50%	pK_a[c]
1,3,4-Thiadiazole-2,5-di-	800	870	20	6.2
2-Acetylamino-1,3,4-thiadiazole-5-	330	440	15	7.2
2-Amino-1,3,4-thiadiazole-5-	25	..	..	7.7
Benzothiazole-2-	730	2500	7	7.8
Thiazole-2-	120	240	10	8.25
4-Methylthiazole-2-	50	70	15	8.35
2-Acetylaminothiazole-5-[b]	15	6	50	8.5
2-Aminothiazole-5-[b]	3	..	..	9.25

[a] *Cf.* Table I. [b] Prepared in this Laboratory by Mr. H. W. Marson using the method of Backer and Buisman, *Rec. trav. chim.*, **63**, 228 (1944). [c] pK_a values were determined by Miss K. S. Howard by electrometric titration or ultraviolet spectral shifts.

liable to be reported. In general, the correlation appeared to be less striking among the other heterocyclic derivatives, although the most active compounds were also among the most acidic. Undoubtedly steric effects and other factors are also important, and they may in many cases overshadow any pK_a relationship.

Several of the heterocyclic sulfonamides are sufficiently more active than sulfanilamide so that, if the relative activities carry over to *in vivo* conditions, they may be of considerable interest, both from the standpoint of elucidating the functions of carbonic anhydrase, and possibly in providing more effective diuretic action than that attainable with sulfanilamide. It would also appear to be of interest to examine the inhibitory action of these compounds with other enzyme systems, such as those involved in the Krebs cycle, for which carbon dioxide or bicarbonate ion serve as substrates.

Experimental

Preparation of Carbonic Anhydrase.—The crude "chloroform enzyme" of Meldrum and Roughton,[12] prepared from beef erythrocytes with the modification of Roughton and Booth,[13] was employed in a majority of the inhibition studies. A light brown powder was obtained by lyophilization of the enzyme solution. This product was stable for more than two years when stored in the freezing compartment of a refrigerator. Some studies were also carried out with a partially purified enzyme obtained by the fractional ammonium sulfate precipitation method of Keilin and Mann[14] applied directly to the crude "chloroform enzyme." Approximately an eight-fold concentration of lyophilized enzyme was obtained by this procedure.

Determination of Enzymic Activity.—For a majority of the inhibitors, the rapid sodium bicarbonate buffer method of Philpot and Philpot[10] was employed. The control reaction time (no enzyme) was approximately sixty seconds. Sufficient enzyme was used to reduce the reaction time to about ten seconds, the working range for inhibition studies being ten to twenty-five seconds. Although the color change at the end-point is quite sharp, it is helpful to use a titration light with a white background. The brom thymol blue indicator was prepared according to the directions of Kolthoff and Laitinen.[15]

For some of the inhibition studies, a veronal buffer was used in a modification of the procedures of Roughton and Booth[11] and Wilbur and Anderson.[16] These procedures have not been evaluated extensively. It was, therefore, necessary to carry out preliminary studies to determine a satisfactory working range for the veronal system. Under the conditions employed, the control end-point in the absence of enzyme was in the neighborhood of one hundred and fifty seconds. The end-points were more gradual than in the bicarbonate system and a titration lamp and white background was much more necessary. In the presence of enzyme, where the time interval was five to twenty seconds, the end-points were considerably sharper. A study of this range indicated that, while a plot of enzyme concentration *versus* time gave a hyperbola, a straight line was obtained for the reciprocal of enzyme concentration (Fig. 1). It is particularly important in this system that all solutions be kept at constant temperature in an ice-bath. Dewar flasks were employed to advantage for this purpose.

Inhibitor Studies—Heterocyclic Sulfonamides.—The relative values, compared with sulfanilamide on a molar basis, for the heterocyclic sulfonamides investigated are given in Tables I and II. The total volume in the bicarbonate buffer system was 17 ml. including 11 ml. of the carbon dioxide saturated sodium bicarbonate solution, 4 ml. of water containing enzyme and test substance and 2 ml. of sodium carbonate-bicarbonate solution, as described by Philpot and Philpot.[10] For crude "chloroform enzyme," 3 ml. or more of a solution containing 7–10 mg. of enzyme per 250 ml. was used. With partially purified carbonic anhydrase the amount was 2–4 ml. of a solution containing 1 mg. of enzyme per 100 ml. Unaccountable variations in enzyme activity from time to time were encountered, an observation which has also been reported by other investigators.[3b,13] It was found that such variations could be minimized by preparing proper dilutions of enzyme the previous day and storing these solutions in the refrigerator.

The results obtained with the Philpot method did not

(12) Meldrum and Roughton, *J. Physiol.*, **80**, 113 (1933).
(13) Roughton and Booth, *Biochem. J.*, **40**, 309 (1946).
(14) Keilin and Mann, *ibid.*, **34**, 1163 (1940).
(15) Kolthoff and Laitinen, "pH and Electro Titrations," 2nd ed., John Wiley and Sons, Inc., New York, N. Y., 1941, p. 28.
(16) Wilbur and Anderson, *J. Biol. Chem.*, **176**, 147 (1948).

TABLE III

CARBONIC ANHYDRASE INHIBITORY CONCENTRATION OF VARIOUS SULFONAMIDES IN TWO DIFFERENT BUFFER SYSTEMS

Sulfonamide	Sodium bicarbonate 50% (10^{-7} M)	Sodium bicarbonate ~100% (10^{-6} M)	Veronal 50% (10^{-7} M)	Veronal ~100% (10^{-6} M)
Sulfanilamide	100–170	34	4.6–7.0	3.4–4.7
1-Methylimidazole-2-	7.3	11	12	
Benzimidazole-2-	12		0.76	
1,2,4-Triazole-3-	60		22	
1-Methyltetrazole-5-	9–11		2.5–3.7	
Thiophene-2-	8–13	5.4	0.6–1.2	4.9–6.1
Thiazole-2-	1.4	0.14	0.15	
2-Acetylaminothiazole-5-	11	5.3	3.6	
Benzothiazole-2-	0.16–0.21	0.013	0.062–0.075	0.014–0.019
1,3,4-Thiadiazole-2,5-di-	0.15–0.19	0.039	0.082	0.025–0.033
2-Acetylamino-1,3,4-thiadiazole-5-	0.52	0.078	0.09–0.11	0.071–0.089
2-Amino-1,3,4-thiadiazole-5-	3.9		0.33	

vary by more than 10–20% in any series of experiments. Because of the unaccountable variations in enzyme activity from time to time, however, values different by ±50% were encountered with the same inhibitor. For this reason, sulfanilamide was used as a control and all results reported as relative values. Differences less than twofold in the activity ratios were considered to be of questionable significance. Data obtained with semi-purified carbonic anhydrase showed about the same variations as those with the crude enzyme. Fifty per cent. end-points were determined after the approximate range had been established by the use of a twofold change in enzyme concentration. The 50% inhibitory end-point was then recorded as that concentration of sulfonamide which reduced the activity of enzyme to the same interval as was observed with one-half as much enzyme in the absence of inhibitor.

Precise values for 100% inhibition were more difficult to determine because the control end-points were less clear-cut. Only some of the compounds more active at 50% inhibition were studied. In these cases the results are reported in terms of the relative molar concentration of sulfonamide required to reduce the activity of the same amount of carbonic anhydrase used for determining 50% inhibition, to the approximate time (within 5–10%) required for the same pH change in the absence of enzyme. Although these observations appeared to be reasonably reproducible, the wider variations in control end-points make them less reliable than the results at 50% inhibition. No meaningful limits of error can be applied to the ~100% values.

The buffer for use in the veronal system (pH 8) was prepared by dissolving 8.096 g. (0.044 mole) of 5,5-diethylbarbituric acid and 0.88 g. (0.022 mole) of sodium hydroxide in distilled water to make one liter of solution (note errors in name of acid and molarity of sodium hydroxide in references 16 and 11, respectively). The total volume in the veronal system was 5 ml., including 2 ml. of buffer containing 10 mg./200 ml. of brom thymol blue, 1 ml. of solution containing 15–30 μg. of crude "chloroform enzyme" and appropriate amounts of inhibitor plus 2 ml. of water saturated with carbon dioxide at 0°. To ensure a constant concentration of carbon dioxide the rate at which the gas bubbled into water in an ice-packed Dewar flask was controlled with a flowmeter. It was also customary to leave the Dewar well packed with ice overnight with carbon dioxide bubbling through the solution at a reduced rate.

During the determinations, all solutions were maintained at constant temperature (±0.05°). The 3 ml. of indicator buffer, enzyme and inhibitor were introduced into a 2 × 15 cm. test-tube immersed in an all-glass ice-bath agitated with an air stream, and allowed to stand for three minutes. At about two and three-quarter minutes a 2-ml. syringe with a 7.5 cm. No. 30 gage needle was removed from a Dewar ice-bath and filled with the 0° saturated carbon dioxide solution. This solution was added quickly to the test-tube at three minutes. The change in color was observed and the end-point taken when it matched a standard solution adjusted to pH 6.3 in a second test-tube in the ice-bath. (The standard solution should, of course, contain the same final concentration of brom thymol blue as the reaction mixture.)

Some of the actual values obtained with different inhibitors in both the veronal and sodium bicarbonate buffer systems are recorded in Table III. When a number of determinations were made on several occasions, the range of inhibitory concentrations is given. A single value represents the average of two or more determinations carried out on the same occasion and not differing by more than 20%. It will be noted that the values in veronal buffer are generally lower than in the bicarbonate system for 50% inhibition, although the ~100% end-points are generally much closer. In some instances, the relative order of activity of the sulfonamides also differs in the two systems. The explanation for these effects is uncertain, although the differences in the inhibitory action of the buffers on the enzyme,[11,16] and the different hydrogen ion concentrations at which the two systems operate (the enzyme and inhibitor equilibrate at about pH 5.5 in the bicarbonate

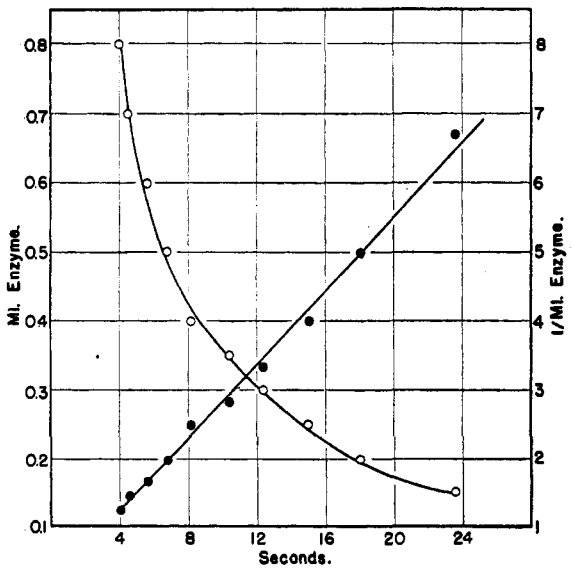

Fig. 1.—Time required for end-points with varying concentrations of carbonic anhydrase in veronal buffer system: —○—, enzyme concentration of solution containing 0.056 mg./ml. of crude "chloroform enzyme"; —●—, reciprocal of enzyme concentration.

buffer and at approximately *p*H 8 in the veronal system) in relation to the Michaelis constants of the enzyme, the equilibrium concentrations of the substrates and the dissociation of the inhibitors may all be factors.

It is of interest to compare the values for sulfanilamide and thiophene-2-sulfonamide with those given in the literature. Thus, Davenport,[7] using a manometric method, obtained values of 7×10^{-7} *M* and 1.6×10^{-7} *M*, respectively, for the 50% inhibitory end-point of these two sulfonamides, and Krebs[3b] reported 9×10^{-7} *M* and 3×10^{-7} *M*, respectively, by use of a similar method. These data agree reasonably well with the results obtained in the veronal buffer system. The values obtained by means of the Philpot method are generally considerably higher for 50% inhibition. From the standpoint of relative values in comparison with sulfanilamide, of course, these differences are less important.

TABLE IV

RELATIVE INHIBITORY EFFECT OF VARIOUS AROMATIC SULFONAMIDES ON CARBONIC ANHYDRASE

Compound	Inhibitory activity (HCO_3^-) 50%	~100%
Sulfanilamide	1	1
o-Toluenesulfonamide[a]	1.8	
Metanilamide	2.5	
Benzenesulfonamide[a]	4.0	
p-Toluenesulfonamide[a]	8.5	
p-Chlorobenzenesulfonamide	18	
p-Nitrobenzenesulfonamide	23	
p-Benzenedisulfonamide	40	170
3,4-Dichlorobenzenesulfonamide	56	160
Prontosil	50	<56 (?)
Neoprontosil	200	<85 (?)

[a] The corresponding values calculated from Krebs[3b] report are 1.4, 2 and 9, respectively.

Aromatic Sulfonamides.—In addition to the heterocyclic sulfonamides, a number of aromatic derivatives were studied. The results obtained with a few of the most active of these are given in Table IV, in terms of their relative activity compared with sulfanilamide on a molar basis in the bicarbonate system. The high activity of Prontosil (4'-sulfamyl-2,4-diaminoazobenzene hydrochloride) and Neoprontosil[17] (disodium 2-(4'-sulfamylphenylazo)-7-acetamido-1-hydroxynaphthalene-3,6-disulfonate) reported by Krebs[3b] was confirmed. While reproducible values for ~100% inhibition were not obtained because of the interfering color of these substances, their high activity did not appear to be maintained at this level. From the standpoint of *in vivo* studies, these compounds would also suffer from the disadvantage that they are rather readily converted to the much less active sulfanilamide.[18]

Summary

The inhibition of carbonic anhydrase by a series of heterocyclic and aromatic sulfonamides has been investigated. Some of the heterocyclic unsubstituted sulfonamides have been found to be about 100–2000 times as active as sulfanilamide. The relationship between acid dissociation constants and inhibitory activity has also been studied.

(17) We are greatly indebted to Dr. C. M. Suter, Sterling-Winthrop Research Institute, Rensselaer, New York, for a supply of these materials.

(18) Fuller, *Lancet*, **I**, 191 (1937); Litchfield, White and Marshall, *J. Pharmacol.*, **72**, 291 (1941).

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Rearrangement of Oxime N-Ethers

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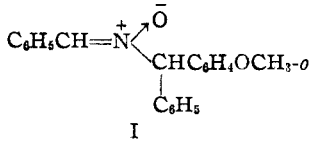
Allyl- and benzyldialkylamine oxides have been found to undergo thermal rearrangement with migration of the allyl or benzyl group from nitrogen to oxygen, forming trialkylhydroxylamines.[2] The N-ethers of oximes possess a semi-polar nitrogen–oxygen bond such as is present in amine oxides, and it therefore seemed possible that N-allyl and N-benzyl oxime ethers would undergo thermal rearrangement to the isomeric O-ethers. Such rearrangements have been observed to occur during acid hydrolysis of certain N-benzyl and N-benzhydryl oxime ethers. Martinoff[3] has reported that heating N-*o*-methoxybenzhydrylbenzaldoxime (I) with 12% hydrochloric acid resulted in formation of the isomeric O-ether together with its hydrolysis products, di-(*o*-methoxybenzhydryl) ether, benzaldehyde and hydroxylamine hydrochloride. Hydrolysis of N-*m*-methoxybenzhydrylbenzaldoxime and N-*p*-methoxybenzhydrylbenzaldoxime with dilute hydrochloric acid also was found to form the products expected from the isomeric O-ethers, which accordingly were probably formed as intermediates. Grammaticakis[4] observed that N-α-(*p*-methoxyphenyl)-propylbenzaldoxime (II) on treatment with concentrated hydrochloric acid formed benzaldehyde, hydroxylamine hydrochloride and anethole. He postulated rearrangement of II to the isomeric O-ether prior to hydrolysis, and formation of anethole by dehydration of α-ethyl-*p*-methoxybenzyl alcohol formed by hydrolysis of the O-ether. An alternate path which would explain the formation of anethole would be decomposition of the N-ether II in a reaction similar to the thermal decomposition of N,N-



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(1) Arthur D. Little Predoctoral Fellow, 1949–1950.

(2) (a) Meisenheimer, *Ber.*, **52**, 1667 (1919); (b) Kleinschmidt and Cope, THIS JOURNAL, **66**, 1929 (1944); (c) Cope and Towle, *ibid.*, **71**, 3423 (1949).

(3) Martinoff, *Ann. chim.*, [11] **7**, 424 (1937).

(4) Grammaticakis, *Compt. rend.*, **205**, 60 (1937).