

N-Substituted Sulfonamide Carbonic Anhydrase Inhibitors with Topical Effects on Intraocular Pressure

Michael W. Duffel,*† I. Silwai Ing,† Tyrone M. Segarra,† John A. Dixon,† Charles F. Barfknecht,† and Ronald D. Schoenwald‡

Divisions of Medicinal Chemistry and Natural Products, and Pharmaceutics, College of Pharmacy, University of Iowa, Iowa City, Iowa 52242. Received August 29, 1985

N-Methylacetazolamide was shown to be active topically in reducing intraocular pressure (IOP) to a small but statistically significant level in the normotensive rabbit eye. In vivo experiments with *N*-methylacetazolamide suggest that ocular metabolism to acetazolamide was responsible for the observed topical activity. Examination of initial rate kinetics of carbonic anhydrase catalyzed *p*-nitrophenyl acetate hydrolysis showed that *N*-methylacetazolamide was a competitive inhibitor, in contrast to noncompetitive inhibition seen with acetazolamide and other primary sulfonamide inhibitors. *N*-Substituted and unsubstituted 4-chlorobenzene- and 4-nitrobenzenesulfonamides were also synthesized, and their biochemical characteristics and in vivo ability to lower IOP when applied topically were determined. The primary sulfonamides were reversible noncompetitive inhibitors of carbonic anhydrase, with no effect on IOP after topical administration. 4-Nitrobenzene- and 4-chlorobenzenesulfonamides containing both *N*-hydroxy and *N*-methyl substituents were model irreversible inhibitors of carbonic anhydrase and exhibited a trend toward topical activity in reducing IOP in normotensive rabbit eyes. Therefore, this paper describes the synthesis and characterization of two types of carbonic anhydrase inhibitors; the *N*-methyl-substituted sulfonamides are reversible competitive inhibitors of carbonic anhydrase, while the *N*-hydroxy-*N*-methyl-substituted sulfonamides are irreversible inhibitors.

Inhibitors of carbonic anhydrase are used in the treatment of glaucoma due to their ability to lower intraocular pressure (IOP) by reducing aqueous humor formation. Although IOP can be controlled by oral administration of carbonic anhydrase inhibitors, systemic side effects severely limit this mode of therapy.¹ These problems with oral dosage have led to a search for carbonic anhydrase inhibitors that would penetrate the cornea and be active in lowering IOP when topically administered to the eye.² In attempting to increase corneal penetration of carbonic anhydrase inhibitors, many structural modifications have been recently made to enhance physicochemical properties.²⁻⁵ However, these derivatives have only included the primary unsubstituted sulfonamides.

Previous investigators have stated that substitutions on the sulfonamide nitrogen destroy in vitro activity of carbonic anhydrase inhibitors.^{6,7} However, when *N*-alkylated sulfonamides were administered by intravenous or oral routes they were converted in vivo to active carbonic anhydrase inhibitors.⁷ The use of metabolism by ocular tissues to convert *N*-alkylated sulfonamides to more active carbonic anhydrase inhibitors, such as the corresponding unsubstituted sulfonamide or other active metabolites, has not been previously investigated.

We have taken the approach of preparing *N*-substituted derivatives of sulfonamides, to act either as carbonic anhydrase inhibitors or as pro-drugs with enhanced corneal penetration for metabolic conversion to carbonic anhydrase inhibitors within ocular tissues. To explore this path to topically active agents, we have prepared the *N*-methyl derivative of acetazolamide. This *N*-substituted sulfonamide was an inhibitor of carbonic anhydrase in vitro and was topically active in lowering IOP in normotensive rabbits maintained on a 0.5% NaCl diet. After initial observation of carbonic anhydrase inhibition due to *N*-methylacetazolamide, we synthesized model sulfonamides with both *N*-methyl and *N*-hydroxy substituents. The *N*-hydroxy-*N*-methyl sulfonamides were of interest since they could either be in vivo metabolites responsible for topical activity of the corresponding *N*-methyl sulfonamides or have independent use as carbonic anhydrase inhibitors. In this report we describe the physicochemical,

Table I. IOP in Normal Rabbits^a (*N* = 12) following Topical Dosing of 1% *N*-Methylacetazolamide

time after dosing, min	IOP change, mmHg	
	aqueous suspension ^b	gel suspension ^c
10	-1.08	
20	-0.92	-1.83
30	-1.30	
40	-0.55	-1.08
50	-1.60	
60	-1.20	-2.0
70	-1.40	
80	-1.40	-1.46
90	-1.90	
100	-1.80	0
110	-0.83	
120	-1.40	-0.75
140		-0.92
160		-1.25
180		-0.88
200		-1.83
220		0.83
240		0.75

^aProcedures for dosing, measurement of IOP change, and statistics are given in Experimental Section. ^bProbability changes in decline in IOP between 10 and 120 min were $p < 0.01$. ^cProbability changes in decline in IOP were < 0.025 except at 180 min ($p < 0.05$) and at 100 min (nonsignificant).

biochemical, and in vivo properties of *N*-methylacetazolamide and two series of model sulfonamides with hydroxyl and methyl substituents on the sulfonamide nitrogen.

Results

***N*-Methylacetazolamide.** *N*-Methylacetazolamide (1) was topically active in lowering intraocular pressure, as seen in Table I. The aqueous suspension of 1 caused a small but statistically significant lowering of IOP that was

- (1) Zimmerman, T. J. *Ann. Ophthalmol.* 1978, 10, 509.
- (2) Woltersdorf, D. W., Jr.; Michelson, S. R.; Sondey, J. M.; Schwam, H. U.S. Patent 4 386 098, 1983.
- (3) Maren, T. H.; Jankowska, L.; Sanval, G.; Edelhauser, H. F. *Exp. Eye Res.* 1983, 36, 457.
- (4) Stein, A.; Pinke, R.; Krupin, T.; Glabb, E.; Podos, S.; Serle, J.; Maren, T. H. *Am. J. Ophthalmol.* 1983, 95, 222.
- (5) Lewis, R. A.; Schoenwald, R. D.; Eller, M. G.; Barfknecht, C. F.; Phelps, C. D. *Arch. Ophthalmol.* 1984, 102, 1821.
- (6) Mann, T.; Keilin, D. *Nature (London)* 1940, 146, 164.
- (7) Maren, T. H. *J. Pharmacol. Exp. Ther.* 1956, 117, 385.

* Divisions of Medicinal Chemistry and Natural Products.

† Division of Pharmaceutics.

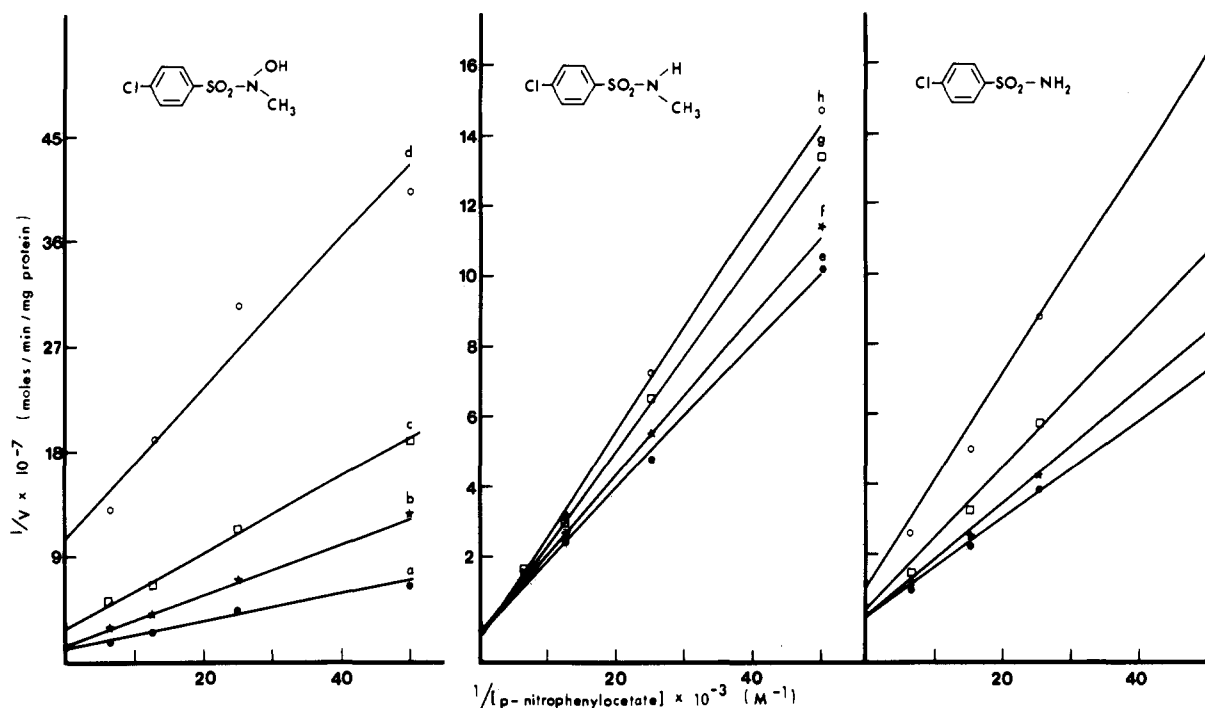


Figure 1. Initial rate data for inhibition of *p*-nitrophenyl acetate hydrolysis catalyzed by carbonic anhydrase: (left) concentrations of inhibitor 8 were a, no inhibitor; b, 0.1 mM; c, 0.2 mM; d, 0.4 mM; (center) concentrations of inhibitor 7 were e, no inhibitor; f, 0.1 mM; g, 0.2 mM; h, 0.3 mM; (right) concentrations of inhibitor 6 were i, no inhibitor; j, 0.1 μ M; k, 0.2 μ M; l, 0.4 μ M.

sustained for the full 2 h of measurement. This effect was in contrast to the parent drug acetazolamide (2), which has been reported to not lower IOP when applied topically.³ Although the normotensive rabbit model showed a statistically significant lowering of IOP for 1, aqueous and gel suspensions of acetazolamide (1% w/v) were tested in the model. The results are summarized in Table II and confirm a complete lack of topical activity of acetazolamide for the aqueous suspension. The gel was responsible for 2 producing an extremely small but statistically significant drop in IOP at 80, 180, and 200 min. This can be attributed to the prolonged retention properties of the gel vehicle.

Physical parameters for 1 and kinetic characteristics of its inhibition of carbonic anhydrase are listed in Table III. Compounds 1 and 2 differ dramatically in K_i values and show completely different types of inhibition.

In order to rule out a contamination of *N*-methylacetazolamide with acetazolamide, the purity of *N*-methylacetazolamide was determined with the HPLC conditions described in the Experimental Section. No acetazolamide was detected in the *N*-methylacetazolamide used for K_i determinations and in vivo dosing. The limits of detection for this HPLC procedure would have allowed qualitative determinations of 0.01 μ M acetazolamide in a solution of 200 μ M *N*-methylacetazolamide.

In Vivo Activity of Model Compounds. Model carbonic anhydrase inhibitors, either with or without substituents on the sulfonamide nitrogen, were synthesized in order to determine their ability to act topically to lower IOP. Compounds 5–8 were tested in vivo with normal rabbits for their ability to lower IOP after topical administration as a 1% (w/v) suspension. As seen in Table IV, compound 5 showed a trend toward lowering IOP from 40 through 80 min. However, conjunctivitis was observed with compound 5 and indirectly could cause a lowering of IOP. Since this toxic response is most likely unrelated to carbonic anhydrase inhibition, and because the effect is small, the lowering of IOP by 5 must remain suspect. Compound 8 also lowered IOP following topical adminis-

Table II. IOP in Normal Rabbits^a ($N = 12$) following Topical Dosing of 1% Acetazolamide

time after dosing, min	IOP change, mmHg	
	aqueous suspension ^b	gel suspension ^c
20	-0.42	0.42
40	-0.50	0.50
60	0.83	0
80	0.50	-1.08
100	-0.16	-0.58
120	0.67	0.08
140	-0.25	-0.50
160	0.92	-0.42
180	0.50	-0.75
200		-0.75
220		1.17
240		-0.17

^a Procedures for dosing, measurement of IOP change, and statistics are given in the Experimental Section. ^b No statistically significant IOP changes occurred. ^c Probability changes in decline in IOP were $p < 0.025$ at 80, 180, and 200 min; at all other times the IOP change was nonsignificant.

tration (Table IV), but the effect was only seen at 60 min and was not observed either at 80 min or thereafter. Both 4-chlorobenzenesulfonamide (6) and *N*-methyl-4-chlorobenzenesulfonamide (7) failed to lower IOP significantly at any time point during the 2 h of measurement (data not shown).

In Vitro Inhibition of Carbonic Anhydrase Activity. Compounds 3 and 4 were synthesized to complete the series of 4-nitrobenzenesulfonamides. Initial rate studies indicated that compounds 3 and 6 (Figure 1) were non-competitive inhibitors, exhibiting inhibition characteristics similar to acetazolamide (2). Methylation of the sulfonamide nitrogen, as in compounds 1, 4, and 7, increased the K_i and altered the type of interaction with the enzyme, since in all cases the inhibition became competitive (Figure 1 and Table III). *N*-Hydroxy-*N*-methyl sulfonamides exhibited decreased K_i values (Table III) and double-reciprocal plots that indicated either noncompetitive or irreversible inhibition; the data for 8 are shown in Figure 1.

Table III. Kinetic and Physical Properties of Carbonic Anhydrase Inhibitors

compd	structure	kinetic data		physical parameters			
		K_i , μM	type of inhibition	$\text{p}K_a$	log DC ^a	solubility, ^b mol/L	P_{app} , ^c cm/s
1		258	competitive	7.52	0.32	1.44×10^{-3}	2.26×10^{-6}
2		0.8	noncompetitive	7.40	-0.45	1.86×10^{-2}	1.28×10^{-6}
3		0.2	noncompetitive				
4		146	competitive				
5		44	irreversible				
6		0.3	noncompetitive	9.95	1.24	2.23×10^{-2}	5.47×10^{-5}
7		584	competitive	10.96	1.92	2.08×10^{-2}	6.51×10^{-5}
8		107	irreversible				

^a Octanol/pH 7.2 phosphate buffer distribution coefficient. ^b pH 7.2 phosphate buffer, 35 °C. ^c Corneal permeability coefficient determined at pH 7.65.

Table IV. IOP in Normal Rabbits following Topical Dosing of 1% *N*-Methylbenzenesulfonamide Analogues^a

time after dosing, min	IOP change, mmHg	
	<i>N</i> -hydroxy- <i>N</i> -methyl-4-nitrobenzenesulfonamide ^b	<i>N</i> -hydroxy- <i>N</i> -methyl-4-chlorobenzene-sulfonamide ^c
10	0.33	
20	-0.33	0.17
30	-0.67	
40	-1.67	1.00
50	-1.83	
60	-1.67	-1.25
70	-2.00	
80	-1.67	0.17
90	0.20	
100	-0.50	0.67
110	-0.33	
120	-1.00	0.33
140		-0.33
160		-0.25
180		-0.25

^a Procedures for dosing, measurement of IOP change, and statistics are given in the Experimental Section. ^b $N = 6$. Although probability changes in decline in IOP were significant only at 50 min ($p < 0.05$), a lowering trend was apparent between 40 and 80 min (where $p < 0.20$ at 40 and 80 min, and $p < 0.10$ at 60 and 70 min); at all other times the IOP changes were nonsignificant. ^c $N = 12$. Probability changes in IOP were $p < 0.01$ at 60 min; all other times were nonsignificant.

Preincubation of the inhibitor and carbonic anhydrase, with subsequent dilution of the inhibitor in a standard assay, was used to distinguish between reversible noncompetitive inhibition and irreversible inhibition. As seen in Figure 2, compound 8 irreversibly inactivated carbonic anhydrase, while compounds 6 and 7 did not affect enzyme activity after preincubation and dilution. Compounds 3–5 were also tested for reversibility of inhibition and yielded results similar to those obtained for 6–8; that is, the *N*-hydroxy-*N*-methyl sulfonamide displayed inhibition of carbonic anhydrase which was not reversible by dilution (Figure 3). The data in Figures 2 and 3 were used to calculate pseudo-first-order rate constants for carbonic anhydrase inactivation by compound 8 ($6.0 \times 10^{-5} \text{ s}^{-1}$) and compound 5 ($1.3 \times 10^{-4} \text{ s}^{-1}$). Inhibition by the primary and *N*-methyl sulfonamides was completely reversible by dilution.

Table V. Acetazolamide Concentrations in Ocular Tissues of Normal Rabbits after Administration of *N*-Methylacetazolamide (Topical) or Acetazolamide (iv)

	drug and route administered	
	<i>N</i> -methylacetazolamide ^a (topical)	acetazolamide ^b (iv)
iris/ciliary body	0.723 ± 0.230^c	0.749 ± 0.129^c
aqueous humor	0.043 ± 0.016^d	0.276 ± 0.071^d
ratio (iris/ciliary to aqueous humor)	16.8	2.7

^a *N*-Methylacetazolamide (196 $\mu\text{g}/\text{mL}$) was administered topically for the 170 min, and tissue concentrations of acetazolamide were determined as in the Experimental Section. ^b Acetazolamide was administered iv at a dose of 8 mg/kg, and tissue samples were taken after 60 min for determination of tissue concentration of acetazolamide. ^c Values are expressed in micrograms of acetazolamide per gram of iris/ciliary and are the mean \pm standard deviation of at least four determinations. ^d Values are expressed in micrograms of acetazolamide per milliliter of aqueous humor and are the mean \pm standard deviation of at least four determinations.

In Vivo *N*-Demethylation of *N*-Methylacetazolamide. One possible mechanism for topical activity of *N*-methylacetazolamide is metabolism to acetazolamide in ocular tissues. To determine if this metabolic conversion was occurring, the concentration of acetazolamide was determined in iris/ciliary body and aqueous humor following topical infusion of *N*-methylacetazolamide (196 $\mu\text{g}/\text{mL}$ for 170 min) to the right eyes of anesthetized rabbits. The concentration of acetazolamide in these tissues was compared to tissue levels of acetazolamide obtained in rabbits that had received acetazolamide iv in a dose (8 mg/kg) sufficient to decrease IOP by $18 \pm 4\%$. The results are seen in Table V. The levels of acetazolamide found in iris/ciliary body after topical infusion of *N*-methylacetazolamide were comparable to levels obtained by iv dosage with acetazolamide. However, concentrations of acetazolamide in aqueous humor were approximately 50-fold less in rabbits receiving topical *N*-methylacetazolamide than in rabbits receiving iv acetazolamide. Formation of acetazolamide was apparently due to ocular metabolism since the contralateral eye contained no detectable acetazolamide. When acetazolamide was administered iv, both eyes contained approximately equal acetazolamide concentrations.

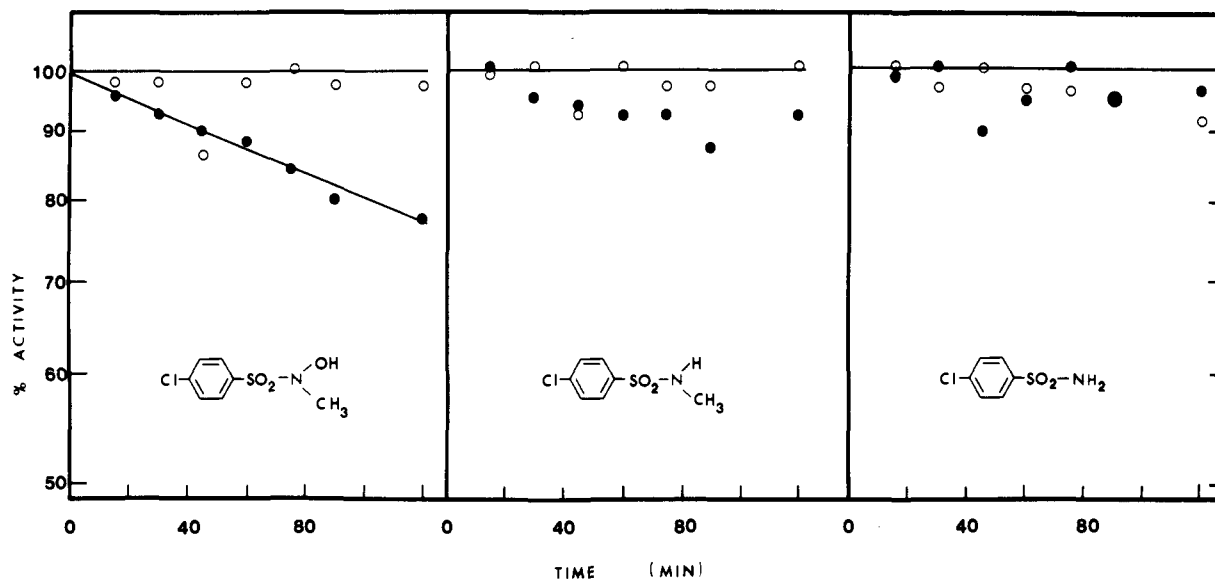


Figure 2. Effect of preincubation with inhibitors on carbonic anhydrase catalyzed hydrolysis of 4-nitrophenyl acetate. Assays were conducted as described in the Experimental Section. Open circles denote control preincubations where no inhibitor was present; closed circles indicate preincubation with inhibitors. Concentrations of inhibitors in the preincubations were as follows: (left) 0.2 mM for compound 8, (center) 0.2 mM for compound 7, and (right) 0.4 μ M for compound 6.

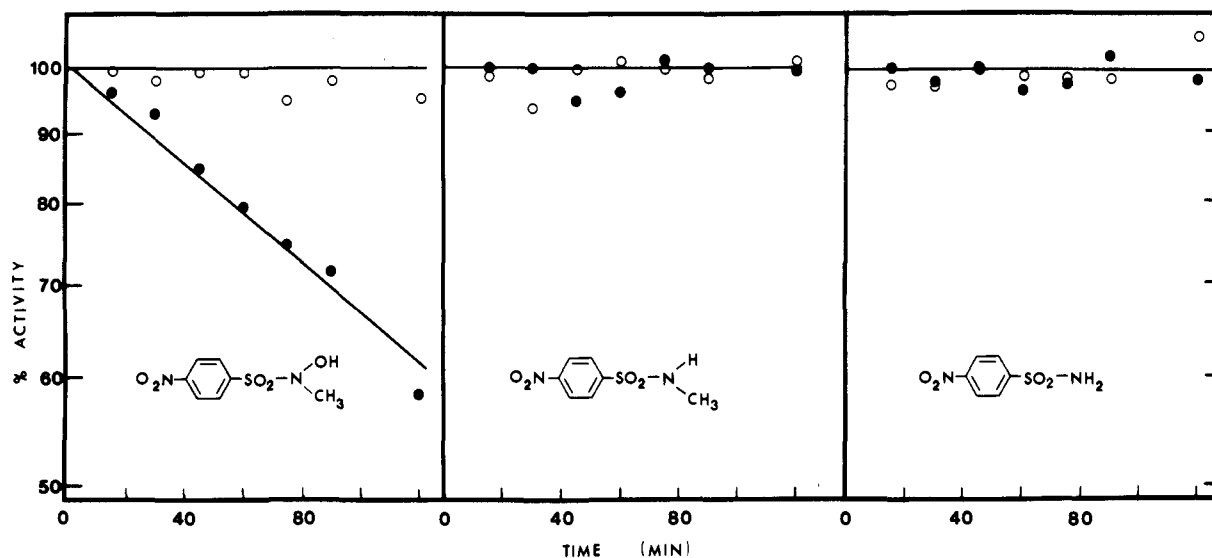


Figure 3. Effect of preincubation with inhibitors on carbonic anhydrase catalyzed hydrolysis of 4-nitrophenyl acetate. Assays were conducted as described in the Experimental Section. Open circles denote control preincubations where no inhibitor was present; closed circles indicate preincubation with inhibitors. Concentrations of inhibitors in the preincubations were as follows: (left) 0.1 mM for compound 5, (center) 0.4 mM for compound 4, and (right) 0.2 μ M for compound 3.

Discussion

The discovery that *N*-methylacetazolamide (1) was active in lowering IOP when administered topically led to a more complete examination of the structural features involved in the activity. There are several possible explanations for the fact that 1 was active topically in aqueous suspension while acetazolamide (2) was not. This may have been due to enhanced transport into or within the eye or to enhanced transport with subsequent conversion to an active metabolite within the eye. Metabolic changes that could occur include *N*-demethylation or *N*-hydroxylation mediated by cytochrome P-450 monooxygenases, which are known to be present in eye tissue.⁸ Model compounds 3–8 were synthesized to determine the molecular properties that dictate carbonic anhydrase in-

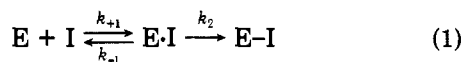
hibition by *N*-substituted sulfonamides. In the process we have defined a new class of reversibly competitive inhibitors and a new type of irreversible inhibitor of carbonic anhydrase.

Results with model compounds indicated that methylation of the sulfonamide nitrogen decreased the binding affinity of the inhibitor by at least 1000-fold and changed the type of inhibition from reversible noncompetitive to reversible competitive. However, additional substitution of a hydroxyl on the nitrogen of an *N*-methyl sulfonamide produced an irreversible inhibitor that was 3–6 times higher in binding affinity than the corresponding *N*-methyl sulfonamide. These results suggested that we were observing fundamental changes in the nature of binding of inhibitors to carbonic anhydrase as we altered the substituents on the sulfonamide nitrogen and that changes in the K_i were not simply due to sterically hindering sulfonamide binding.

One of the most interesting cases where a change in type of inhibition occurred was with the *N*-methyl-*N*-hydroxy

(8) Shichi, H.; Nebert, D. W. In *Extrahepatic Metabolism of Drugs and Other Foreign Compounds*; Gram, T. E., Ed.; Spectrum: Jamaica, New York, 1980.

sulfonamides. These inhibitors caused irreversible inhibition of the carbonic anhydrase. Irreversible inactivation by preincubation with compounds 5 and 8 was not detectable during the first 3 min, yet inhibition was readily observed in initial rate studies. Therefore, it is possible that there is an initial association between enzyme and inhibitor followed by covalent modification of the enzyme as seen in eq 1. Thus, there would be an initial equilib-



rium described by $K_1 = (k_{-1}/k_{+1})$, followed by a slower irreversible step described by a first-order rate constant, k_2 . The exact mechanism for this final irreversible step is uncertain at this time.

Studies on the in vivo metabolism of *N*-methylacetazolamide indicated that it is converted to acetazolamide in ocular tissue. Furthermore, the concentrations of acetazolamide present in iris/ciliary body after topical infusion with *N*-methylacetazolamide are similar to those obtained after iv dosage with acetazolamide. However, the ratio of drug in iris/ciliary body to aqueous humor, in which distribution equilibrium is assumed, is much greater for topical infusion than iv administration. Conversion of *N*-methylacetazolamide to acetazolamide and subsequent retention within the ciliary process may be sufficient to account for carbonic anhydrase inhibition leading to lower IOP. Also, since there is a relatively small difference in corneal permeability between 1 and 2, factors such as distribution, rate of metabolism, and relative retention of *N*-methylacetazolamide and acetazolamide within ocular tissues (i.e., ocular disposition) may be more important determinants of topical activity than corneal penetration. Further investigation of these aspects of topically active *N*-methyl sulfonamides is in progress.

It is evident from the in vitro data, and from previous work with ethoxolamide,^{9,10} that the model compounds 3-8 have not yet been optimized for the best physical chemical parameters for use as topical agents to lower IOP. Furthermore, although *N*-demethylation of *N*-methylacetazolamide is implicated in its topical activity in lowering IOP, the involvement of an *N*-hydroxylated metabolite has not yet been ruled out.

Further studies will also be necessary to determine the role of isoenzymes of carbonic anhydrase in rabbit ocular tissue. In contrast to human ciliary body,¹¹ rabbit ciliary body contains both isoenzyme forms of carbonic anhydrase.¹² Therefore, a complete understanding of the mechanism of IOP lowering by topical administration of *N*-methyl sulfonamides in the rabbit model will require additional biochemical studies on the C-type carbonic anhydrase, as well as the previously mentioned studies on rate of *N*-demethylation, distribution, and retention of the drugs in ocular tissues.

Experimental Section

Materials. Human erythrocyte carbonic anhydrase B, *p*-nitrophenyl acetate, and acetazolamide were obtained from Sigma Chemical Company. New Zealand white rabbits (Morrison Rabbitry, West Branch, IA) weighing 1.7-2.2 kg were housed in the University of Iowa Animal Care Unit for a week or less prior to use. Rabbits were maintained on a standard laboratory diet

with the exception that the drinking water contained an additional 0.3% (w/v) sodium chloride, to provide no less than 0.5% total dietary NaCl, since it has been shown that a diet deficient in sodium ion produces a variable IOP response to sulfonamide drugs.¹³

Mass spectra were obtained on a Finnigan 3200 GC/MS; elemental analyses were performed by Galbraith Laboratories, TN. Melting points were determined in capillary tubes in a Mel-Temp apparatus (Laboratory Devices, Cambridge, MA) and are uncorrected.

Intraocular Pressure. Rabbits were maintained on a diet containing 0.5% sodium chloride for 3 weeks prior to testing. During this period numerous IOP determinations were made to familiarize the rabbits with the procedure. IOP was determined with a Digilab applanation pneumatonograph. Base-line IOP measurements were made prior to drug administration. Drugs (1% w/v) were administered topically; one eye, chosen randomly, received 50 μ L of a 1% (w/v) suspension of solution every 2 min for a total of three doses, and the contralateral eye received a solution vehicle. Acetazolamide and *N*-methylacetazolamide (both 1% w/v) were formulated in a gel vehicle, which is known to promote drug absorption into the eye.^{14,15} With gel a single 50- μ L dose was applied randomly to one eye, whereas the contralateral (control) eye received the gel vehicle. In all experiments the observer was masked. Changes in IOP are expressed as follows:

$$\Delta\Delta = \text{IOP (dosed eye)}_t - \text{IOP (dosed eye prior to dosing)}_{t=0} - \text{control eye} \quad (2)$$

IOP change is reported as the mean of values from 6 or 12 rabbits. Significance of the change in IOP from $t = 0$ is based on a two-sided t test.

Determination of Physical Properties. Solubility. Enough drug was added to 5 mL of a pH 7.2 phosphate buffer to form a suspension. The suspension, sealed in a 5-mL glass ampule, was placed in a shaking water bath precalibrated to 37 $^{\circ}$ C and shaken overnight. At the end of the time period a sample was removed, filtered, and assayed by HPLC.

pK_a. Determination of pK_a values for *N*-methylacetazolamide and the model compounds was conducted by use of pH solubility and potentiometric methods.¹⁶

Distribution Coefficient. Distribution coefficients were determined by adding the compound of interest to either octanol (presaturated with buffer) or pH 7.2 phosphate buffer (presaturated with octanol). Aqueous solutions of each compound were partitioned at about 1/2 to 3/4 their solubility. The phases were mixed by inverting the tubes, which were then placed in a centrifuge for 1 h to separate the phases. The aqueous phase was carefully withdrawn and assayed for drug. The difference in drug content in the aqueous phase before and after distribution with the organic phase represented the amount present in the organic phase. The distribution experiments were repeated at different drug concentrations to be certain that dimerization or complexation was not occurring. The results are expressed as log DC (distribution coefficient) or log [(concentration in octanol)/(concentration in aqueous)].

Corneal Permeability. The excised cornea procedure used in this study has been described elsewhere.^{17,18} When the quantity of drug crossing the cornea (q) is plotted vs. time (t), the linear slope of each plot represents the steady-state permeability rate ($\Delta q/\Delta t$). The least-squares slope of each individual experiment is determined and averaged ($N = 4$ is usually sufficient). Each averaged slope value is divided by an averaged corneal surface area (A), which is 1.089 cm² for a 1.5-2.0-kg rabbit. In order to compare the results independent of the initial concentrations, each

- (9) Schoenwald, R. D.; Eller, M. G.; Dixson, J. A.; Barfknecht, C. F. *J. Med. Chem.* 1984, 27, 810.
 (10) Eller, M. G.; Schoenwald, R. D.; Dixson, J. A.; Segarra, T.; Barfknecht, C. F. *J. Pharm. Sci.* 1985, 74, 155.
 (11) Wistrand, P. J.; Garg, L. C. *Invest. Ophthalmol. Visual Sci.* 1979, 18, 802.
 (12) Kishida, K.; Ochi, N. *Experientia* 1980, 36, 42.

- (13) Kinsey, V. E.; Camacho, E.; Cavanaugh, G. A.; Constant, M.; McGinty, D. A. *Arch. Ophthalmol.* 1955, 53, 680.
 (14) Schoenwald, R. D.; Boltralik, J. J. *Invest. Ophthalmol.* 1979, 18, 61.
 (15) March, W. F.; Stewart, R. M.; Mandell, A. I.; Bruce, L. A. *Arch. Ophthalmol.* 1982, 100, 1270.
 (16) Albert, A.; Serjeant, E. P. *The Determination of Ionization Constants*, 2nd ed.; Chapman and Hall: London, 1971.
 (17) Hull, D. S.; Hine, J. E.; Edelhofer, H. F.; Hyndiuk, R. A. *Invest. Ophthalmol.* 1974, 13, 457.
 (18) Schoenwald, R. D.; Huang, H. S. *J. Pharm. Sci.* 1983, 72, 1266.

averaged slope is also divided by the initial concentration (C_0). The final values represent an apparent permeability coefficient (P_{app}) with units of centimeters per second,¹⁹ as shown in eq 3.

$$P_{app} = q/\Delta t(3600)(C_0)(A) \quad (3)$$

HPLC Assay Procedure. High-pressure liquid chromatography (HPLC) was used to determine concentration and purity of each analogue. Because of the structural similarities in the compounds, it was possible to assay each analogue using a reverse-phase μ Bondapak C-18 column (Waters Associates) and a mobile phase consisting of 1% acetic acid in deaerated double-distilled water and varying concentrations of methanol. All compounds were detected by optical absorbance at 254 nm. Retention times were between 3 and 8 min, and linear calibration curves were observed for all compounds. This HPLC procedure was not applicable to compounds 5 and 8 due to their slow decomposition in the acidic mobile phase.

In Vitro Assays of Carbonic Anhydrase Inhibition. Carbonic anhydrase inhibitors were characterized by using initial rate kinetic analysis of the ester hydrolysis reaction catalyzed by carbonic anhydrase. This reaction parallels the CO_2 hydration activity of the enzyme²⁰ and has the added feature of being conducted at a single buffered pH. This is important since the activity of the enzyme varies greatly between pH values of 7–9.²⁰ Carbonic anhydrase activity was monitored continuously by measuring the enzyme-dependent increase in absorbance of the *p*-nitrophenylate ion at 400 nm. Reactions were conducted at 25 °C in temperature-controlled cells in a Pye-Unicam SP8-100 double-beam spectrophotometer. Assay mixtures (1.0-mL total volume) contained 0.02 M sodium phosphate at pH 7.5, 25–75 μg of carbonic anhydrase, the indicated amount of inhibitor in 0.1 mL of distilled water, and the appropriate amount of *p*-nitrophenyl acetate in 20 μL of acetonitrile. Reaction was initiated by addition of carbonic anhydrase. Linear rates were obtained for at least 3 min, and any nonenzymatic rate (always less than 25% of the enzymatic rate) was subtracted to yield the enzyme-dependent rate of *p*-nitrophenyl acetate hydrolysis. Plots of $1/\text{velocity}$ vs. $1/(\text{concentration of } p\text{-nitrophenyl acetate})$ at various levels of inhibitors yielded straight lines (by least-squares analysis), which gave an initial indication of the type of inhibition, i.e., competitive or noncompetitive. Replots of the slopes of the lines obtained from initial velocity studies yielded inhibition constants (K_i) for each inhibitor.²¹

Further experiments were used to distinguish between non-competitive and irreversible inhibition for the model compounds. Carbonic anhydrase (1 mg/mL) and a concentration of inhibitor causing approximately 50% inhibition were incubated at 25 °C in 0.02 M sodium phosphate, pH 7.5. Control mixtures without inhibitor were incubated in parallel. Aliquots (50 μL) were withdrawn at various time intervals and added to a standard assay containing 40 μM *p*-nitrophenyl acetate, 0.02 M sodium phosphate at pH 7.5, and water to a final assay volume of 1.0 mL. Control experiments indicated that when the inhibitors were diluted to the final concentration in the assay, they did not significantly alter the rate of carbonic anhydrase catalyzed ester hydrolysis. Furthermore, the inhibitors tested did not decompose on standing at 25 °C in 0.02 M sodium phosphate, pH 7.5. This was determined by thin-layer chromatography on silica gel using two developing solvents (A, toluene/ethanol, 9:1; and B, ethyl acetate/hexane, 4:1).

Topical Infusion of N-Methylacetazolamide. N-Methylacetazolamide was administered to rabbits by constant application of the drug using a topical infusion technique, as described previously.²² One eye received N-methylacetazolamide and the contralateral eye served as control. The concentration of 1 was 196 $\mu\text{g}/\text{mL}$ in isotonic potassium phosphate at pH 7.65, and each

corneal well contained a volume of 0.7 mL. After 170 min, the wells were removed, the cornea rinsed with 10–15 mL of normal saline, and the rabbits sacrificed by injection of a bolus of air into the marginal ear vein. Aqueous humor samples were removed with a 24-gauge needle inserted through the corneal-scleral junction. Iris/ciliary tissue was obtained by dissection. Aqueous humor samples and iris/ciliary tissue were immediately frozen in dry ice/acetone and stored at –70 °C until extraction and analysis.

Determination of Acetazolamide in Ocular Tissues. Extraction of acetazolamide from aqueous humor was accomplished by addition of 0.2 M potassium phosphate at pH 6.0 to the sample of aqueous humor, followed by three extractions with ethyl acetate, evaporation of the combined organic layers to dryness under nitrogen, and dissolution of the sample in 0.2 mL of mobile phase for HPLC analysis. HPLC analysis was carried out as described above, except that the mobile phase was 32% methanol in 0.01 M phosphate at pH 5.5.

Acetazolamide concentrations in iris/ciliary body were determined by homogenization of the tissue in 1.0 mL of 0.2 M potassium phosphate, pH 6.0, in a ground-glass tissue homogenizer. After the homogenate was treated at 100 °C for 3 min, the aqueous layer was extracted 3 times with ethyl acetate and analyzed by HPLC as described for aqueous humor samples. Recovery of acetazolamide carried through the extraction procedure with iris/ciliary tissue was 80%; extraction efficiency with aqueous humor was 86%. All values of acetazolamide concentration are uncorrected for extraction efficiency.

Synthesis of N-Methylacetazolamide (1). 5-Amino-1,3,4-thiadiazole 2-mercaptan (1.0 g, Aldrich Chemical Co.) was heated with acetic anhydride (0.5 mL) and acetic acid (1.0 mL) to form a yellow paste. Upon addition of water the product separated and was filtered and washed with two portions of water. The solid 5-acetamido-1,3,4-thiadiazole was dissolved in 10% sodium carbonate solution and reprecipitated with cold dilute acetic acid to give the product in 90% yield: mp 290–293 °C; mass spectrum, m/e 175 (M^+).

To 1.0 g of 5-acetamido-1,3,4-thiadiazole 2-mercaptan was added sodium hydroxide (0.25 g) in 10 mL of water to form a solution. This solution and 15 mL of sodium hypochlorite solution (5.25%, 3 equiv) were added simultaneously with constant stirring to 35 mL of 40% aqueous methylamine that had been cooled to –10 °C. The stirred reaction mixture was maintained below 10 °C, and sufficient diluted acetic acid was added to bring the pH to 7. The product, 5-acetamido-1,3,4-thiadiazole-2-methylsulfenamide, precipitated and was collected by filtration and dried. Oxidation to the sulfonamide was accomplished by dissolving the sulfenamide in dimethoxyethane and combining the resulting solution with 3.0 g of *m*-chloroperoxybenzoic acid (2.5 equiv) also dissolved in dimethoxyethane. The solutions were maintained at 0 °C and stirred for 24 h. The solvent was removed by vacuum distillation, and the excess peroxy acid was neutralized with 5% sodium bicarbonate. The solid product was filtered and washed several times with water before drying under vacuum: yield 75%; mp 256–258 °C, lit. 256–258 °C.⁷

Synthesis of 4-Nitrobenzenesulfonamide (3). Concentrated ammonium hydroxide was reacted with 4-nitrobenzenesulfonyl chloride following the procedure of Drozdov and Ignatyuk-Maistrenko.²³ The crude product was purified by column chromatography on silica gel and eluted with methylene chloride to give a 65.8% yield of pure 4-nitrobenzenesulfonamide: mp 181–183 °C, lit. 176 °C.²³

Synthesis of 1-[(N-Methylamino)sulfonyl]-4-nitrobenzene (4). This was prepared by a previously described procedure;²³ the crude product was purified by column chromatography on silica gel and eluted with chloroform to give a 62% yield: mp 100–102 °C, lit. 107–107.5 °C.²³ Anal. ($\text{C}_7\text{H}_8\text{N}_2\text{O}_4\text{S}$) C, H, N.

Synthesis of 1-[(N-Hydroxy-N-methylamino)sulfonyl]-4-nitrobenzene (5). N-Methylhydroxylamine hydrochloride (0.75 g, 0.009 mol) was dissolved in a mixture of methanol (10 mL) and pyridine (0.73 mL) at 0 °C. 4-Nitrobenzenesulfonyl chloride (1.0

(19) Kim, J. H.; Green, K.; Martinez, M.; Paton, D. *Exp. Eye Res.* 1971, 12, 231.

(20) Pocker, Y.; Stone, J. T. *J. Am. Chem. Soc.* 1965, 87, 5497.

(21) Segel, I. H. *Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems*; Wiley: New York, 1975.

(22) Eller, M. G.; Schoenwald, R. D.; Dixon, J. A.; Segarra, T.; Barfknecht, C. F. *J. Pharm. Sci.* 1985, 74, 525.

(23) Drozdov, N. S.; Ignatyuk-Maistrenko, V. A. *J. Appl. Chem. USSR (Engl. Transl.)* 1939, 12, 1065; *Chem. Abstr.* 1940, 34, 3266.

g, 0.0045 mol) was added in portions over a 15-min period. The reaction was removed from the ice bath and stirred for 15 h at room temperature. Water (75 mL) was added and the solution cooled in an ice bath. The precipitated product was collected by vacuum filtration and purified by column chromatography using silica gel with 9:1 toluene/ethyl acetate as the eluent to give 0.7 g (67% yield) of 1-[(*N*-hydroxy-*N*-methylamino)sulfonyl]-4-nitrobenzene: mp 147-149 °C; mass spectrum, *m/e* 232 (*M*⁺). Anal. (C₇H₈N₂O₅S) C, H, N.

Synthesis of 4-Chlorobenzenesulfonamide (6). The general method used for the synthesis of the 4-nitro analogue (3) was followed: mp 136-138 °C, lit. 144 °C.²⁴ Anal. (C₆H₆NO₂SCI) C, H, N.

Synthesis of 1-[(*N*-Methylamino)sulfonyl]-4-chlorobenzene (7). The general method used for the synthesis of the

4-nitro analogue 4 was followed: mp 56-58 °C, lit. 59 °C.²⁵

Synthesis of 1-[(*N*-Hydroxy-*N*-methylamino)sulfonyl]-4-chlorobenzene (8). The general method used for the synthesis of the 4-nitro analogue 5 was followed: mp 81-83 °C. Anal. (C₇H₈NO₃SCI) C, H, N.

Acknowledgment. This research was supported by NIH Research Grant EY 03297.

Registry No. 1, 68300-47-0; 3, 6325-93-5; 4, 6319-45-5; 5, 94592-96-8; 6, 98-64-6; 7, 6333-79-5; 8, 94592-95-7; 5-amino-1,3,4-thiadiazole 2-mercaptan, 2349-67-9; 5-acetamido-1,3,4-thiadiazole 2-mercaptan, 32873-56-6; 5-acetamido-1,3,4-thiadiazole-2-methylsulfenamide, 94515-21-6; 4-nitrobenzenesulfonyl chloride, 98-74-8; *N*-methylhydroxylamine hydrochloride, 4229-44-1; 4-chlorobenzenesulfonyl chloride, 98-60-2.

(24) Allen, C. F. H.; Frome, G. F. *J. Org. Chem.* 1942, 7, 15.

(25) Baxter, R.; Chattaway, F. *J. Chem. Soc.* 1915, 107, 1814.

2-Alkyl-Substituted 1,1-Bis(4-acetoxyphenyl)-2-phenylethenes. Estrogen Receptor Affinity, Estrogenic and Antiestrogenic Properties, and Mammary Tumor Inhibiting Activity

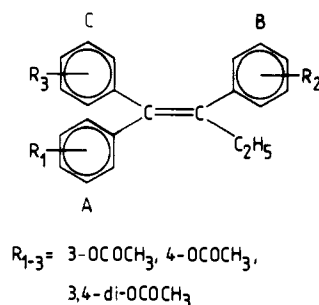
Martin R. Schneider

Institut für Pharmazie, Lehrstuhl Pharmazeutische Chemie II, Universität Regensburg, Universitätsstrasse 31, D-8400 Regensburg, F.R.G. Received October 17, 1985

1,1-Bis(4-acetoxyphenyl)-2-phenylethenes that are substituted with H, CH₃, C₂H₅, *n*-C₃H₇, *i*-C₃H₇, or CH₂CF₃ in position 2 were synthesized in order to study the influence of the alkyl side chain on estradiol receptor affinity, estrogenic and antiestrogenic properties, and inhibition of the hormone-dependent MXT mammary carcinoma of the mouse. Furthermore, the double bond of 1,1-bis(4-acetoxyphenyl)-2-phenylbut-1-ene was hydrogenated or epoxidated to yield the corresponding ethane and oxirane derivative. Compounds 14 (R = H), 15 (R = CH₃), and 16 (R = C₂H₅) had the best binding affinities. Lengthening the side chain, hydrogenation, or epoxidation decreased the RBA values. In the immature mouse assay, 15 (R = CH₃) and 19 (R = CH₂CF₃) had the highest uterotrophic activity. There was no correlation between receptor affinity and estrogenic properties. Compounds 14 (R = H), 17 (R = *n*-C₃H₇), the ethane 20, and the oxirane 21 had some antiuterotrophic activity in a low dosage. The MXT tumor was best inhibited by compounds 15 (R = CH₃), 16 (R = C₂H₅), and 18 (R = *i*-C₃H₇) without significant elevation of the uterine weight determined at the end of the experiment. The antitumor effect of 15, 16, and 18 was significantly better than that of tamoxifen. In this series, a certain estrogenic potency in the immature mouse test seems to be necessary for a good antitumor activity, as all compounds with antiuterotrophic and low uterotrophic properties did not exert any significant tumor-inhibiting effect.

Tamoxifen, a compound of the triarylethylene type, is now routinely used for the treatment of the advanced, hormone-dependent mammary carcinoma.¹ In three former publications,²⁻⁴ we have presented studies on structure-activity relationships in the class of 1,1,2-triphenyl-1-enes, i.e., triphenylethylenes with an ethyl side chain, which are substituted on the three aromatic rings with all possible combinations of 3-acetoxy, 4-acetoxy, and/or 3,4-diacetoxy groups. The pharmacological properties of these compounds with regard to estrogen receptor affinity, estrogenic and antiestrogenic effects, and tumor-inhibiting activities on hormone-dependent tumor models strongly depend on the applied mode of substitution of the phenyl rings (Scheme I).²⁻⁴

Scheme I



Of major interest are compounds with a substitution in ring A and C, as they had strong tumor inhibiting properties and only low estrogenic side effects.²⁻⁴ A β-aminoethoxy group attached on ring C as in tamoxifen and other triphenylethylene antiestrogens is not absolutely essential for antiestrogenic and tumor-inhibiting activity.²⁻⁴

Several other molecular modifications of triphenylethylenes have been described. However, no systematic structure-activity study concerning the variation of the substituent in position 2 was carried out in the class of

- (1) Jordan, V. C. In *Hormone Antagonists*; Agarwal, M. K., Ed.; de Gruyter: Berlin, New York, 1982; pp 109-128.
- (2) Schneider, M. R.; von Angerer, E.; Schönenberger, H.; Michel, R.-Th.; Fortmeyer, H. P. *J. Med. Chem.* 1982, 25, 1070.
- (3) Schneider, M. R.; Ball, H.; Schönenberger, H. *J. Med. Chem.* 1985, 28, 1880.
- (4) Schneider, M. R.; Ball, H.; Schiller, C.-D. *J. Med. Chem.*, in press.