Biochemical Pharmacology, Vol. 33, No. 5, pp. 731–738, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00

THE BINDING OF SULFONAMIDES TO HORSE LIVER ALCOHOL DEHYDROGENASE

ANDRÉ WESTERN, CHRISTIAN SYVERTSEN and JOHN S. MCKINLEY-MCKEE
Biochemical Institute, University of Oslo, Norway

(*Received* 25 *July* 1983; *accepted* 3 *October* 1983)

Abstract—The binding of sulfonamides to the active site of horse liver alcohol dehydrogenase has been studied by their effect on affinity labelling and steady state kinetics. Affinity labelling with iodoacetate and BIP has been used to study binding to free enzyme. The unsubstituted sulfonamide, sulfanilamide (I), shows very weak binding compared to the other sulfonamides tested. Most important for binding is the type of substituent attached to the parent sulfonamide, particularly when as in sulfathiazole this is a heterocycle which binds to the catalytic zinc atom of the enzyme. For sulfathiazole the dissociation constant from the enzyme is pH dependent showing two pK_a values. The lower at pH 7 is the pK_a of the drug itself, while that at pH 9 agrees with the ionization of water bound to the catalytic zinc ion.

Steady state kinetics have been carried out at pH 7.0 and 10.0 to examine sulfonamide binding to the enzyme when coenzyme is attached. Both NAD^+ and NADH induce substrate competitive sulfonamide binding. Likewise sulfathiazole accelerates the dissociation of NADH from the enzyme and so V_{max} for alcohol oxidation. The latter like stimulation of the affinity labelling reaction with iodoacetate is considered to result from binding of the thiazole ring to the catalytic zinc ion. With all the sulfonamides examined hydrophobic binding and charge are important in determining affinity to the active site and the mode of binding. Sulfonamides containing pyrazole or imidazole rings can be important in alcohol therapy.

Sulfonamides are extensively used as antibacterial or diuretic drugs. They are one of the few groups of drugs whose mechanism of antimicrobial action is known at the enzyme level. The mode of action of the sulfonamides is characterized by a competitive antagonism with *p*-aminobenzoic acid, which is an essential building block of folic acid [1].

Many aromatic and heterocyclic sulfonamides are powerful and specific inhibitors of the zinc metalloenzyme carbonic anhydrase [2]. The metal ion surrounded by three histidines and a water moelcule, is essential for strong sulfonamide binding [3]. The sulfonamide group is considered to be located within the primary coordination sphere of the metal ion [4, 5], and to bind to the metal ion in the anionic form $-SO_2NH^-$ [6, 7], nitrogen being the coordinating atom [8, 9]. Proton dissociation occurs if the sulfonamide–enzyme complex is formed at a pH below the pK_a of the sulfonamido group [10].

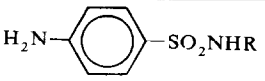
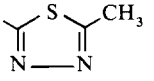
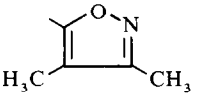
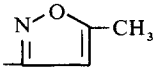
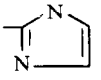
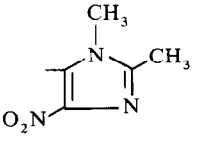
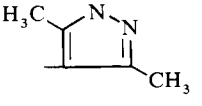
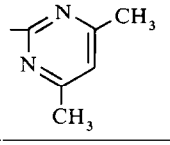
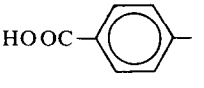
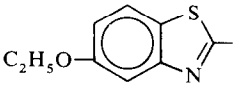
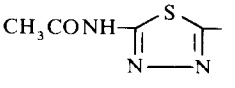
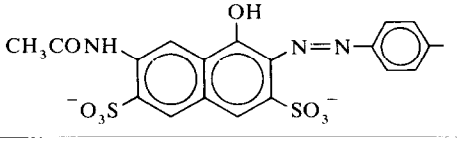
Like carbonic anhydrase and alkaline phosphatase, also inhibited by sulfonamides [11, 12], horse liver alcohol dehydrogenase (LADH), is also a zinc metalloenzyme. In LADH the catalytic zinc atom is bound to the three protein ligands; Cys-46, Cys-174 and His-67, and a water molecule [13]. It is therefore of interest to compare the binding of sulfonamides to these enzymes. This is also the case because appropriate sulfonamides, used medically, may be able to affect tissue alcohol oxidation. This is particularly so because many sulfonamides contain heterocyclic rings such as substituted pyrazoles which are known to influence liver alcohol dehydrogenases and have been used against alcohol poisoning [14]. The heterocycles alone are unable to pass through the blood–brain barrier, but sulfonamides can pass this barrier [15]. By substituting the sulfonamides with appropriate heterocycles, the latter should be able to reach the brain. An additional advantage with sulfonamides is that they are easily absorbed from the intestine, and into the blood stream.

To study the binding of sulfonamides to liver alcohol dehydrogenase, their effect on the affinity labelling of the enzyme with iodoacetate and BIP has been studied. Both these reagents are specific for the active site and inactivate the enzyme by modification of Cys-46. Being anions the affinity labels form reversible complexes with the anion binding site which is important for coenzyme binding, while BIP also coordinates the catalytic zinc ion by its imidazole group [16, 17]. Competition with BIP thus can show sulfonamide binding to the active site zinc of the free enzyme. Stimulation of iodoacetate inactivation can also indicate this. In addition steady state kinetics have been performed to study binding to LADH with coenzyme attached and the influence of sulfonamides and various heterocycles on enzyme catalyzed alcohol oxidation.

Address for correspondence, John McKinley-McKee, Biokjemisk Institutt, Universitetet i Oslo, Post boks 1041, Blindern, Oslo 3, Norway

Abbreviations: BIP, (R,S)-2-bromo-3-(5-imidazolyl)-propionic acid; Mes, 4-morpholineethanesulfonic acid; Pipes, 1,4-piperazinediethanesulfonic acid; Epps, 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid; Bicine, *N*,*N*-bis(2-hydroxyethyl)glycine; O, NAD^+, oxidized nicotinamide-adenine dinucleotide; R, NADH, reduced nicotinamide-adenine dinucleotide; LADH, horse liver alcohol dehydrogenase (EC 1.1.1.1).

Table 1. Sulfonamides used

Compound	R	Name
		
I	—H	Sulfanilamide
II		Sulfathiazole, *N*1-thiazole-2-yl-sulfanilamide
III		Sulfamethizole, *N*1-(5-methyl-1,3,4-thiadiazole-5-yl)-sulfanilamide
IV		Sulfisoxazole, *N*1-(3,4-dimethyl-isoxazole-5-yl)-sulfanilamide
V		Sulfamethoxazole, *N*1-(5-methyl-isoxazole-3-yl)-sulfanilamide
VI		*N*1-imidazole-2-yl-sulfanilamide
VII		*N*1-(1,2-dimethyl-4-nitroimidazole-5-yl)-sulfanilamide
VIII		*N*1-(3,5-dimethylpyrazole-4-yl)-sulfanilamide
IX		Sulfamethazine, *N*1-(4,6-dimethyl-pyrimidine-2-yl)-sulfanilamide
	$R—SO_2NH_2$ R	
X		*p*-Carboxybenzensulfonamide
XI		Ethoxzolamide, 6-ethoxy-2-benzothiazole sulfonamide
XII		Acetazolamide, 5-acetamido-1,3,4-thiadiazole-2-sulfonamide
XIII		Azosulfamide, 6-(acetylamino-3-((4-(aminosulfonyl)-phenyl)azo)-4-hydroxy-2,7-naphthalene-disulfonic acid disodium salt

MATERIALS AND METHODS

Enzyme. Horse liver alcohol dehydrogenase (EC 1.1.1.1) was obtained from Boeringer Mannheim. The crystalline suspension was dialysed against two changes of phosphate buffer, pH 7.0 (I=0.1M) and then against three changes of 30 mM Epps/NaOH buffer, pH 8.2. Assay was as previously described [16].

Buffers. Mes, Pipes, Epps, Bicine and glycine were obtained as free acids from Sigma. Borax was from Merck. Before use the buffers were titrated with alkali to the desired pH. The buffers affect the affinity labelling reaction, and concentrations around 20–30 mM were therefore used [18].

Other reagents. The coenzymes βNAD$^+$ and βNADH were purchased from Sigma (grade III) and used without further purification. 2-Iodoacetic acid and BIP were from Sigma. Before use iodoacetate was titrated with alkali to pH 5 and BIP to pH 7. Iodoacetate was recrystallized from petroleum ether and water. Thiazole, pyrazole and isoxazole were from Fluka. Double quartz distilled water was used for all solutions.

Sulfonamides. Table 1 lists the sulfonamides used. I, X and XI were generous gifts from Prof. E. Grell, Max-Planck-Institut for Biophysik, Frankfurt, F.R.G. VI and VII were gifts from F. Hoffman-La Roche & Co, while VIII was a gift from Bayer AG. The rest were from Sigma.

Enzyme inactivations were carried out at 23.5°, using enzyme concentrations around 5 μM. All the sulfonamides were tested at pH 8.2 in 30 mM Epps buffer. During inactivation, the reaction was followed by withdrawing aliquots (20–50 μl) for enzyme assay.

Initial rates of alcohol oxidation were followed at 340 nm by measuring the production of NADH with a Pye Unicam SP6-550 spectrophotometer, coupled to a Kontron W+W 1100 recorder. The steady state kinetic measurements were carried out at pH 7.0 in Pipes buffer and at pH 10.0 in glycine buffer, either with excess NAD$^+$ and varied ethanol concentrations or vice versa. Reaction was started by adding enzyme to a final concentration of about 10 nM.

Programware. First-order plots of log(activity) against time were treated by linear regression analysis. For derivative-free nonlinear regression analysis of inactivation data the program BMDPAR (Copyright 1979, University of California, LA) was used. Computations were carried out on the DEC-10 machine of the University of Oslo.

RESULTS

Affinity labelling. Iodoacetic acid and BIP inactivate liver alcohol dehydrogenase in a reaction which is first-order with respect to enzyme, and consistent with the affinity labelling mechanism of scheme (1) [16].

$$E + I \overset{K_{EI}}{\rightleftharpoons} EI \overset{k_2}{\rightarrow} E' \text{ (inactive)} \qquad \text{(scheme1)}$$

I is the inactivator or affinity label, EI the reversible enzyme–inactivator complex, K_{EI} the dissociation constant and k_2 the maximum first-order rate constant.

When adding a ligand, such as a sulfonamide, the rate of inactivation can be affected, according to scheme (2):

$$\begin{array}{ccc} E + I \overset{K_{EI}}{\rightleftharpoons} EI & \overset{k_2}{\longrightarrow} & E' \\ + & & + \\ L & & L \\ K_L \updownarrow & & \updownarrow \alpha K_L \\ EL + I \underset{\alpha K_{EI}}{\rightleftharpoons} EIL & \underset{\beta k_2}{\longrightarrow} & E' \end{array} \qquad \text{(scheme 2)}$$

Here L denotes the sulfonamide ligand, K_L the dissociation constant for the binary enzyme–sulfonamide complex, α a cooperativity or interaction constant and β a constant affecting the maximum rate. Depending on how the ligand binds, it will be classified with $\beta<1$ as a competitive, non-, un- or mixed-competitive inhibitor and with $\beta>1$ as an activator. $\alpha<1$ means there is a positive interaction or cooperativity in the binding of I and L to the enzyme, and that I binds stronger to EL than to E, and L stronger to EI than to E. $\alpha>1$ indicates negative cooperativity in the binding of I and L.

Equation (1) is an expression for the observed first-order rate constant, k_{obs}, for scheme (2)

$$k_{obs} = \frac{k_2[I](\alpha K_L + \beta[L])}{[L][I] + \alpha(K_L[I] + K_I[L] + K_L K_I)} \qquad (1)$$

Loading BMDPAR with this expression using k_{obs}, L and I as variables, allowed determination of the parameters α, β and K_L. The expression also allows determination of the parameters k_2 and K_I, but since these have been determined previously, they were kept at their fixed values [18].

The effect of sulfonamides on affinity labelling with iodoacetate. Figure 1 shows a semi-logarithmic, first-order plot of enzyme activity against time for various concentrations of iodoacetate, using ethoxzolamide (XI) as a ligand at pH 8.2.

The results of regression analysis for the inactivation of liver alcohol dehydrogenase with iodoacetate in the presence of various sulfonamides are summarized in Table 2a. All the sulfonamides were mixed type inhibitors with respect to iodoacetate except VIII which activated the inactivation reaction with iodoacetate.

Sulfanilamide (I) up to 10 mM did not affect the affinity labelling reaction with iodoacetate at pH 8.2. At pH 10.0 nearly 50% exists in the anion form and competitive inhibition with respect to iodoacetate was observed. The K_L was 45 mM which indicates weak binding.

Figure 2 depicts the half-time of inactivation against the reciprocal of the iodoacetate concentration at pH 8.2 for azosulfamide (XIII) which shows partial mixed inhibition. Partial inhibition means that the replot of the slope or the intercept on the ordinate versus the inhibitor concentration is nonlinear. β will therefore lie between 1 (competitive inhibition) and 0 (linear inhibition). For sulfisoxazole (IV) and sulfamethoxazole (V) β approaches zero, α is large and there is linear mixed inhibition. Only VIII in Table 2a has $\beta>1$ meaning that the ternary EIsulfonamide complex inactivates the enzyme

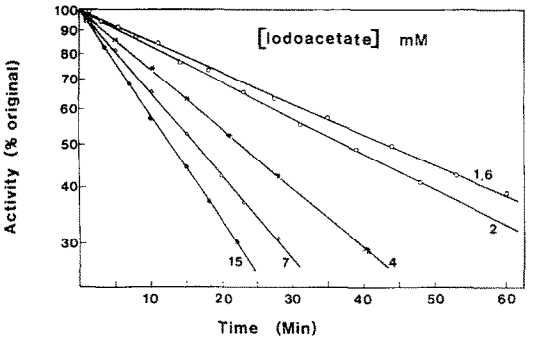


Fig. 1. Semi-log plots for the inactivation of liver alcohol dehydrogenase with iodoacetate in the presence of 0.2 mM ethoxzolamide (XI). Buffer: pH 8.2, 20 mM Epps–NaOH. The concentration of iodoacetate is as indicated.

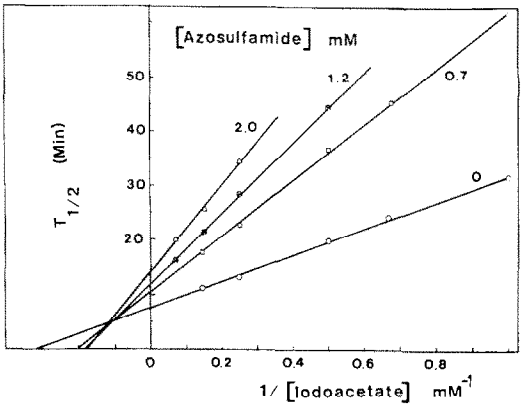


Fig. 2. Effect of azosulfamide (XIII) on the inactivation of liver alcohol dehydrogenase with iodoacetate. Plots of inactivation half-time versus the reciprocal concentration of iodoacetate. Buffer: pH 8.2, 20 mM Epps–NaOH. The concentration of azosulfamide is as indicated.

faster than the EI complex. Since α is also < 1 there is positive cooperativity in the binding of iodoacetate and VII to the enzyme.

Figure 3 shows the plot of log K_L, where K_L is the dissociation constant for sulfathiazole (II) versus pH. The data indicate two pK_a values around 7 and 9.3 respectively. As seen from the curve sulfathiazole has less affinity to the enzyme on the acidic side of the lower pKa and on the basic side of the higher pK_a. Replotting slope versus inhibitor concentration gave a nonlinear pattern at most pH values, which increased the standard deviation of the K_L values. At all pH values α is > 1 and there is negative cooperativity in the binding of sulfathiazole and iodoacetate to the enzyme. At pH 6.2 and 6.9, β is 3.1 and 1.1 respectively, which demonstrates that inactivation is accelerated by sulfathiazole. Figure 4 shows the half-time of inactivation, $t_{\frac{1}{2}}$, versus the reciprocal concentration of iodoacetate at pH 6.2. Activation is shown by the intercept on the ordinate in the presence of sulfathiazole being below that in its absence. Because the interaction constant α is large, in Fig. 4 inhibition occurs at low iodoacetate concentration.

The effect of sulfonamides on affinity labelling with BIP. Table 2b summarizes the results from regression analysis of inactivations with BIP in the presence of the sulfonamides. Sulfathiazole (II) was tested on both sides of pH 7.1 its pK_a value [15]. The β values for sulfathiazole and VIII are 1 showing competitive inhibition. In contrast XIII with $\alpha = 1$ is non-competitive with respect to BIP.

Steady state kinetics. Effect of sulfonamides on alcohol oxidation. Table 3a summarizes the type of inhibition for the sulfonamides tested and the dissociation constants determined. Figure 5 is a Lineweaver–Burk plot for pH 10.0 and varied ethanol concentrations, in the presence of ethoxzolamide (XI) a non-competitive inhibitor.

Table 2. Binding of sulfonamides to horse liver alcohol dehydrogenase studied by affinity labelling with (a) iodoacetate and (b) BIP

Compound	pH	K_L (μM)	α	β
(a) Iodoacetate inactivations				
I	8.2	∞		
IX	8.2	7400		
VII	8.2	3900 ± 1000	10	~1
X	8.2	3800 ± 1100	3.4 ± 0.8	0.48 ± 0.04
XII	8.2	1700 ± 500	1.7 ± 0.1	0.28 ± 0.03
VIII	8.2	1520 ± 290	0.5 ± 0.1	1.22 ± 0.07
V	8.2	900 ± 60	4.2 ± 0.6	0.05 ± 0.04
IV	8.2	600 ± 30	7.6 ± 1.4	~0
III	8.2	520 ± 30	6.8 ± 1.1	0.25 ± 0.05
XIII	8.2	460 ± 30	2.5 ± 0.3	0.28 ± 0.03
II	8.2	110 ± 15	3.6 ± 1.0	0.35 ± 0.08
XI	8.2	80 ± 9	4.1 ± 0.8	0.76 ± 0.08
(b) BIP Inactivations				
VII	8.2	2160 ± 210	1.4 ± 0.2	0.29 ± 0.03
VIII	8.2	1064 ± 174	3 ± 0.3	1
XIII	8.2	391 ± 5	1	0.25 ± 0.03
II	6.1	700 ± 30	15 ± 3	1
II	8.9	80 ± 5	13 ± 2	1

Buffers: pH 6.1, 30 mM Mes. pH 8.2 and 8.9, 30 mM Epps. K_L is the dissociation constant of the sulfonamides from the enzyme according to scheme (2), α an interaction constant and β a constant affecting the maximum rate of inactivation.

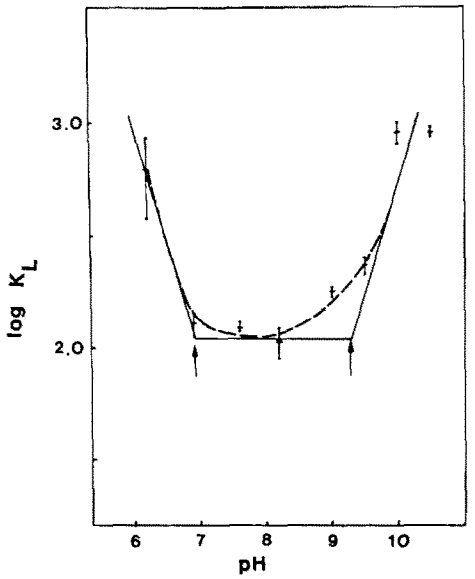


Fig. 3. pH dependence of the dissociation constant for sulfathiazole (II) from the enzyme. Affinity label: iodoacetate.

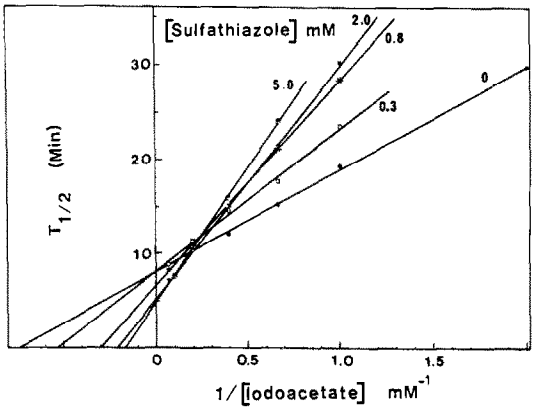


Fig. 4. The effect of sulfathiazole (II) on the inactivation of liver alcohol dehydrogenase with iodoacetate. Plots of inactivation half-time versus the reciprocal concentration of iodoacetate. Buffer: pH 6.2, 25 mM Mes–NaOH. The concentration of sulfathiazole is as indicated.

Sulfanilamide (I) at pH 7.0, showed a biphasic Lineweaver–Burk plot with varied ethanol, but at higher ethanol concentrations inhibition was competitive with a K_i or $K_{EO,I}$ of 20 mM for binding to the enzyme–NAD^+ complex. Using acetaldehyde as substrate and NADH as coenzyme sulfanilamide was competitive against acetaldehyde (Fig. 6). K_i or $K_{ER,I}$ is 12 mM and measures binding to the enzyme–NADH complex.

Sulfathiazole (II) at pH 7.0 accelerates the rate of reaction at ethanol concentrations higher than 1 mM, with the maximum velocity, V_{max}, increasing three

Table 3. Effect of (a) sulfonamides and (b) heterocycles on the initial rate of alcohol oxidation with horse liver alcohol dehydrogenase

Compound	pH	Varied species	Type of effect*	K_i (mM)
(a) Sulfonamides				
I	7.0	Acetaldehyde	C	12
		Ethanol	C	20
I	10.0	NAD^+	M	7.5
		Ethanol	M	21
II	7.0	NAD^+	A	1.0
		Ethanol	A	4.2
II	10.0	NAD^+	C	0.6
		Ethanol	N	5.1
XI	10.0	NAD^+	C	0.1
		Ethanol	N	2.3
XIII	7.0	NAD^+	C	
		Ethanol	—	0.2
XIII	10.0	NAD^+	M	0.3
		Ethanol	N	< 1
(b) heterocycles				
Thiazole	7.0	Ethanol	C	10
Thiazole	10.0	Ethanol	C (N-L)	30
		NAD^+	C	32
Isoxazole	7.0	Ethanol	C	14
Isoxazole	10.0	Ethanol	M	> 100
		NAD^+	M	125
Pyrazole	7.0	Ethanol	C	0.3×10^{-3}
Pyrazole	10.0	Ethanol	C	$\sim 3 \times 10^{-3}$

pH 7.0: 30 mM Pipes buffer. NAD^+ 1.7 mM; ethanol varied. Ethanol 8.0 mM; NAD^+ varied.
pH 10.0: 30 mM glycine buffer. NAD^+ 0.5 mM; ethanol varied.
Ethanol 8 mM; NAD^+ varied. K_i results from a replot of slope vs. concentration of sulfonamide.
*: C, competitive, M, mixed-competitive, N, non-competitive, A, activation, N-L, non-linear plot.

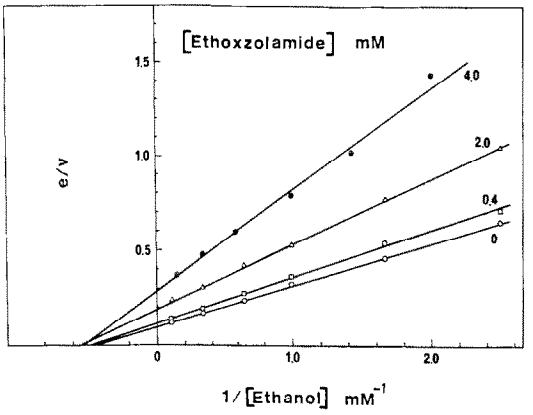


Fig. 5. The effect of ethoxzolamide (XI) on the initial rate of alcohol oxidation. Plot of e/v versus the reciprocal concentration of ethanol. Buffer: pH 10.0, 30 mM glycine–NaOH. NAD^+: 0.4 mM. Enzyme concentration: about 10 nM. The concentration of ethoxzolamide is as indicated.

fold. At pH 10.0 sulfathiazole is non-competitive against ethanol (Fig. 7a), but competitive against the coenzyme (Fig. 7b).

Theorell and McKinley-McKee found that at pH 7.0 substrate inhibition occurs at ethanol concentrations higher than 7–8 mM [19]. Figure 8 confirms this and also shows that substrate inhibition is lowered in the presence of sulfathiazole. However, at pH 10.0 under conditions of substrate inhibition increasing concentrations of sulfathiazole only increased the inhibition of ethanol oxidation.

Effects of heterocycles on alcohol oxidation. Unlike sulfathiazole the individual heterocycles thiazole (Fig. 9), pyrazole and isoxazole exhibit competitive inhibition with respect to ethanol at pH 7.0. Inhibition is due to formation of an E–NAD^+–heterocycle (EOI) complex. At pH 10.0 pyrazole was competitive against ethanol. Isoxazole at the same pH was mixed-competitive against both ethanol and

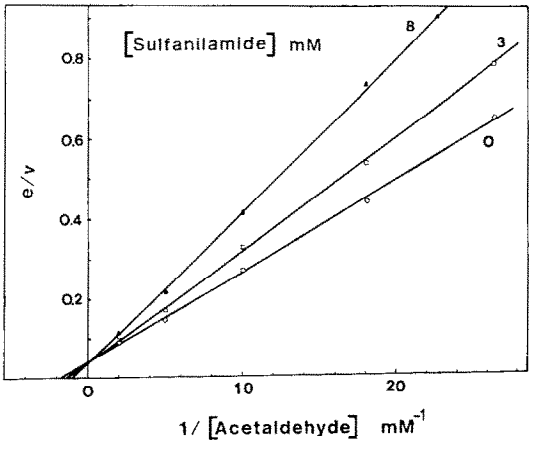


Fig. 6. The effect of sulfanilamide (I) on the initial rate of aldehyde reduction. Plots of e/v versus the reciprocal concentration of acetaldehyde. Buffer: pH 7.0, 30.0 mM Pipes–NaOH. NADH: 0.2 mM. Enzyme concentration: about 10 nM. The concentration of sulfanilamide is as indicated.

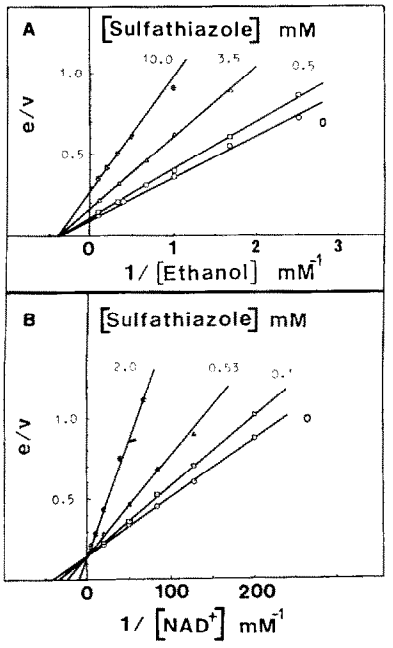


Fig. 7. The effect of sulfathiazole (II) on the initial rate of alcohol oxidation. (A) Plots of e/v versus the reciprocal concentration of ethanol. NAD^+: 0.5 mM. (B) Plots of e/v versus the reciprocal concentration of NAD^+. Ethanol: 8 mM. Buffer: pH 10.0, 30.0 mM glycine–NaOH. Enzyme concentration: about 10 nM. The concentration of sulfathiazole is as indicated.

NAD^+. Thiazole, which showed competitive inhibition against NAD^+, gave a nonlinear Lineweaver–Burk plot against ethanol. Table 3b lists the K_i values determined. K_i measured from the slope represents the dissociation constant for binding to the same enzyme species that the variable substrate is bound to. With increase in pH, $K_{EO.I}$ for each heterocycle increases just as the Michaelis constant for alcohol, K_m (alcohol), increases from 0.37 at pH 7.0 to 3.4 at pH 10.0.

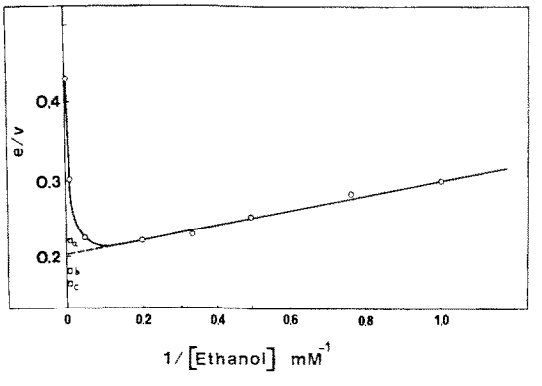


Fig. 8. Effect of sulfathiazole (II) on substrate inhibition of ethanol oxidation at ethanol concentrations higher than 7–8 mM. Buffer: pH 7.0, 30 mM Pipes–NaOH. NAD^+: 1.7 mM. Enzyme concentration: about 10 nM. ○ Plot of e/v versus the reciprocal concentration of ethanol. □ 100 mM ethanol: sulfathiazole: 5 mM (a), 10 mM (b), 15 mM (c).

DISCUSSION

In the case of carbonic anhydrase an unsubstituted sulfonamido group attached to a conjugated ring system is required for inhibition [20].

In the present work two different types of sulfonamides have been tested. Those having a free sulfonamido group ($-SO_2NH_2$) and substituted sulfonamides ($-SO_2NHR$) with a R group attached to the N-1 nitrogen atom of the sulfonamido group.

Table 2 shows no correlation between K_L and whether the substituent is in the sulfonamido group or not. Thus with horse liver alcohol dehydrogenase the free sulfonamido group is not necessary for inhibition. Indeed compared to the other sulfonamides tested K_L is large for sulfanilamide (I) and an R group is essential for high affinity to the active site of the free enzyme.

Most of the sulfonamides, L, showed a mixed competitive type of inhibition in affinity labelling with iodoacetate, I, with the formation of ternary, EIL, complexes. Large negative interactions ($\alpha \gg 1$) occur with the simultaneous binding of L and I.

Sulfathiazole. For sulfathiazole (II) the pH profile of K_L shows one pK_a around pH 7.0, which agrees with that of the drug itself (15). This pK_a is a result of the ionization of the sulfonamido group. On the acidic side of this value, where sulfathiazole is neutral, it is competitive against BIP (Table 2b). Since BIP binds through its imidazole ring to the catalytic zinc [17], and thiazole is also considered to bind to the catalytic zinc (see below), sulfathiazole is concluded to bind to the metal via the nitrogen atom in its thiazole ring. Binding to the catalytic zinc ion is also supported by sulfathiazole accelerating the maximum rate of inactivation with iodoacetate (Fig. 4). Several nitrogen containing heterocycles including thiazole have been shown to accelerate inactivation with iodoacetate [21, 22]. Essential for activation is a reactive ternary enzyme–iodoacetate-activator complex in which the activator is bound to the catalytic zinc ion.

Sulfathiazole becomes an anion on the basic side of its pK_a of 7.1. As it is also competitive against BIP at higher pH values, metal binding is again indicated. The increase in affinity to the free enzyme on the basic side of the pK_a value is a result of interactions with the anion binding site. The second pK_a around pH 9.3 in Fig. 3 is considered due to the ionization of the zinc-bound water which has a pK_a around 9 in the free enzyme [19, 23]. The decrease in the affinity of sulfathiazole to the enzyme above pH 9 results from either electrostatic repulsion between hydroxyl ion and the sulfathiazole anion, or to hydroxyl being more tightly bound to the metal than is a water molecule.

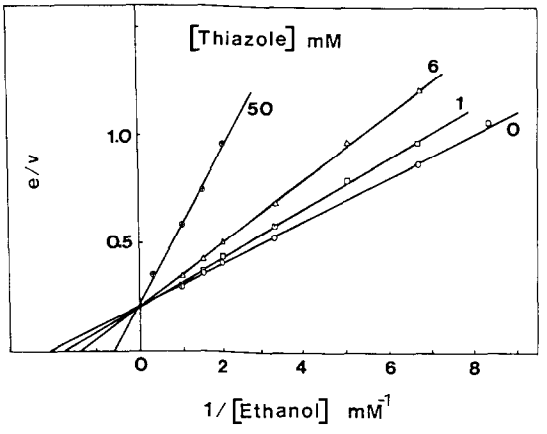


Fig. 9. The effect of thiazole on the initial rate of alcohol oxidation. Plots of e/v versus the reciprocal concentration of ethanol. Buffer: pH 7.0, 30 mM Pipes–NaOH, NAD^+: 1.7 mM. Enzyme concentration: about 10 nM. The concentration of thiazole is as indicated.

Activation by sulfathiazole of both the affinity labelling reaction with iodoacetate and the oxidation of ethanol is only observed below pH 7 and implicates the neutral form of sulfathiazole. In steady state kinetics substrate inhibition occurs through formation of a ternary ERalcohol complex, which is more stable and from which R dissociates more slowly than from the rate limiting binary ER complex. Likewise the activation of ethanol turnover at pH 7 by sulfathiazole occurs through formation of a ternary ERsulfathiazole complex which is more labile than ER [24], and which competitively prevents formation of the more stable and inhibiting ERalcohol complex. Figure 8 shows this as at higher alcohol concentrations reciprocal activity for both 10 and 15 mM sulfathiazole is beneath the base line. At lower ethanol concentrations inhibition with sulfathiazole arises due to competition between sulfathiazole and ethanol for the EO complex in the first phase of reaction.

The strict competition between the heterocycle thiazole and ethanol (Fig. 9) support that in the ternary ERsulfathiazole complex, sulfathiazole is bound to zinc via its thiazole ring.

Other sulfonamides. Pyrazole-sulfanilamide (VIII) also activates the affinity labelling reaction with iodoacetate. Here $\alpha < 1$ and $\beta > 1$ means that activation or promotion is found for all concentrations of iodoacetate. As in the case of pyrazole [25], it is the unprotonated nitrogen atom in the ring which binds to zinc. This is reinforced by the fact that VIII is competitive against BIP and that pyrazole itself shows competitive inhibition against ethanol at both pH 7.0 and 10.0. It is also the only sulfonamide that facilitates binding of iodoacetate to the enzyme ($\alpha < 1$), and indicates that VIII is neutral at pH 8.2.

As seen from Table 2a IV and V exhibit greater dissociation constants than II and III. Thioethers bind metal ions poorly [26], which again supports the nitrogen atom in the thiazole ring being responsible for binding to the metal. Sulfamethizole (III) with a less basic ring than thiazole, binds weaker to the enzyme than sulfathiazole (II).

The oxygen containing heterocyclic rings have low pK values, and this, with the strong inductive effect between the oxygen and the nitrogen atoms, can explain their weaker binding. The position of the nitrogen atom in the isoxazole-ring and the orientation of the methyl groups are considered to account for the difference in affinity of the two isoxazole sulfonamides (IV and V) to the enzyme.

In the case of carboxybenzenesulfonamide (X) its affinity to LADH is similar to that found for small anions [27] and benzoate [28]. This is to be expected since X is an anion and also contains benzoate.

The substrate binding site of liver alcohol dehydrogenase is a hydrophobic barrel which tends to strongly bind ligands with long hydrophobic chains [29]. In agreement with this, XI, which is the least water-soluble of the sulfonamides tested, has the smallest dissociation constant from the free enzyme, and gives rise to an ERethoxzolamide complex at pH 10.0.

Azosulfamide (XIII) on the other hand is competitive against NAD^+ at pH 7.0, and at pH 8.2 and below appears to bind at the anion binding site in the coenzyme binding region, rather than in the hydrophobic barrel. That XIII is non-competitive against BIP and shows very little effect against ethanol supports this. The dissociation constants found from the different methods for this compound are in good agreement with each other. At pH 7.0 only binding to the free enzyme is observed, but at pH 10.0 binding as an anion [30] to ER also occurs.

For sulfanilamide (I) the reported dissociation constants at pH 7.0 for the complex with Co(II) carbonic anhydrase are 22 and 4.5 μM for the human B and C forms respectively [31]. At pH 7 sulfanilamide with a pK_a of 10.5 [15] is neutral but it is bound in the anionic form to carbonic anhydrase [8]. For liver alcohol dehydrogenase, the high dissociation constant for sulfanilamide in competition with the affinity label iodoacetate indicates that this unsubstituted sulfonamide binds poorly to the free enzyme. The experiments at lower pH show that the drug binds in its anion form to the anion binding site. This is very different from the situation with carbonic anhydrase where RSO_2NH^- is bound to zinc. However in steady state kinetics at pH 7 there is strict competition between sulfanilamide and both ethanol and acetaldehyde (Table 3a, Fig. 6) with the sulfonamido group binding in the presence of the coenzyme to the catalytic zinc of LADH.

In liver alcohol dehydrogenase the most interesting sulfonamides seem to be those with a heterocyclic substituent which can combine with the catalytic zinc ion. Then as with sulfathiazole (II) they can form binary complexes with the enzyme or ternary complexes with the enzyme–coenzyme complexes which may result in the inhibition or activation of alcohol metabolism. In the latter case the sulfonamido group is optimally in the neutral state, because then binding to the anion binding site and competition with the coenzymes is avoided.

Inhibition by substituted pyrazoles in particular 4-methyl pyrazole has been used medically against alcohol poisoning [14]. Sulfonamides containing pyrazole rings substituted in the 4-position may therefore have medical importance. Indeed imidazole- or 4-pyrazole-sulfonamides accelerating or inhibiting ethanol metabolism or inhibiting the metabolism of alcohol poisons such as methanol or glycol can have a place in alcohol therapy.

REFERENCES

1. D. D. Wood, *Br. J. exp. Path.* **21**, 74 (1940).
2. T. H. Maren, *Physiol. Rev.* **47**, 595 (1967).
3. A. Meister, *Adv. Enzym.* **47**, 149 (1978).
4. S. Lindskog, *J. biol. Chem.* **238**, 945 (1963).
5. K. Fridborg, K. K. Kannan, A. Liljas, J. Lundin, B. Strandberg, R. Strandberg, B. Tilander and G. Wirén, *J. molec. Biol.* **25**, 505 (1967).
6. R. F. Chen and J. C. Kernohan, *J. biol. Chem.* **242**, 5813 (1967).
7. K. Kumar, R. W. King and P. R. Carey, *Biochemistry* **15**, 2195 (1976).
8. J. L. Evelhoch, D. F. Boican and J. L. Sudmeier, *Biochemistry* **20**, 4951 (1981).
9. K. Kanamori and J. D. Roberts, *Biochemistry* **22**, 2658 (1983).
10. J. E. Coleman, *A. Rev. Pharmac.* **15**, 221 (1975).
11. G. H. Price, *Clin. Chim. Acta* **94**, 211 (1979).
12. G. H. Price, *Clin. Chim. Acta* **101**, 313 (1980).
13. C. I. Brändén, H. Jörnvall, H. Eklund and B. Furugren, in *The Enzymes* (Ed. P. D. Boyer), 3rd. edn, Vol. 11, p. 103. Academic Press, London (1975).
14. A. Blomstrand and H. Theorell, *Life Sci.* **9**, 631 (1970).
15. T. Struller, *Arzneimittel Forsch.* **12**, 389 (1968).
16. C. H. Reynolds and J. S. McKinley-McKee, *Eur. J. Biochem.* **10**, 474 (1969).
17. K. H. Dahl and J. S. McKinley-McKee, *Eur. J. Biochem.* **81**, 223 (1977).
18. C. Syvertsen and J. S. McKinley-McKee, *Eur. J. Biochem.* **117**, 165 (1981).
19. H. Theorell and J. S. McKinley-McKee, *Acta Chem. Scand.* **15**, 1811 (1961).
20. P. W. Taylor, R. W. King and A. S. V. Burgen, *Biochemistry* **9**, 2638 (1970).
21. K. H. Dahl and J. S. McKinley-McKee, *Eur. J. Biochem.* **120**, 451 (1981).
22. C. Syvertsen and J. S. McKinley-McKee, *Archs Biochem. Biophys.* **223**, 213 (1983).
23. P. Andersson, J. Kvassman, A. Lindström, B. Oldén and G. Petterson, *Eur. J. Biochem.* **113**, 425 (1981).
24. H. Theorell and J. S. McKinley-McKee, *Acta Chem. Scand.* **15**, 1835 (1961).
25. H. Theorell and T. Yonetani, *Biochem Z.* **338**, 537 (1963).
26. L. G. Sillén and A. E. Martell, in *Stability Constants of Metal-ion Complexes*, Special publications No. 17 and 25, The Chemical Society, London (1964, 1971).
27. B. Oldén and G. Pettersson, *Eur. J. Biochem.* **125**, 311 (1982).
28. P. Andersson, J. Kvassman, B. Oldén and G. Petterson, *Eur. J. Biochem.* **118**, 119 (1981).
29. A. D. Winer and H. Theorell, *Acta Chem. Scand.* **14**, 1729 (1960).
30. P. R. Carey and R. W. King, *Biochemistry* **18**, 2834 (1979).
31. S. A. Cockle, S. Lindskog and E. Grell, *Biochem. J.* **143**, 703 (1974).