Inhibition of Carbonic Anhydrase in *Neisseria*: Effects on Enzyme Activity and Growth

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SUMMARY

The presence of carbonic anhydrase in strains of *Neisseria* has been detected and quantified. Each of ten sulfonamide carbonic anhydrase inhibitors tested antagonized activity of bacterial carbonic anhydrase. Nine of these inhibited growth of carbonic anhydrase-producing strains of *Neisseria*. The more active drugs inhibited growth in concentrations as low as 0.005 μg/ml. Organisms lacking carbonic anhydrase were unaffected. Carbon dioxide markedly limited the antibacterial activity of these drugs. *p*-Aminobenzoate had no effect on their activity. It is concluded that bicarbonate is a growth requirement for strains of *Neisseria* that contain carbonic anhydrase, and that the mechanism of antibacterial activity of these drugs is reduction in the rate of bicarbonate formation.

INTRODUCTION

In 1963 Veitch and Blankenship (1) reported that certain strains of *Neisseria sicca* produced high concentrations of carbonic anhydrase. Small amounts of this enzyme were found in strains of other *Neisseria* species and in one strain of *Streptococcus salivarius*. Other bacterial strains did not appear to produce enzyme under similar conditions of growth. Forkman and Laurell (2) then showed that the specific carbonic anhydrase inhibitor, acetazolamide, inhibited the growth of many strains of *N. gonorrheae*, *N. meningitidis*, and *N. flava*. Minimum concentrations inhibiting growth ranged from 0.5 to 114 μg/ml, and this effect was not reversed by *p*-aminobenzoic acid (PABA). The purpose of this study was to determine the nature and spectrum of the antibacterial activity of acetazolamide and its congeners. In the course of the study numerous related problems arose: the role of the enzyme in the organism's growth; relative susceptibility of the bacterial and mammalian type enzyme to inhibition; and the relationship of antibacterial activity and ability of drug to diffuse into cells.

MATERIALS AND METHODS

Microorganisms. Nonpathogenic *Neisseria* strains were freshly isolated on 5% sheep's blood agar from the upper respiratory tract of human subjects. Strains of *N. meningitidis* were recently isolated on Thayer-Martin medium (Baltimore Biological Laboratories—BBL) from the nasopharynx or spinal fluid of human subjects. *N. gonorrheae* strains were recently isolated on Thayer-Martin medium from patients with illness resembling gonorrhea.[1] Bacteria other than *Neisseria* were isolated from clinical specimens submitted to the Microbiological Laboratories of the Shands Teaching Hospital, Gainesville, Florida. Strains of all bacteria were identified by conventional morphological, cultural, biochemical, and serological techniques (3). Stock cultures of *Neisseria* were main-

[1] Cultures of *N. meningitidis* and *N. gonorrheae* were kindly supplied by Dr. John Boring, Communicable Disease Center, Atlanta, Georgia.

tained on cysteine-trypticase agar with phenol red indicator (BBL) and were propagated on Mueller-Hinton agar (BBL) prior to testing. Strains of other bacteria were maintained and propagated on either trypticase-soy (BBL) or Mueller-Hinton agar. Cultures of *N. gonorrheae* were grown and maintained at 37° in a CO_2 candle jar. All other cultures were grown at 37° in air.

Pharmacologic agents. Drugs were selected on the basis of a wide variety of chemical and physical properties (4, 5) and known activity against carbonic anhydrase (5, 6). Acetazolamide, methazolamide, N^4-acetylsulfanilamide, CL-13,850, CL-13,580, and CL-17,262 were supplied by the American Cyanamid Company; benzolamide (formerly CL-11,366) by Wallace Laboratories; ethoxzolamide by Upjohn; chlorothiazide, hydrochlorothiazide, and dichlorphenamide by Merck, Sharp & Dohme; N^7-acetylchlorothiazide as described by Maren and Wiley (7). Structures and characteristics of the carbonic anhydrase inhibitors are presented in Table 3; those of the inactive homologs CL-13,580, CL-17,262 are discussed in Section 2 of Results. Stock solutions of the drugs were prepared as their sodium salts in 0.1 M potassium phosphate buffer by addition of 1–2 *M* equivalents of sodium hydroxide.

Enzyme activity. Carbonic anhydrase activity was assayed by the changing pH method, using the barbital buffer system (8, 9). The bacteria were grown on Mueller-Hinton agar plates for 18–24 hr and washed off with distilled water. There was no blood or other carbonic anhydrase-containing substance in the media. Approximately 10^9 organisms were suspended in each milliliter of distilled water, and assay of enzyme activity was made using this preparation. It was assumed that the enzyme had been lysed from the cells by this procedure. This was confirmed by finding equivalent enzyme activity in suspensions (10^9 cells per ml isotonic phosphate-buffered saline) exposed to ultrasound (Raytheon Sonicator, Model DF101, 10 kc for 6 and 20 min), and little or no enzyme activity in similar suspensions, not subjected to ultrasound. Enzyme activity is expressed in units, as described previously (8, 9). One unit of enzyme is that amount which will double the uncatalyzed rate of hydration of CO_2 in the system used. Dog erythrocytes, used as a standard of comparison in Table 3, contain 4700 units/ml.

Partition coefficients. Ether partition coefficients listed in Table 3 are data from Wistrand, Rawls, and Maren (10) or were determined using that method. Drugs were prepared in phosphate (0.01–0.1 *M*)-saline (0.16 M) buffer in concentrations ranging from 25 to 2000 μg/ml. The higher concentrations were employed for drugs anticipated to be poorly soluble in ether. In several instances, partitions were studied over a 40-fold range of original concentrations of drug in buffer, without materially affecting the results. With some drugs, additional NaOH was required to achieve solubility in buffer, but the final solution maintained a pH of 7.4–7.5. Ethyl ether was washed in the phosphate-saline buffer for 1 hr. Twenty milliliters of washed ether was then added to an equal volume of drug solution and shaken for 1 hr on a Burrell shaker. The two phases were then separated. Ether was evaporated at 40°, and the residue was dissolved in 0.1 N NaOH. Alternatively, several milliliters of the NaOH was added to the ether before evaporation. Aliquots of ether and aqueous phase were analyzed for drug in triplicate by either the Bratton-Marshall (11) or the carbonic anhydrase method (8, 9).

Determination of activity of drugs against bacterial and red cell carbonic anhydrase. The preparation described above was used as the source of the bacterial enzyme. Drug activity was measured according to the usual procedure of this laboratory previously described for red cells and tissues (8, 9). Enzyme and drug were added to a saturated solution of CO_2 at 0° and equilibrated for a time experimentally determined for each drug (15 sec to 10 min). Barbital buffer was added, and the time required to titrate the buffer by the conversion of CO_2 to H_2CO_3 was

then measured. This method is designated SEI (substrate-enzyme-inhibitor). The SEI_{50} was calculated as the concentration of drug that reduced the catalyzed rate 50%, and SEI_{90} was the concentration required for 90% reduction.

Determination of antibacterial activity. Assay of antibacterial activity was performed by the plate dilution method utilizing solid medium (Mueller-Hinton, BBL). Solid medium was chosen because many *Neisseria* are unable to grow in liquid culture (3). Each series of 15 plates, prepared in duplicate, contained 2-fold dilutions of the agents to be tested. Range of concentration of carbonic anhydrase inhibitors was 100.0–0.005 μg/ml. Higher concentrations of drug were employed with some strains that appeared to be resistant to the 100 μg/ml dilution. Final pH of medium containing the various dilutions ranged from 7.2 to 7.4. Medium was poured into plastic petri dishes, 90 mm in diameter. Depth of medium was 6 mm. Suspensions of 1.0 to 6.0×10^8 organisms to be tested were prepared in 0.9% saline. A loopful (0.01 ml platinum loop) of this suspension was spread over 1–2 cm^2 of the surface of the medium. Plates, containing nonpathogenic *Neisseria* and meningococci were then incubated at 37° in air. Those streaked with gonococci were incubated in a candle-jar, except as noted below. Minimum inhibitory concentration (MIC) was determined when control plates revealed a clearly visible lawn of bacterial growth (20–24 hr incubation for most organisms, 24–48 hr for *N. gonorrheae*). The lowest concentration of drug that completely inhibited growth of the test organism in duplicate plates was considered to be the minimum inhibitory concentration. Results of duplicate determinations agreed within one dilution of test drug. Results of replicate determinations performed on separate days agreed within at least two dilutions.

Susceptibility of nonpathogenic *Neisseria* to the antibacterial activity of sulfadiazine was determined by the disk-sensitivity method (BBL) or by plate dilution method; susceptibility of strains of *N. meningitidis* was determined by the plate dilution method; in which sulfadiazine of sulfanilamide was added to the Mueller-Hinton medium to yield a final concentration of 0.1 or 1.0 mg/100 ml.

Determination of the effect of CO_2 concentration and PABA on antibacterial activity of carbonic anhydrase inhibitors. Minimum inhibitory concentrations of test drugs were determined when organisms were grown in air, or in 3, 5, or 10% CO_2. Minimum inhibitory concentrations were determined when 0 (0.1 ml phosphate buffer), 2.0, and 20.0 μg of PABA were added per milliliter of medium.

RESULTS

1. *Enzyme Activity and Inhibition by Drugs*

Of 13 *Neisseria* strains examined, 12 were shown to possess carbonic anhydrase (Table 1). Among these, there was a 30-fold variation in the concentration of enzyme. Whether this variation reflects differences in growth characteristics of individual strains from day to day, or dif-

TABLE 1

Carbonic anhydrase content of 13 strains of Neisseria

Carbonic anhydrase activity was determined by the changing pH method, using barbital buffer (8, 9).

Neisseria strain		Enzyme units/10^9 organisms
A	*N. perflava*	0.55
B	*N. perflava*	0.08
C	*N. perflava*	0.68
E	*N. perflava*	2.92
J	*N. catarrhalis*	0
N	*N. perflava*	0.70
O	*N. sicca*	0.10
P	*N. perflava*	1.00
R	*N. mucosa*	0.40
T	*N. perflava*	0.85
Z	*N. sicca*	0.50
2	*N. perflava*	0.23
14	*N. perflava*	0.39

ferences in stable characteristics among the strains is not clear. However, with repeated preparations the concentration of enzyme detected in strains J, E, and T remained the same. Table 2 shows that the carbonic anhydrase in all strains tested was inhibited by ethoxzolamide and acetazolamide. Variation in susceptibility of enzyme from these strains to ethoxzolamide was 35-fold. The response to acetazolamide was more uniform. Inhibitory activity of acetazolamide under these conditions the strongest E $\cong$ I. Within about a 2-fold margin of error, the same applied to the assay against the bacterial enzyme.

2. *Failure of R-SO_2 NH-substituted Homologs to Inhibit Bacterial Enzyme*

Three drugs, each a close homolog of one of the inhibitors in Table 3, but devoid of activity against red cell enzyme (6), were also inactive against the bacterial enzyme (not shown). These drugs (CL-17,262; CL-13,850, and N^7-acetylchloro-

TABLE 2

Ethoxzolamide and acetazolamide: I_{50} for carbonic anhydrase and MIC for growth of 8 strains of Neisseria

Neisseria strain	Ethoxzolamide[a]		Acetazolamide[a]	
	I_{50} ($\times 10^7$ M)	MIC ($\times 10^7$ M)	I_{50} ($\times 10^7$ M)	MIC ($\times 10^7$ M)
A *N. perflava*	5.0	3.9	3.2	35.1
C *N. perflava*	1.1	3.9	3.7	35.1
E *N. perflava*	1.6	3.9	2.9	9.0
N *N. perflava*	0.3	3.9	3.2	9.0
P *N. perflava*	2.8	1.9	1.3	9.0
R *N. mucosa*	5.6	3.9	1.9	9.0
T *N. perflava*	2.8	0.8	6.4	35.1
Z *N. sicca*	11.0	15.5	2.1	9.0

[a] I_{50} is the concentration of drug which inhibits 50% of the bacterial enzyme. MIC is the lowest concentration of drug which completely inhibits bacterial growth.

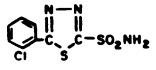
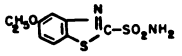
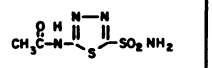
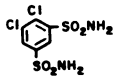
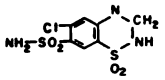
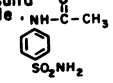
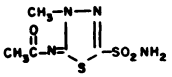
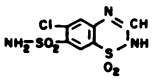
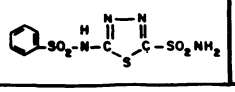
(SEI) was about one-twentieth of that for dog or human red cells (Table 3). Table 3 shows the activity of 10 carbonic anhydrase inhibitors against the strain of *Neisseria* (E) that produced most enzyme. Included for comparison is their activity in this test against enzyme from dog red blood cells. Using either the I_{50} or I_{90} as a criterion, there was fairly close correlation in the order of activity of the drugs against enzymes from the two sources. All drugs were less active against the bacterial enzyme. The ratio I_{90}:I_{50} for the dog red cells approximates 10 for the weaker inhibitors and 2 for the strongest, which conforms with the theoretical relationship for the simple reaction (E) + (I) $\rightleftharpoons$ (EI), in which for weaker drugs E $\ll$ I and for thiazide) are homologs of methazolamide, acetazolamide, and chlorothiazide, respectively. The methazolamide and acetazolamide homologs each contain a tertiary-butyl substituent on one of the protons of the sulfonamide group. The lack of effect of these drugs on bacterial growth is shown in Table 4.

3. *Comparison of Antibacterial Activities of Carbonic Anhydrase Inhibitors in Vitro*

Minimum inhibitory concentrations of 10 known sulfonamide carbonic anhydrase inhibitors, 3 inactive homologs, and sulfadiazine for each of 4 strains of *N. perflava* are shown in Table 4. The magnitude and relative order of antibacterial activity of

TABLE 3

Activity of carbonic anhydrase inhibitors against enzyme and growth of Neisseria perflava, strain E[a]

DRUG	MIC Strain E ($\times 10^{-7}$ M)	I_{50} / I_{90}		MIC/I_{90} Neisseria E	$_{p}ka$	Ether/Buffer Partition Coefficient
		Neisseria E ($\times 10^{-7}$ M)	Dog RBC ($\times 10^{-7}$ M)			
CL-13,580 	0.36	0.5 2.6	0.02 0.13	.16	6.6	79
Ethoxzolamide 	4	1.6 5.5	0.02 0.05	.7	8.1	140
Acetazolamide 	9	2.9 12.5	0.10 0.50	.7	7.4	0.14
Dichlorophenamide 	50	14 94	0.4 1.5	.5	8.3	9
Hydrochlorothiazide 	530	833 4000	225 671	.12	8.8	0.4
Sulfanilamide 	907	166 6200	48 914	.15	10.4	0.15
N^4-Acetylsulfanilamide 	37	200 1335	13 166	.03	9.7	0.1
Methazolamide 	130	2 13	0.12 0.92	10	7.2	0.62
Chlorothiazide 	33000	133 1100	14 96	30	6.7	0.013
Benzolamide 	>31000	2.8 7.4	0.01 0.03	>4500	3.2	0.001

[a] Strain E is similarly designated in Tables 1, 2, 3, 4, and 8. MIC is the lowest concentration of drug which completely inhibits bacterial growth. I_{50} and I_{90} are those concentrations of drug that inhibit 50 and 90% of bacterial enzyme, respectively.

these drugs was roughly comparable for each of the strains. Since benzolamide was the only known carbonic anhydrase inhibitor that failed to prevent growth of these strains, it was tested against 8 additional carbonic anhydrase-producing, acetazolamide-sensitive strains (not shown). In concentrations up to 1000 μg/ml, no antibacterial activity could be detected.

4. *Relationship between Enzyme Inhibition and Suppression of Growth*

Using data obtained for *Neisseria* E in Sections 1 and 3 above, the relationship between enzyme inhibition and suppression of growth was assessed for each of the 10 carbonic anhydrase inhibitors listed in Tables 3 and 4. This relationship is expressed as a ratio, MIC:SEI_{90}, for each drug (MIC/I_{50} in Table 3). Among the first 6 drugs, potency of activity against both enzyme and growth varied over a 3000-fold range. However, the relationship between these two functions (MIC:SEI_{90}) varied only 6-fold. The 4 remaining drugs did not fit this pattern. The significance of these observations is presented in point 7 of the Discussion.

5. *Spectrum of Drug Activity against Growth of Various Strains of Neisseria*

Minimum inhibitory concentrations of ethoxzolamide, acetazolamide, and methazolamide for a variety of strains of nonpathogenic *Neisseria* and *N. meningitidis* grown in air are shown in Table 5. Growth of 14 nonpathogenic and 5 meningococcal strains was assayed simultaneously with all 3 drugs. Each organism was most susceptible to ethoxzolamide and least susceptible to methazolamide. Of the 4 strains resistant to 100 μg/ml or more of methazolamide (Table 5), one (strain 0, *N. sicca*) was inhibited by 25 μg/ml of acetazolamide and the 3 others (1 *N. catarrhalis;* and 2 *N. perflava*) were equally resistant to acetazolamide. Strain 0 was shown to produce relatively little carbonic anhydrase (Table 1), and the others produced no carbonic anhydrase (see Section 8 of Results). These strains were not tested for susceptibility to ethoxzol-

TABLE 4

Minimum inhibitory concentrations of 10 carbonic anhydrase inhibitors, 3 inactive congeners, and sulfadiazine for 4 prototype strains of Neisseria perflava[a]

Strain of *N. perflava*	Minimum inhibitory concentration (μg/ml)			
	S	T	E	W
CL-13,580	0.1	0.05	0.01	0.05
Ethoxzolamide	0.05	0.02	0.1	0.05
Acetazolamide	1.56	0.78	0.2	0.4
Dichlorphenamide	3.12	1.56	1.56	3.12
Hydrochlorothiazide	125.	31.2	15.6	31.2
Sulfanilamide	31.2	15.6	15.6	15.6
N^4-Acetylsulfanilamide	3.12	1.56	0.78	3.12
Methazolamide	12.5	3.12	3.12	3.12
Chlorothiazide	>1000.	1000.	1000.	1000.
Benzolamide	>1000.	>1000.	>1000.	>1000.
CL-17,262	>1000.	>1000.	>1000.	>1000.
CL-13,850	>1000.	>1000.	>1000.	>1000.
N^7-Acetylchorothiazide	>1000.	>1000.	>1000.	>1000.
Sulfadiazine	3.9	125.	15.6	3.9

[a] Minimum inhibitory concentration is the lowest amount of drug which completely inhibits bacterial growth. Strains T and E are similarly designated in Tables 1 and 2.

TABLE 5

Minimum inhibitory concentrations of ethoxzolamide, acetazolamide, and methazolamide for nonpathogenic Neisseria and N. meningitidis strains grown in air

Concentration of drug (μg/ml):	>100	100	50	25	12.5	6.25	3.12	1.56	0.8	0.4	0.2	0.1	0.05	0.02	0.01	0.005	<0.005[a]
	Number of strains																
Ethoxzolamide																	
Nonpathogenic *Neisseria* (14)[b]	0	0	0	0	0	0	0	0	0	0	0	4	6	4	0	0	0
N. meningitidis (22)	0	0	0	0	0	0	0	0	0	0	1	1	2	5	5	0	8
Acetazolamide																	
Nonpathogenic *Neisseria* (39)	3	0	0	1	0	1	2	3	15	9	5	0	0	0	0	0	0
N. meningitidis (10)	0	0	0	0	0	0	0	3	0	2	2	2	1	0	0	0	0
Methazolamide																	
Nonpathogenic *Neisseria* (39)	4	0	1	1	3	7	13	7	1	1	1	0	0	0	0	0	0
N. meningitidis (5)	0	0	0	0	1	1	2	1	0	0	0	0	0	0	0	0	0

[a] Organisms were sensitive to 0.005 μg/ml. Lower concentrations were not tested.

[b] Number of strains tested given in parentheses.

TABLE 6
Minimum inhibitory concentrations of ethoxzolamide, acetazolamide, and methazolamide determined for Neisseria gonorrheae grown in CO_2 and in air

Concentration of drug (μg/ml):	>1000	1000	500	250	125	62.5	31.2	15.6	7.8	3.9	3.9–0.05	<0.05[a]
Growth in CO_2 candle-jar												
Ethoxzolamide (10 strains)	0	0	0	2	0	1	0	3	2	2	0	0
Acetazolamide (7 strains)	0	0	0	0	1	3	3	0	0	0	0	0
Methazolamide (11 strains)	1	1	4	0	0	2	0	0	1	2	0	0
Growth in air												
Ethoxzolamide (3 strains)	0	0	0	0	0	0	0	0	0	0	0	3

[a] Organisms were sensitive to 0.05 μg/ml. Lower concentrations were not tested.

amide. Growth of all meningococcal strains tested was inhibited by 12.5 μg/ml or less of one or more of these drugs. Their degree of susceptibility appeared to be unrelated to serotype of strain tested.

Minimum inhibitory concentrations of these drugs for strains of *N. gonorrheae* grown in a candle-jar (CO_2 concentration 3–5%) are shown in Table 6. Only 3 of the 10 strains tested in CO_2 with ethoxzolamide were capable of growth in air. When tested in air (2–4 weeks of incubation at 37° in high humidity), the minimum concentration of ethoxzolamide necessary to inhibit growth fell strikingly (Table 6).

6. *Effects of Carbonic Anhydrase Inhibitors on Growth of Bacteria Varying in Susceptibility to Sulfadiazine*

Among strains of *Neisseria*, no correlation was found between susceptibility to ethoxzolamide and susceptibility to sulfadiazine (Table 7). Most significantly, strains highly resistant to sulfadiazine were as susceptible to ethoxzolamide as were the more sensitive strains. Other bacteria, presumed not to contain carbonic anhydrase (1), were not inhibited by 100 μg/ml or more of ethoxzolamide, regardless of their degree of sensitivity to sulfadia-

TABLE 7
Comparison of minimum inhibitory concentrations of ethoxzolamide and sulfadiazine for Neisseria and other bacteria

Parameter	Minimum inhibitory concentration of sulfadiazine: Less than 1 μg/ml	1–10 μg/ml	Greater than 10 μg/ml
Nonpathogenic *Neisseria*			
Number of strains	(11)	(4)	(5)
Median MIC ethoxzolamide (μg/ml)	0.1	0.1	0.1
Range MIC	0.02–25.0	0.05–0.2	0.05–0.2
N. meningitidis			
Number of strains	(16)	(4)	(10)
Median MIC ethoxzolamide (μg/ml)	0.01	0.05	0.01
Range MIC	0.005–0.5	0.005–0.05	0.005–0.2
Other bacteria[a]			
Number of strains	(4)	(4)	(4)
Median MIC ethoxzolamide (μg/ml)	>100	>100	>100
Range MIC	–	–	–

[a] Each group of 4 consisted of one diphtheroid, one coliform, one *S. albus*, and one *S. aureus*, presumed not to contain carbonic anhydrase (1).

zine. In identical experiments, in which acetazolamide or methazolamide were substituted for ethoxzolamide, similar results were obtained (not shown).

7. *Comparison of Effects of Supplementation of Media with PABA on Antibacterial Activities of Acetazolamide, Sulfanilamide, and Sulfadiazine*

Three strains of *Neisseria perflava*, equally sensitive to acetazolamide in the absence of supplemental PABA, were chosen for study. Addition of 2.0 and 20.0 μg/ml of PABA to the test medium did not significantly alter the minimum inhibitory concentrations of acetazolamide and sulfanilamide for the 3 strains (Table 8). However, supplemental PABA markedly reduced the antibacterial activity of sulfadiazine, assayed with the same organisms (Table 8).

TABLE 8

Effect of PABA and CO_2 on antibacterial activity of acetazolamide, sulfanilamide, and sulfadiazine

Parameter	Minimum inhibitory concentration (μg/ml)		
	Strain S[a]	Strain E	Strain W
Acetazolamide			
No PABA, in air	0.39	0.39	0.39
No PABA, in 3% CO_2	>1000.	>1000.	>1000.
20.0 μg/ml PABA, in air	0.39	0.39	0.39
20.0 μg/ml PABA, in 3% CO_2	>1000.	>1000.	>1000.
Sulfanilamide			
No PABA, in air	31.2	62.5	62.5
No PABA, in 3% CO_2	>1000.	>1000.	>1000.
20.0 μg/ml PABA, in air	62.5	62.5	62.5
20.0 μg/ml PABA, in 3% CO_2	>1000.	>1000.	>1000.
Sulfadiazine			
No PABA, in air	3.9	15.6	3.9
No PABA, in 3% CO_2	3.9	15.6	7.8
20.0 μg/ml PABA, in air	>1000.	>1000.	>1000.
20.0 μg/ml PABA, in 3% CO_2	>1000.	>1000.	>1000.

[a] Organisms S, E, and W are strains of *N. perflava*. Strain E is similarly designated in Tables 1–4. Strains S and W are so designated in Table 4.

8. *Effect of CO_2 Concentration on the Antibacterial Activity of Carbonic Anhydrase Inhibitors*

Carbon dioxide concentrations of 3, 5, and 10% completely abolished the antibacterial activity of acetazolamide, methazolamide, and ethoxzolamide when tested with 12 strains of *Neisseria* (10 *N. perflava*, 1 *N. sicca*, 1 *N. meningitidis*) that were sensitive to these drugs in air (not shown). In an attempt to demonstrate residual antibacterial activity in the presence of added CO_2, concentrations of drug as high as 1000 μg/ml were employed for 3 of the strains, but no inhibition of growth could be observed (Table 8). This contrasts with the observations made above (Table 6), that 3–5% CO_2 reduces, but does not abolish, the activity of ethoxzolamide against strains of *N. gonorrheae*. As shown in Table 8, 3% CO_2 also markedly reduced the activity of sulfanilamide against growth of three strains of *Neisseria*.

9. *Effect of Acetazolamide and Sulfadiazine on Strains of Neisseria and Other Bacteria Lacking Carbonic Anhydrase*

Three strains of *Neisseria* and 2 strains of *Staphylococcus* were demonstrated in this laboratory not to produce carbonic anhydrase. None of these strains was susceptible to the antibacterial activity of acetazolamide in concentrations up to 1000 μg/ml. One *Neisseria* (Strain J) and

one *Staphylococcus* were sensitive to 2.5 μg/ml sulfadiazine, while the other organisms were resistant to 1000 μg/ml of this drug.

Fifty-two other bacteria (from the genera *Staphylococcus, Bethesda, Aerobacter, Streptococcus, Mima, Bacillus, Escherichia, Pseudomonas, Proteus, Salmonella, Shigella, Klebsiella,* and *Serratia*), presumed not to produce carbonic anhydrase (1), were tested for susceptibility to acetazolamide. In concentrations up to 1000 μg/ml, no inhibition of growth was observed (not shown).

DISCUSSION AND CONCLUSIONS

The data presented characterize both the nature and spectrum of the antibacterial activity of the sulfonamide carbonic anhydrase inhibitors. The finding of Veitch and Blankenship (1), that most *Neisseria* produce carbonic anhydrase, has been confirmed. Similarly, the observation of Forkman and Laurell (2), that acetazolamide is capable of inhibiting growth of certain *Neisseria* strains has been confirmed. It has been established that each of ten sulfonamide carbonic anhydrase antagonists inhibits the bacterial enzyme, and that nine of these are capable of inhibiting growth of enzyme-producing *Neisseria* strains. Of 13 *Neisseria* strains tested, 12 produced carbonic anhydrase. The growth of each of these 12 was inhibited by one or more of the carbonic anhydrase inhibitors studied, while several were resistant to the antibacterial activity of sulfadiazine. Growth of strain J (*N. catarrhalis*), which produced no enzyme (Table 1), was not inhibited by acetazolamide, but was by sulfadiazine (Results Section 9). These findings suggested that the presence of carbonic anhydrase was a necessary prerequisite for growth inhibition by the sulfonamide carbonic anhydrase inhibitors and that the mechanism of action of these drugs was distinct from that of sulfadiazine. Further evidence to support this conclusion is summarized as follows:

1. The antibacterial activity of sulfanilamide is known to be dependent upon integrity of the free amino group para to the sulfonamide radical. Replacement of a proton on the free amino group by any substituent abolishes the ability of this molecule to interfere with bacterial synthesis of folic acid (12, 13), while preserving its anti-carbonic anhydrase activity (14). Conversely, placement of a substituent on the sulfonamide moiety preserves activity against bacterial folic acid synthesis (12, 13) and abolishes activity against carbonic anhydrase (14). Clearly, a free aryl amino group is required for inhibition of folic acid synthesis. There is no free aryl amino radical on the sulfonamide carbonic anhydrase inhibitors employed in these studies, except for sulfanilamide itself. Thus, a difference in mechanism of antibacterial activity between the sulfonamide carbonic anhydrase inhibitors and sulfadiazine could have been predicted solely from a consideration of chemical structures.

2. The sulfonamide carbonic anhydrase antagonists inhibited growth of sulfadiazine resistant strains of Neisseria and vice versa. (Table 7 and Results Section 9).

3. PABA antagonized the antibacterial activity of sulfadiazine, but not that of the sulfonamide carbonic anhydrase inhibitors. It is noteworthy in this context that the characteristics of sulfanilamide inhibition of growth of *Neisseria* (e.g., lack of reversal by PABA, and antagonism of activity by 3% CO_2, Table 8) were identical to those of acetazolamide, not to those of sulfadiazine.

4. Homologs of acetazolamide, methazolamide, and chlorothiazide, lacking activity against mammalian or bacterial carbonic anhydrase, were devoid of antibacterial activity (Table 4).

5. Strains of bacteria other than *Neisseria,* demonstrated or presumed to lack carbonic anhydrase, were resistant to the antibacterial activity of the sulfonamide carbonic anhydrase inhibitors (Results Section 9).

6. Progressive increases in CO_2 concentration in the medium abolished activity of the carbonic anhydrase inhibitors

against growth of nonpathogenic *Neisseria* and meningococci,[2] and markedly reduced activity against gonococci (Results Section 8, Table 8). It appears that, at the concentrations of CO_2 employed, the uncatalyzed rate of hydration of CO_2 was sufficiently accelerated to overcome partially or completely the organism's dependence upon carbonic anhydrase as the rate determining factor for this reaction.

7. A quantitative relationship between enzyme inhibition and suppression of growth in air exists for six of the drugs shown in Table 3. These drugs are listed in order of activity, which is the same for the two functions. Although the potency of these drugs varies over a 3000-fold range for both activities, the ratio MIC:SEI_{90} varies only 6-fold. All 6 drugs have a moderate lipid-solubility, as shown by the ether/buffer partition coefficients, which range from 0.14 to 140. It is presumed that these drugs permeate the cell quite readily, so that enzyme inhibition in the growing organism bears a relationship to inhibition in the cell-free system. This relationship is not exact, presumably because of critical differences in the milieu, i.e., the enzyme assay is done at 0° with a high CO_2 concentration. The effect of such variations in the milieu is not yet known for bacterial enzyme, but is documented for mammalian red cell carbonic anhydrase (6). However, from the relationship between SEI_{90} and MIC for these six drugs, it would appear that growth is inhibited when some 80% of enzyme is inactivated.

Four of the drugs do not fit into this pattern. Two (chlorothiazide and benzolamide) are relatively lipid insoluble and ionized, benzolamide almost entirely so. It can only be assumed at present that these drugs are partially or completely excluded from the cell by virtue of these characteristics. This implication is particularly striking for benzolamide, which is as active against the enzyme as ethoxzolamide, but more than 10^5 times less active against growth. Moreover, the difference in ether solubility for these two drugs is also of the order of 10^5. Methazolamide is about 10-fold less active against growth than might be predicted from the enzyme assay; N^4-acetylsulfanilamide is approximately 10-fold more active. These differences cannot be readily explained, but the most reasonable possibility is that alluded to above, i.e., conditions of enzyme assay are necessarily too far removed from those of growth to allow one to predict, in all cases, the quantitative activity of a given drug. It is now known (6) that all these drugs do not respond similarly to alterations in the procedure for study of enzyme inhibition.

These seven points indicate that the antibacterial activity of the sulfonamide carbonic anhydrase inhibitors results from their ability to inhibit bacterial carbonic anhydrase, and thus impede hydration of CO_2, which is curiously vital for growth and multiplication of most of the *Neisseria* group of organisms. Whether bicarbonate is a necessary one-carbon fragment for synthetic pathways or plays a role in maintenance of a stable intracellular pH, or both, is yet undetermined.

[2] Since the preparation of this manuscript, Forkman and Laurell have reported [*Acta Pathol. Microbiol. Scand.* 67, 542 (1966)] that 10% CO_2 markedly diminishes the antibacterial activity of acetazolamide against carbonic anhydrase-producing strains of *Neisseria*. This observation conforms with that reported in the present study.

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