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INTERACTION OF ENZYMES WITH SULFANILAMIDE DISULFIDES*

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Abstract—Sulfanilamide disulfides strongly inhibit YADH and YLDH,‡ but are unable to inhibit LADH and HLDH. The inhibition of YADH and YLDH is pH dependent, with optima of pH of around 9·0–10·0. The inhibition is of non-competitive type with respect to the coenzyme, and of mixed type with respect to the substrate concentrations. The inhibition is connected with the reaction between disulfide and free thiol groups of the enzyme; in this reaction an inactive stable mixed disulfide En—SS—R is formed, which cannot be split by reducing agents like cysteine.

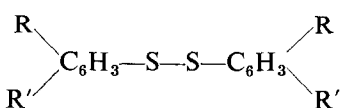
RNAase was insensitive to these aromatic disulfides. Carbonic anhydrase is inhibited by the sulfonamidic group, but the presence of the disulfide bond contributes, and the mechanism appears to be different from that suggested for the inhibition of the dehydrogenases.

FREE thiol groups in the molecule are essential for the activity of most oxidoreductive enzymes; moreover, many of them require the presence in the medium of an activating compound, like cysteine or GSH; on the contrary, in some cases, cystine and GSSG act as inhibitors, because they oxidize the thiol groups of the enzyme to inactive disulfides.

While the aliphatic disulfides cystine, cystamine, GSSG and similar compounds, and more recently, the alkyl thiuram disulfide derivatives have been extensively studied, it appears from the recent literature that aromatic disulfides have been disregarded as enzyme inhibitors, though some compounds were employed for determining the free —SH groups in the proteins.[1, 2] Sulfanilamides, also, have been found to inhibit several enzymes, but studies of the effects of these compounds on the oxidoreductive enzymes are few and often unconfirmed.[3]

It seemed interesting, therefore, to study some aromatic disulfides containing sulfonamidic groups, which in preliminary experiments showed a strong inhibitory effect on YADH and YLDH .It must be noted, however, that the same compounds have a protective action on HLDH and LADH exposed to ultraviolet, roentgen and γ radiations.[4]

These compounds, first prepared and studied by Pappalardo,[5] have the general formula



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‡ Abbreviations: YADH, yeast alcohol dehydrogenase; LADH, liver alcohol dehydrogenase; YLDH, yeast lactic dehydrogenase; HLDH, heart lactic dehydrogenase; RNAase, pancrease ribonuclease; GSH and GSSG, reduced and oxidized glutathione.

in which R is the sulfonamidic group (or a substituted derivative) and R′ is the amino-group; R′ may be absent.

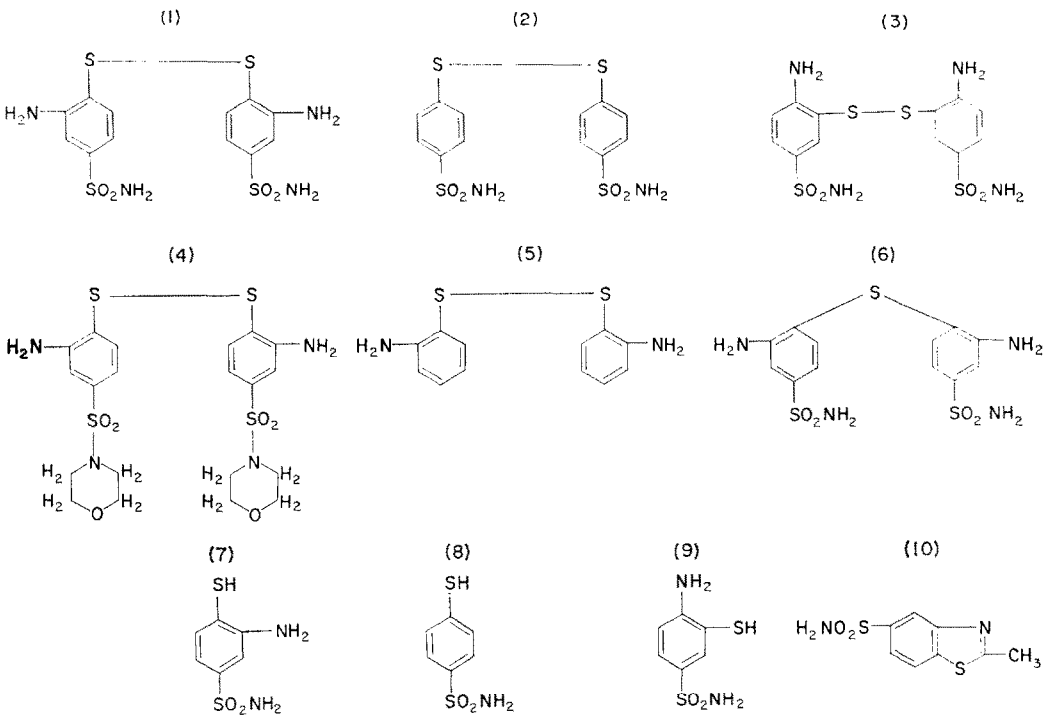
These disulfides (as well as the corresponding thiol, monosulfide and benzothiazole derivatives) were tested on some typical sulfhydril enzymes, such as alcohol and lactic dehydrogenases obtained from either a yeast or an animal source, since their behaviour appeared quite different in the preliminary experiments. Other enzymes were studied for comparison, namely the RNAase and the carbonic anhydrase, which are not dependent for their activity upon the presence of free thiol groups; on the contrary, the latter is known to be inhibited by several sulfanilamide derivatives.

Some results of the experiments were previously reported at the meetings of the Societa Italiana di Biologia Sperimentale.[6, 7]

MATERIALS AND METHODS

Sulfanilamide disulfides and derived compounds

These compounds were prepared and kindly supplied by Dr. Pappalardo. The structural formulae of these disulfides and of the compounds which were tested for comparison are shown below:



All the compounds, with the exception of the thiols, are sparingly soluble in water, but more soluble in methanol and ethanol. As ethanol showed no significant effect on all the enzymes under study, ethanolic solutions were used.

Enzymes

Yeast alcohol dehydrogenase. Purified and crystallized according to Racker.[8] Preparations with a specific activity around 120,000 were used. Tests were carried out at room temperature by the method described by Theorell and Bonnichsen as reported by Bonnichsen and Brink[9] for LADH. In the reverse reaction acetaldehyde → ethanol, a buffer phosphate 0·1 M pH 7·0 was used. The reaction ethanol → acetaldehyde was initiated by the addition of DPN since the substrate was also the solvent of the disulfides, which, as it could be seen later on, must be preincubated with the enzyme. Control experiments showed that no change in the reaction rate occurred when the reaction was initiated by the addition of ethanol or DPN.

Liver alcohol dehydrogenase. Prepared from horse liver according to Bonnichsen and Brink.[9] Highly purified, but not crystallized preparations were used. Tests of activity were performed by the method of Theorell and Bonnichsen as reported by Bonnichsen and Brink;[9] the reaction was initiated by the addition of DPN.

Yeast lactic dehydrogenase. Prepared by the method of Boeri and Rippa;[10] the preparation had a TN of about 15,000. Tests were performed according to Boeri *et al.*[11] The reaction was initiated by the addition of ferricyanide.

Heart lactic dehydrogenase. Crystallized and tested according to Neilands.[12] A preparation with a TN of 15,000 was used.

Pancreas ribonuclease. A crystalline preparation made by Sigma Chemical Company was used. Activity tests were performed according to McDonald.[13]

Carbonic anhydrase. Prepared from bovine red blood cells and tested in veronal–CO_2 buffer as described by Waygood;[14] the specific activity of the preparation was of 800 E.U./mg protein.

Reagents and apparatus

For the enzymatic preparations only pure reagents (analytical grade) from primary manufacturers were used. DPN, DPNH were obtained from Sigma Chemical Company; lactic acid and veronal from Bayer Farbenindustrie; ethanol from Carlo Erba S.p.A.; test buffers were prepared with reagents of Merck GmbH.

Tests for determining dehydrogenase activity and spectra were carried out using either a DU or a DK-2 Beckman spectrophotometer; in some experiments the activity was tested through the fluorescence changes of DPNH, using a double monochromator Farrand spectrofluorimeter at 340 and 440 mμ wavelength, respectively, for the exciting and the fluorescent light.

RESULTS

Experiments on YADH

The first experiments showed that the disulfides under study inhibit YADH in relation to the concentration, but the results were scarcely reproducible. A possible effect of the time of incubation was presupposed. In fact, as shown in Fig. 1, the inhibitory effect of the disulfide No. 1 increased with the incubation time. Therefore, for all the successive experiments a standard incubation time of 2 min was chosen. At this time the more intensively studied compound (No. 1), at the final concentration of 10 μM, has an inhibitory effect of around 60 per cent. The temperature of incubation was usually 2–4°.

All the studied disulfides have a strong inhibitory effect, both those with free and substituted sulfonamidic groups. Figure 2 shows the inhibition curves of some of the disulfides in the reaction ethanol → acetaldehyde. It may be seen that, although very low concentrations (μM) cause a marked inhibition, for no concentration there is an inhibitory effect of 100 per cent. The disulfides also have a similar inhibitory effect

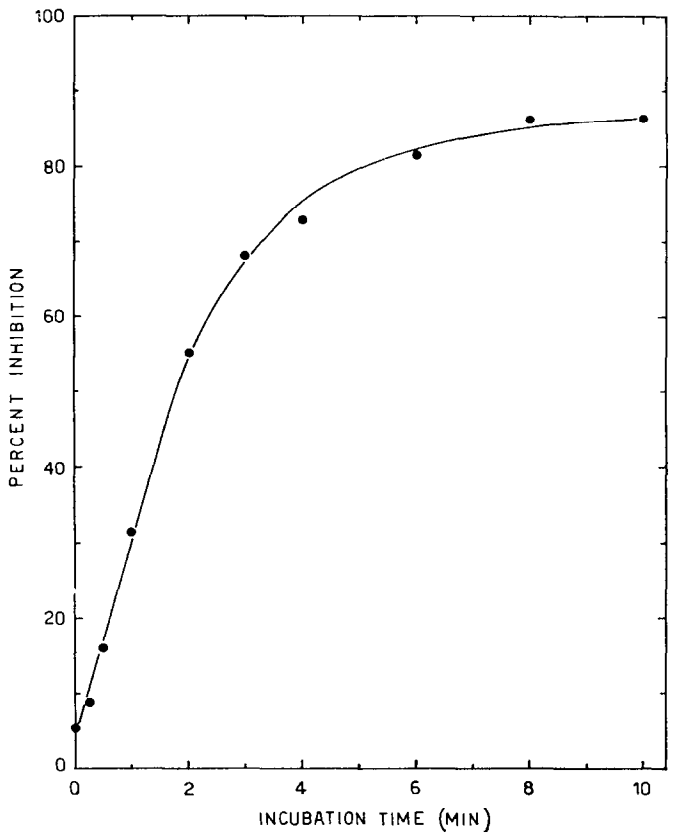


FIG. 1. Influence of the incubation time on the inhibitory effect of the disulfide on YADH. Reaction ethanol → acetaldehyde. Disulfide 1 (1×10^{-5} M final) was added in the enzymatic solution at the selected time before the addition of DPN.

in the reverse reaction acetaldehyde → ethanol (Fig. 3). Controls contained the same amount of ethanol as that present in the sample with the greater concentration of disulfide.

The compounds without —S—S— bridges, like sulfanilamide, monosulfide (No. 6) and benzothiazole derivative (No. 10), did not act as inhibitors. Also the mercapto-compounds (Nos. 7, 8, 9) showed practically no inhibitory effect.

The inhibitory effect of the disulfides, diminishes when the compounds are treated before the incubation with the enzyme, with reducing agents, like sodium boro-hydride, Na_2S, cysteine or GSH (Table 1). Cystine and GSSG influenced neither the activity of the enzyme nor the inhibitory effects of the disulfides. The effects of the

TABLE 1. REMOVAL OF THE INHIBITORY EFFECT OF THE DISULFIDES ON YADH BY PART OF REDUCING AGENTS

Inhibitor		Reducing agent		% inhibition
disulfide 1	$3{\cdot}3 \times 10^{-5}$ M		none	84
disulfide 1	$3{\cdot}3 \times 10^{-5}$ M	Na_2S	$6{\cdot}6 \times 10^{-4}$ M	4
disulfide 1	$3{\cdot}3 \times 10^{-5}$ M	GSH	$3{\cdot}3 \times 10^{-5}$ M	72
disulfide 1	$3{\cdot}3 \times 10^{-5}$ M	GSH	$4{\cdot}4 \times 10^{-5}$ M	14
disulfide 2	$3{\cdot}3 \times 10^{-5}$ M		none	86
disulfide 2	$3{\cdot}3 \times 10^{-5}$ M	Na_2S	$6{\cdot}6 \times 10^{-4}$ M	15
disulfide 3	$3{\cdot}3 \times 10^{-5}$ M		none	81
disulfide 3	$3{\cdot}3 \times 10^{-5}$ M	Na_2S	$6{\cdot}6 \times 10^{-4}$ M	8
disulfide 3	$1{\cdot}0 \times 10^{-5}$ M		none	62
disulfide 3	$1{\cdot}0 \times 10^{-5}$ M	GSH	$2{\cdot}2 \times 10^{-5}$ M	5
disulfide 4	$1{\cdot}0 \times 10^{-5}$ M		none	89
disulfide 4	$1{\cdot}0 \times 10^{-5}$ M	GSH	$2{\cdot}2 \times 10^{-5}$ M	7

The reducing agents have been added to the disulfide in the test solution 2 min before the addition of the enzyme. Tests started with DPN after 2 min of incubation

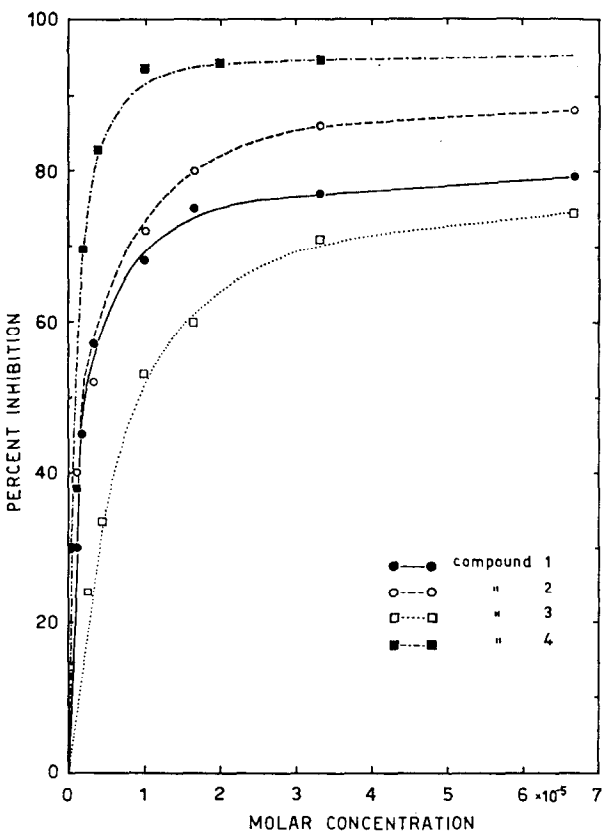


FIG. 2. Effect of the concentration of the disulfides on the activity of YADH in the reaction ethanol → acetaldehyde.

reducing agents were observed only when they were preincubated with the disulfides before their addition to the enzymatic solution.

The pH of the incubating solution greatly affects the inhibitory action of the disulfides. Strong inhibition occurs only at alkaline pH. The experiments were performed by testing the enzymatic activity after the incubation of the enzyme in a separate tube at

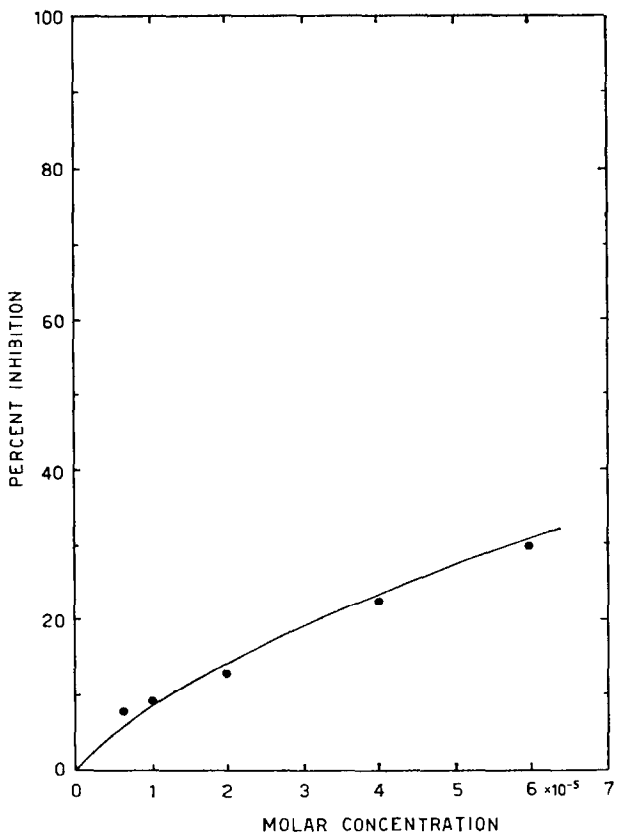


FIG. 3. Effect of the concentration of the disulfide 1 on the activity of YADH in the reaction acetaldehyde → ethanol.

the selected pH. From Fig. 4 it is seen that pH optima for inhibition are around 9·0–10·0; the other disulfides have similar pH optima. The inhibition curves were practically unchanged when the tests were made at a pH the same as that of the incubation solution. Figure 5 shows the same dependence upon pH in the case of the reaction acetaldehyde → ethanol.

It was also tested whether the effect of alkali, which is known[15] to decompose the aliphatic disulfides to mercaptocompounds and sulfinic acids, may depend upon the formation of this latter compound. Neither the sulfinic derivative, prepared separately, nor the disulfide previously treated with NaOH and added to the enzyme after neutralization, had inhibitory effect.

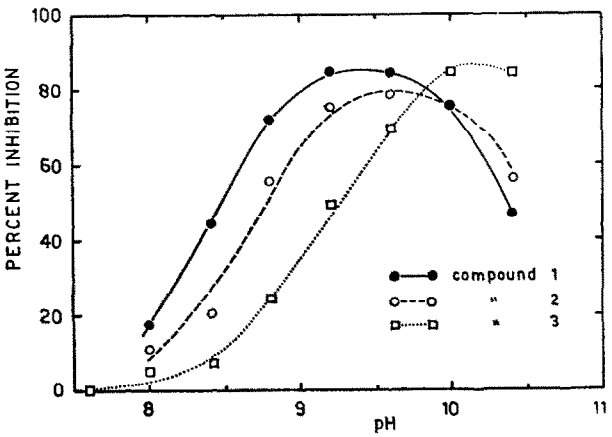


FIG. 4. Influence of pH on the inhibitory effect of the disulfides on YADH in the reaction ethanol → acetaldehyde.
Disulfides were incubated with the enzyme at the selected pH, 2 min before the test carried out at pH 9·6. Final concentration of the disulfides 2×10^{-5} M.

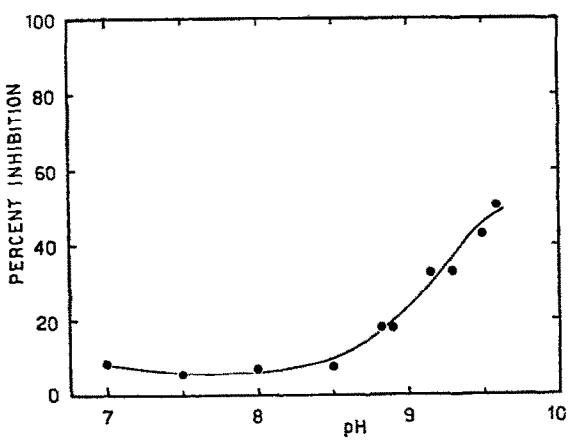


FIG. 5. Effect of pH on the inhibition by disulfide 1 on YADH in the reaction acetaldehyde → ethanol.
Disulfide was incubated with the enzyme at the selected pH, 2 min before the test carried out at the pH 7·0. Final concentration of the disulfide 1×10^{-5} M.

The study of the type of inhibition, carried out by the method of the Lineweaver–Burk plots, was performed both for the reaction ethanol → acetaldehyde and acetaldehyde → ethanol, with respect either to the coenzyme or to the substrate concentrations (Fig. 6 A, B, C, D). The inhibition appears to be of the non-competitive type with respect to the coenzyme, and of the mixed type with respect to the substrate concentrations. By plotting 1/V against i* (according to Dixon,[16]) or $i\alpha/1 - \alpha$ against S† (according to Hunter and Dowins,[17]) curved lines are obtained, confirming that

* i, inhibitor concentration.

† i, S, inhibitor and substrate concentrations; α is the ratio V_i/V, in which V_i and V are the velocities in the presence and in the absence of inhibitor.

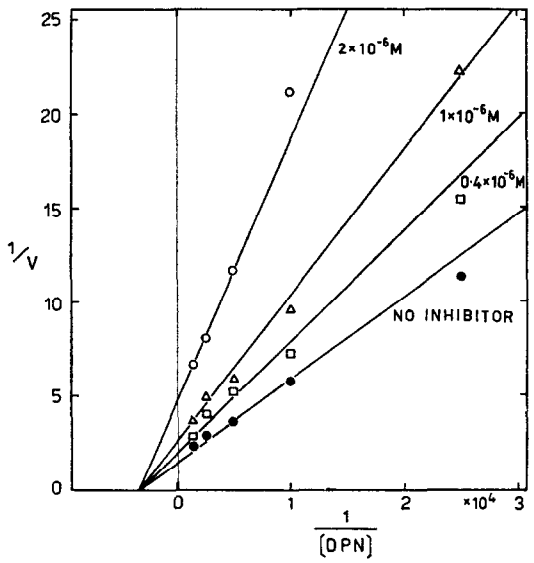


FIG. 6(A)

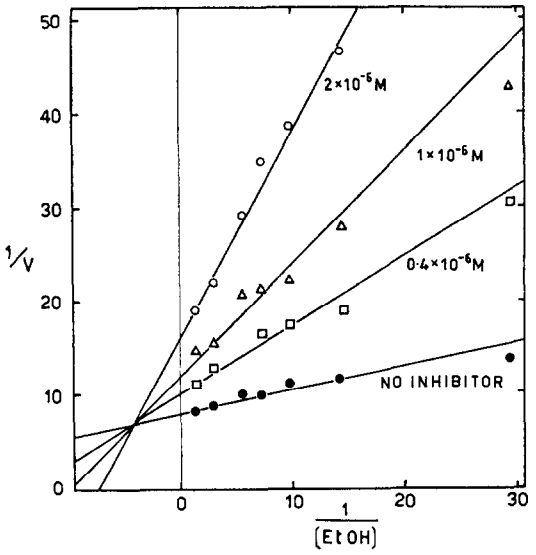


FIG. 6(B)

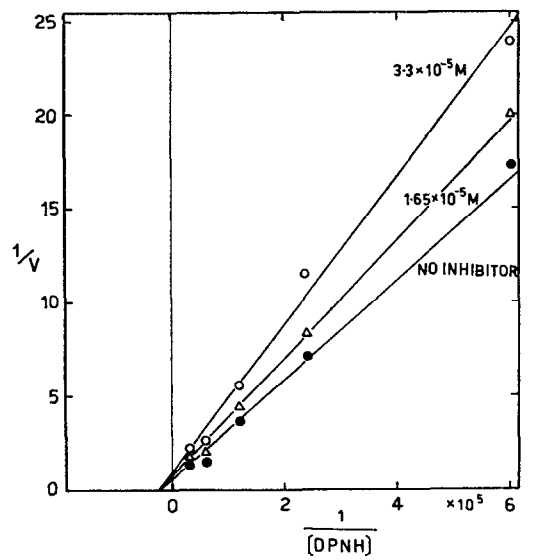


FIG. 6(C)

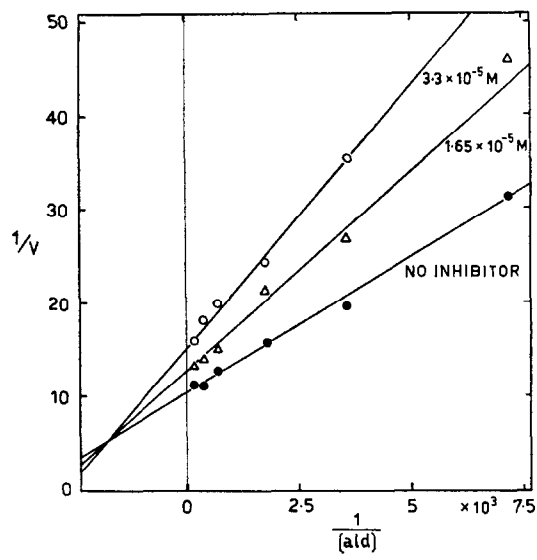


FIG. 6(D)

FIG. 6. Lineweaver-Burk plots for YADH:
(A and B) for the reaction ethanol → acetaldehyde, with respect to the coenzyme and the substrate concentrations;
(C and D) for the reaction acetaldehyde → ethanol, with respect to the coenzyme and the substrate concentrations.
Tests for reaction acetaldehyde → ethanol were performed with the fluorimetric technique.

neither pure competitive nor pure non-competitive inhibition occurs with respect to the substrate concentrations.

Zinc is essential for ADH activity.[18] By adding this metal into the incubation sample, the inhibitory effect of the disulfide is not modified, showing that the compound does not act by binding the metal.

Experiments on YLDH

As was observed in the experiments on YADH, the inhibitory effect of the disulfides on YLDH depended upon the time of incubation, therefore a standard time of 2 min was chosen for all the experiments.

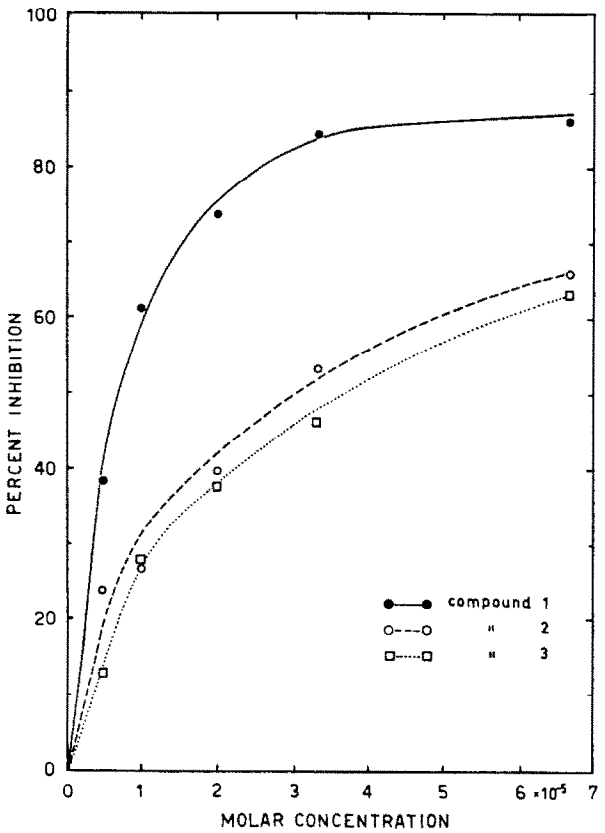


FIG. 7. Effect of the concentration of the disulfides on the activity of YLDH.

TABLE 2. REMOVAL OF THE INHIBITORY EFFECT OF THE DISULFIDE ON YLDH BY PART OF CYSTEINE

Inhibitor		Cysteine	% inhibition
disulfide 1	$3{\cdot}3 \times 10^{-5}$ M	none	79
disulfide 1	$3{\cdot}3 \times 10^{-5}$ M	$3{\cdot}3 \times 10^{-5}$ M	61
disulfide 1	$3{\cdot}3 \times 10^{-5}$ M	$6{\cdot}6 \times 10^{-5}$ M	33
disulfide 1	$3{\cdot}3 \times 10^{-5}$ M	$1{\cdot}0 \times 10^{-4}$ M	20

Conditions as described in Table 1.

Figure 7 shows the effect of increasing concentrations of some disulfides. The corresponding mercaptocompounds (Nos. 7, 8, 9) and the other derivatives without —S—S— bridges (Nos. 6, 10) were ineffective, and when the disulfide was previously treated with cysteine or other reducing agents, its inhibitory effect disappears (Table 2). Figure 8 shows the effect of pH on the inhibition by disulfides; it is seen that the maximum of inhibition is around pH 9·5.

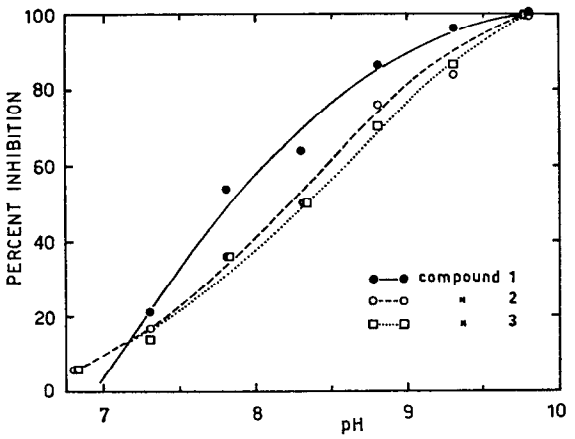


FIG. 8. Influence of pH on the inhibitory effect of the disulfides on the activity of YLDH. Disulfides were incubated with the enzyme at the selected pH, 2 min before the test carried out at pH 8·2. Final concentration of the disulfides 2×10^{-5} M.

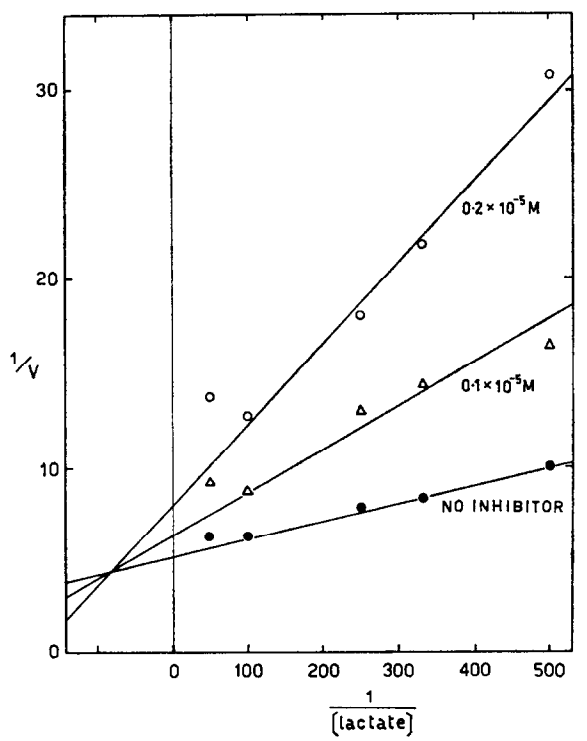


FIG. 9. Lineweaver-Burk plots for YLDH with respect to the substrate concentrations.

The study of the type of inhibition was carried out by the method of the Lineweaver–Burk plots with respect to the substrate concentrations only for the direct reaction. Figure 9 shows that the inhibition is of the mixed type.

Experiments on LADH and HLDH

When both LADH and HLDH were incubated with these disulfides and mercapto-compounds, they were not inhibited, either at neutral or alkaline pH. Only some inhibition (35 per cent) on HLDH was observed when this enzyme was incubated for longer times (more than 1 hr) at alkaline pH, room temperature and a very high concentration (1×10^{-4} M) of inhibitor (No. 1), that is, more than 20 times the concentration necessary to obtain the same effect on YLDH after 2 min incubation at low temperature. Also in the reverse reaction acetaldehyde → ethanol, LADH was not inhibited by part of these compounds. This very low sensitivity was utilized for the studies on the protection exerted by the disulfides on these enzymes submitted to ionizing radiations.[4]

Experiments on RNAase

This enzyme failed to be inactivated by these disulfides also after 1 hr incubation and at a concentration of the compound up to 1×10^{-4} M. On the contrary, RNAase was shown to be protected by the compounds against the effects of ionizing radiations.[4]

TABLE 3. EFFECT OF SOME SULFANILAMIDE DISULFIDES ON RED BLOOD CELLS CARBONIC ANHYDRASE

Inhibitor		% inhibition
disulfide 1	$2{\cdot}5 \times 10^{-5}$ M	88
disulfide 1	$2{\cdot}5 \times 10^{-6}$ M	60
disulfide 2	$1{\cdot}0 \times 10^{-5}$ M	91
disulfide 3	$1{\cdot}0 \times 10^{-4}$ M	72
disulfide 3	$2{\cdot}5 \times 10^{-5}$ M	56
disulfide 4	$2{\cdot}5 \times 10^{-4}$ M	40
disulfide 5	$2{\cdot}5 \times 10^{-4}$ M	37
sulfanilamide	$2{\cdot}5 \times 10^{-5}$ M	60

The enzyme was preincubated with the disulfides 2 min before the addition to the test solution. The concentrations are expressed as final concentrations in the test solution.

Experiments on carbonic anhydrase

Table 3 shows the effects of some disulfides on carbonic anhydrase, which is very sensitive to the sulfonamidic derivatives. All of the compounds with free sulfonamidic groups are inhibitors. Smaller but well evident effects are also shown by the disulfides having no sulfonamidic group or with this group completely substituted. The reduction of the —S—S— bridge by part of cysteine causes a slight but evident reduction of the inhibitory effect (Table 4).

Interaction between disulfides and thiol groups

The inhibition of the yeast dehydrogenases caused by the disulfides under study, and the ability of cysteine and GSH to prevent this inhibition, suggest that an interaction occurs between the disulfides and the —SH groups of both the enzyme and the reducing agents.

TABLE 4. EFFECT OF CYSTEINE ON THE INHIBITORY ACTION OF THE DISULFIDES ON CARBONIC ANHYDRASE

Inhibitor		Cysteine	% inhibition
disulfide 1	$5{\cdot}0 \times 10^{-6}$ M	none	84
disulfide 1	$5{\cdot}0 \times 10^{-6}$ M	$1{\cdot}0 \times 10^{-4}$ M	75
disulfide 1	$2{\cdot}5 \times 10^{-6}$ M	none	60
disulfide 1	$2{\cdot}5 \times 10^{-6}$ M	$1{\cdot}0 \times 10^{-4}$ M	43

Cysteine was added to the disulfide before the preincubation of this with the enzyme. Other conditions as described in Table 3.

The spectrum of the disulfide 1 (Fig. 10, curve A) changes when it is reduced. The position and height of the obtained bands are the same as that of the mercapto-compound (Fig. 10, curve B), either when the used reducing agent is the borohydride (reaction 1), the cysteine or GSH (reaction 2).

$$R—SS—R + 2H^+ + 2e^- \longrightarrow 2\ R—SH \tag{1}$$

$$R—SS—R + 2\ X—SH \longrightarrow 2\ R—SH + X—SS—X. \tag{2}$$

The reaction 2, as stated by Bersin and Steudel,[19] Eldjarn and Pihl[20] and others, occurs in two steps:

$$R—SS—R + X—SH \longrightarrow R—SH + R—SS—X \tag{2a}$$

$$R—SS—X + X—SH \longrightarrow R—SH + X—SS—X; \tag{2b}$$

for the aromatic disulfides another step possibly exists

$$2\ R—SS—X \longrightarrow R—SS—R + X—SS—X \tag{2c}$$

because the aromatic disulfides have a tendency toward a symmetrical structure.[21]

When the disulfide is treated with YADH or YLDH the spectrum of the mercapto-compound is also obtained, but the bands are half in height (Fig. 10, curve C), showing that only reaction (2a) occurs. This reaction alone could be sufficient to explain the inhibitory effect of the disulfide, but it might be possible that the reaction still goes further according to the reaction (2b), because with a large excess of the enzyme the bands have a greater height than those obtained when only reaction (2a) occurs. The complex En—SS—R is greatly stable, and cannot be split by addition of cysteine or

GSH, even if the complex is dialized to obtain a solution completely free from traces of disulfide. Similar spectral changes are observed by treating the other disulfides with reducing agents or YADH or YLDH.

The band differences between the disulfides and the corresponding mercapto-compounds allowed to titrate the disulfides with a sulfhydril compound. The titration

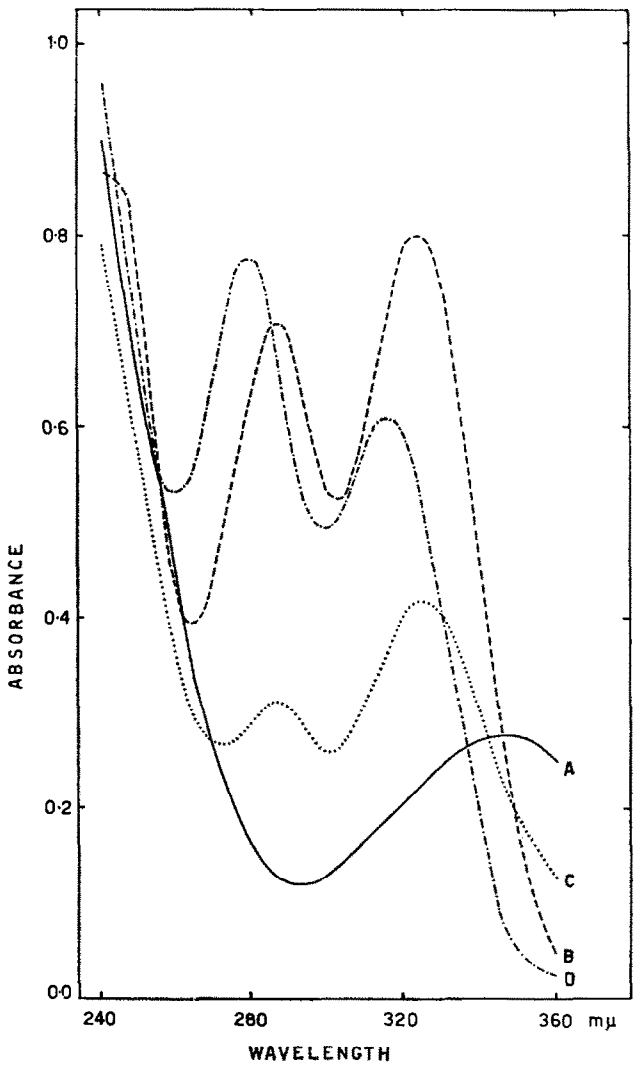


FIG. 10. Spectra of the disulfide 1 after various treatments:
Curve A: disulfide (4×10^{-5} M) untreated;
Curve B: disulfide reduced with sodium borohydride (or cysteine);
Curve C: disulfide treated with YADH;
Curve D: disulfide in alkaline solution (pH 13·0).

curve of a disulfide with a thiol compound X—SH followed by the appearance of the band of the mercaptocompound at 323 mμ in the case of compound 1 (Fig. 11) shows that an equilibrium occurs, and a greater amount of X—SH is necessary than that calculated to reduce a determined amount of disulfide. The pH of the medium influences this reaction; in fact, at alkaline pH the titration occurs more quickly (Fig. 11, curve B).

It is noteworthy that pH influences also the structure of these disulfides, as may be seen from the change in the spectrum. Curve D in Fig. 10 shows the bands of the di-

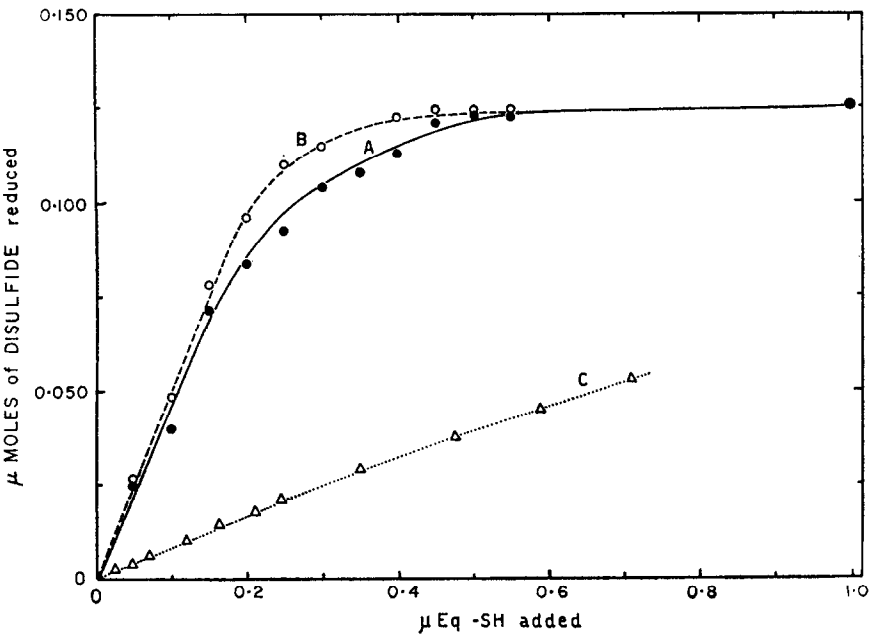


FIG. 11. Titration curves of disulfide 1 with GSH and YADH:
Curve A: GSH at pH 7·0;
Curve B: GSH at pH 8·5;
Curve C: YADH at pH 7·0.
The reduction of the disulfide was calculated by the increase of the band at 323 mμ.
Disulfide in the Beckman cell was 0·125 μmole. Free thiol groups in YADH were calculated with *p*-chloromercuribenzoate.

sulfide in solution at pH 13·0. There appear two bands similar to those of the mercaptocompound, but slightly shifted towards the lower wavelengths; the height of the band at 316 mμ is only threequarters of the band at 323 mμ of the mercaptocompound corresponding to the disulfide No. 1. The rate of this reaction is dependent upon the pH. It begins between pH 9·0 and 9·5, according to the type of disulfide. It is likely that the reaction (3)

$$R—SS—R + 4\ OH^- \longrightarrow 3\ RS^- + R—SO_2^- + 2\ H_2O \qquad (3)$$

occurs. This reaction, described by Schiller and Otto,[15] when aliphatic disulfides are involved, requires high concentrations of alkali and a high temperature. With these disulfides, if the reaction really occurs, these strong conditions are not necessary. It has been seen that a temperature of 2–4° and a pH slightly alkaline are sufficient.

The titration of the disulfides can be carried out also with the enzymes which are inhibited by them (YADH and YLDH) and other proteins (like beef serum albumin, apoferritin). As it may be seen from Fig. 11, curve C, the reduction of the disulfide by part of YADH requires a much greater amount of enzyme than that calculated by the number of —SH groups, as determined with *p*-chloromercuribenzoate, showing that not all of the thiol groups in the protein have the same reactivity toward these disulfides.

No changes in the spectrum of these disulfides were observed when these compounds were treated with LADH or HLDH, which are not inhibited by them. No modifications of the spectra were obtained, also, by treating the disulfides with carbonic anhydrase, showing that no complex enzyme-disulfide is formed, or that it is not of the type En—SS—R.

DISCUSSION

The results of the experiments show: (i) there is a great difference in the sensitivity towards the sulfanilamide disulfides between the yeast and animal dehydrogenases; (ii) the inhibition is of the non-competitive type or of the mixed type with respect to the coenzyme or the substrate concentrations respectively; (iii) pH is an important factor for the inhibiting effect of the compounds; (iv) the inhibition is connected with the reaction between disulfides and free —SH groups of the enzymes.

The difference in the sensitivity towards the reactants of the thiol groups shown by the dehydrogenases has already been described: for instance, while HLDH is insensitive to many —SH inhibitors, with the exception of *p*-chloromercuribenzoate (Neilands[22]), YLDH is inhibited by several thiol blocking agents;[11] iodoacetate inhibits YADH and not LADH.[9] The different behaviour towards the studied disulfides of the yeast and animal alcohol and lactic dehydrogenases can be explained by the latter, by considering the great structural differences. In fact, YLDH is a flavocytochrome b_2 not requiring DPN as cofactor for the electron transfer from the substrate to the acceptor. HLDH, which has no heme groups in the molecule, requires, instead, the DPN as coenzyme for the electron transport. On the contrary, less great structural differences exist between the YADH and LADH. It would appear that the behaviour of the two enzymes may be consistent with a finer structural, chemical and physicochemical differences. This view is supported by the fact that the two enzymes do not bind the DPNH in the same way.[23]

The experiments on YADH and YLDH show that by increasing either the concentration of the disulfides or the incubation time a complete inhibition is never reached. This behaviour is characteristic of inhibition of the non-competitive type. The study of the type of inhibition according to the method of the Lineweaver–Burk plots confirmed that, with respect to the coenzyme, in the case of YADH, the inhibition is of the non-competitive type (Fig. 6 A and C), showing that the disulfide binds itself at a site sufficiently far away from the coenzyme-binding site, so that it has no influence on the binding of the coenzyme on the protein. The mechanism of inhibition with respect to the substrate is more complex. The Lineweaver–Burk plots (Fig. 6 B and D)

show that a mixed type of inhibition occurs, which, according to Dixon and Webb[24] may be determined by a combination of a partially competitive type and a non-competitive type; there is an intermediate case between a competitive and a non-competitive inhibition, as described in general by Friedenwald and Maengwin-Davies.[25] At the same time the enzyme binds the substrate and the inhibitor, which affects the affinity of the enzyme for the substrate and prevents the breakage of the complex enzyme-substrate. The inability of cysteine and other similar reducing agents to split the complex En—SS—R, seems to show that a II-a is combined with a I-b type of inhibition according to the classification of Dixon and Webb.[24] The mechanism of the mixed type of inhibition, with respect to the substrate, presented by the disulfides on YLDH (Fig. 9) seems to be of a similar nature.

With respect to the other enzymes considered, the non-inhibitory effect of the disulfides on RNAase seems to depend upon the lack of free —SH groups in the molecule of this enzyme. Carbonic anhydrase shows a great sensitivity to the studied compounds. The inhibition is dependent to a large measure upon the presence in the compounds of two sulfonamidic groups, which are notoriously inhibitors of this enzyme. However, the inhibition seems to depend also upon the —S—S— bridges, since the compound with no sulfonamidic group (No. 5) and that with this group blocked (No. 4) give a measureable inhibition, and by reducing the —S—S— bridge (Table 4) the inhibition is decreased. It is known that carbonic anhydrase contains some sulfur, but free thiol groups from cysteine were found only in the plant enzyme,[26] so that the inhibition probably cannot depend upon the formation of a mixed disulfide En—SS—R. The failure to obtain the spectrum of the reduced compound by treating the disulfide with carbonic anhydrase confirms this hypothesis. It seems also possible that somehow the disulfide bridge may bind itself to a site influencing the active group of the enzyme, preventing or impairing its activity.

For both YADH and YLDH the inhibition may occur also at a neutral pH, but the maximum is around pH 9·0–10·0. It would seem that the mechanism consists in the change of the structure of the disulfide operated by alkali according to reaction 3, combined with the ionization of the thiol groups in the enzyme. The increase of the reducing power of GSH and cysteine on the disulfides depending upon the increase of pH, is further evidence of this fact. The ineffectiveness of the mercaptocompounds in inhibiting the enzymes confirms that the inhibition occurs only when the thiol groups of the enzymes react with the oxidizing disulfide, and not through other mechanisms.

The reaction between aromatic disulfides and sulfhydril groups, either of simple compounds (cysteine, GSH) or bound to proteins, were described by several authors.[1, 2, 27] The present experiments confirm the general property of the aromatic disulfides to bind at the free —SH groups and to give some changes in their spectra. It is an especially interesting fact that, while the reaction with sulfhydril compounds (cysteine, GSH) occurs complete at relatively low ratio between thiol and disulfide, the reaction with the enzyme requires a large amount of free enzymatic —SH groups. This confirms that the thiol groups in the enzymes are not equally reactive and titratable. As far as the reaction between sulfhydril groups and these disulfides is concerned, it appears that, while with GSH or cysteine reaction (2) occurs complete, essentially only reaction (2a) occurs with YADH and YLDH, showing the formation of a mixed disulfide En—SS—R. As is the case of the reaction between ovoalbumin and an aromatic disulfide, described by Klotz *et al.*,[27] it is probable that the stability of this mixed

disulfide depends upon some not well-determined stabilizing interactions of the organic residue (R) with the protein. This would be confirmed also by the fact that the binding between enzyme and disulfide is stable, and reducing agents (GSH) are unable to restore the activity when the unreacting disulfide is carried out by dialysis.

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