

CHAPTER 76

The Effect of Selected Industrial Biocides on Lactate Metabolism in *Desulfovibrio desulfuricans*

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Two commercially available biocides based on the isothiazolone structure and 2-mercaptobenzothiazole were the only ones effective from eight tested which inhibited lactate metabolism with whole cells, lysed cells, and partially purified lactate dehydrogenase. Only isothiazolones which had chlorine at position 4 and/or 5 or with no substituents at position 2 inhibited partially purified lactate dehydrogenase. The most effective biocide, 2-mercaptobenzothiazole, possibly inhibits lactate dehydrogenase additionally by complexing an essential divalent cation.

INTRODUCTION

Attempts to control environmentally damaging microorganisms such as members of the anaerobic sulfate-reducing group are frequently empirical, often unpredictable, and always a challenge. Postgate (1979) in his monograph gives some of the reasons. They include variability in growth requirements among members of the group as well as the fulfillment of some stringent nutritional needs, e.g., a negative redox below -100 mV, both for their cultivation and recovery.

A rational selection of a biocide should include some knowledge of its mode of action. Armed with this information, there is a possibility for the development of compounds with greater activity and specificity. One approach to mode-of-action studies is the search for a sensitive bacterial site in species proven susceptible to a given biocide. This site should play a major role in the metabolism of that species.

The enzyme system that metabolizes lactate in *Desulfovibrio desulfuricans* is important for energy production in the cell. The oxidation of lactate to pyruvate has not been well studied due to instability of lactate dehydrogenase (LDH), but the metabolism of pyruvate to acetate and the enzymes involved in sulfate reduction are well known and characterized (Siegal 1975). Czechowski and Rossmore (1980) were able to stabilize LDH and study it. This enzyme is membrane-bound but is solubilized with detergents. The solubilized enzyme is specific for d(-1) lactate, is sensitive to sodium azide, sodium cyanide, and EDTA, which suggests the presence of an iron-containing moiety.

A number of biocides with implied or stated activity against *D. desulfuricans* by the chemical manufacturer were evaluated for their

effect on lactate metabolism. This study had a dual purpose not only to uncover some clue to mechanism of biocide action but also concomitantly to reveal some intimacy of enzyme function hitherto unknown.

MATERIALS AND METHODS

Desulfovibrio desulfuricans (ATCC 7757) was grown at 30 C on Postgate's medium (Postgate 1966), modified by using 0.0001% instead of 0.01% FeSO_4 /liter.

Preparation of whole cells - Cells were harvested between 48 and 72 h of incubation and were washed twice in 0.1 M sodium phosphate buffer (pH 7.5) and resuspended in 15 ml of the same buffer.

Preparation of lysed cells - Cells (1.5 g) were washed, suspended in 0.1 M sodium phosphate buffer (pH 7.5) with 0.1 mM mercaptoethanol, and disrupted in a French pressure cell (18,000 to 24,000 psi). The lysed cells were then centrifuged at 8,000 rpm (7,710 $\times g$) in a SR25 rotor (Servall Type RC-2) for 10 min to remove whole cells. The supernatant was used for the biocide studies.

Preparation of partially purified lactate dehydrogenase - Ammonium sulfate (1.7 g/10 ml) was added to supernatant of lysed cells to precipitate the membrane fraction. This suspension was centrifuged at 16,000 rpm for 30 min. The pellet was resuspended in 0.1 M sodium phosphate buffer (pH 7.5) with 1 mM mercaptoethanol, and sodium cholate was added to give a final detergent concentration of 4%. This was mixed for 20 h and then centrifuged at 16,000 rpm (30,900 $\times g$) for 20 min. The enzyme in the supernatant then was precipitated between 30% and 50% ammonium sulfate concentration, and resuspended in 10 ml of buffer to which was added 10 ml of cold (4 C) chloroform. This was mixed for 10 min and centrifuged at 16,000 rpm for 30 min. The upper, slightly turbid layer was LDH activity and was used for biocide studies.

Assay procedure - Lactate reduction was determined by the method of Thunberg (Umbreit et al. 1957). The assay mixture consisted of 4 ml of 3% sodium lactate in 0.1 M sodium phosphate buffer (pH 7.5), 0.4 ml of 0.01% methylene blue, 0.2 ml of bacteria or bacterial extract, and the appropriate concentration of biocide. The biocides and their concentration in the assay mixtures are listed in Tables 2 and 3. The pH of the assay mixture never varied by more than ± 0.3 units which did not affect lactate metabolism. Lactate metabolism is reported by using methylene blue as electron acceptor and monitoring reduction by absorbance decrease/min at 660 nm in a Cary 118 spectrophotometer. An experiment was run without lactate and with lactate but with denatured bacterial sample to determine whether the bacterial sample may chemically reduce methylene blue. The results were negative. The percent inhibition of biocide is calculated as follows:

$$\frac{(\text{Methylene blue reduction without biocide}) - (\text{Methylene blue reduction with biocide})}{\text{Methylene blue reduction without biocide}} \times 100$$

Methylene blue reduction without biocide

The isothiazolone compounds were provided by Rohm and Haas Co. Other biocides used and the company from which each was received are shown in Table 1. Sodium lactate (Lot 743569) was purchased from Fisher Laboratory Chemical. Variation in the quality of sodium lactate was found in the product from different manufacturers and among the different lot numbers of each manufacturer. This gave different rates for lactate metabolism. This also was mentioned by LeGall and Forget (1978).

RESULTS AND DISCUSSION

The first experiment evaluated the effects of a variety of chemical types, all available commercially, on lactate metabolism. Results with both whole and lysed cells (Table 2) shows that only the isothiazolone derivatives affected the lactate system in preparations from *D. desulfuricans*. However, the benzisothiazolin compound appeared to be less effective on lysed cells.

The next series of experiments involved primarily isothiazolone derivatives similar to the commercial product which was initially most successful in the first trial. Table 3 shows the results of these compounds on lysed cell preparations. All of the compounds examined had some inhibitory effect on lactate metabolism, as measured by methylene blue reduction. Two other compounds, 2-mercaptobenzothiazole and a substituted thiazole, both of which are used as industrial biocides, also were tested for inhibitory action.

In the final experiment (Table 4), the isothiazolones and the mercaptobenzothiazole were evaluated against a partially purified LDH with most interesting results. Only the isothiazolone with chlorine in the 4 or 5 position, or those with no substituents in the 2 position, showed any degree of inhibition of methylene blue reduction. Moreover, the two compounds constituting the commercial mixture (Table 1), 2-methyl and the 5-chloro-2-methyl, were decidedly more effective when mixed than when evaluated alone. This is especially true at the lowest level of the commercial mixture, which is the recommended dose by the manufacturer for uses involving control of *D. desulfuricans*. The effectiveness of the 2-mercaptobenzothiazole is outstanding. The possibility exists that this compound, with a free sulfhydryl group, acts by complexing some essential divalent cation (Albert et al. 1947), such as iron, a suspected essential part of the LDH complex.

The inhibition of lactate metabolism by both mercaptobenzothiazole and the thiazolyl derivative (Tables 3 and 4) suggested the possibility that these compounds and those based on isothiazolone may have been competing with the thiazole portion of thiamine. A mode of action for the thiazolyl derivative has been published (Allen and Gottlieb 1970) showing that the compound inhibited a wide variety of oxydases from fungal mycochondria. Notwithstanding, an experiment was carried out to test the value of thiamine hydrochloride as an antagonist of isothiazolone inhibition. The commercial isothiazolone mixture (Table 1 and 2-mercaptobenzothiazole, with equivalent and 10-fold amounts of thiamine hydrochloride, proved unrewarding and not only was there no prevention of inhibitory effect from the biocides, thiamine itself proved inhibitory (about 25%).

How specifically the 4-isothiazolin-3-one compounds affect LDH function has not been revealed. These have been short-term experiments in which measurements of methylene blue reduction were made within

TABLE 1. Commercial compounds with reported activity against *Desulfovibrio desulfuricans*

Chemical Name	Trade Name	Recommended Dose	Company
Hexahydro-1,3,5-triethyl- s-triazine (95%)	Vancide TH	500-1,000 ppm	R. T. Vanderbilt
Hexahydro-1,3,5-tris (2-hydroxy- ethyl)-s-triazine (78%)	Grotan	1,500 ppm	Lehn & Fink
4-(2-nitrobutyl)-morpholine (70%) 4,4'-(2-ethyl-2-nitroethylene)- dimorpholine (20%)	Bioban P-1487	100-3,000 ppm	IMC Chemical Group
6-acetoxy-2, 4-dimethyl-m- dioxane (100%)	Dioxane (Givguard DXN)	200-500 ppm	Givaudan
1,2-benzisothiazolin-3-one (30-35%)	Proxel CRL	100-600 ppm	ICI United States
5-chloro-2-methyl-4 isothiazolin- 3-one (8.6%) 2-methyl-4-isothiazolin-3-one (2.6%)	Kathon 886 MW	100 ppm	Rohm and Haas
Sodium 2-pyridinethiol-n-oxide powder (90%); aqueous solution (40%)	Sodium Omadine	25-115 ppm	Olin
Tertiary butylamine 2-pyridine- thiol-n-oxide		ca 50 ppm	Olin
4-4 dimethylloxazolidine 3,4,4-trimethyl oxazolidine	Amine CS 1135	1,000 ppm	IMC Chemical Group
2-mercapto benzothiazole	Nacap		R. T. Vanderbilt
Thiabenzazole 2-(4'-thiazolyl) benzimidazole	Metasol TK-100	1/2 lb-2 lb per 100 gal	Merck

TABLE 2. Effect of selected industrial biocides on lactate metabolism

Chemical Name	% Inhibition of Methylene Blue Reduction					
	10 ⁻² M		10 ⁻³ M		10 ⁻⁴ M	
	W ^a	L ^b	W	L	W	L
Hexahydro-1,3,5-triethyl-s-triazine	15	0	0	0	0	0
4-(2-nitrobutyl)-morpholine; 4,4'-(2-ethyl-2-nitrotrimethylene)-dimorpholine	15	0	0	0	-	-
6-acetoxy-2, 4-dimethyl-m-dioxane	0	0	0	0	-	-
Hexahydro-1,3,5-tris (2-hydroxyethyl)-s-triazine	15	0	0	0	0	-
1,2-benzisothiazolin-3-one	38	19.4	19	8	10	-
5-chloro-2-methyl-4 isothiazolin-3-one; 2-methyl-4-isothiazolin-3-one	70	64	37.5	32	20	16
Sodium 2-pyridinethiol-n-oxide	12.5	0	0	0	-	-
Tertiary butylamine 2-pyridine-thiol-n-oxide	0	0	0	0	-	-
4-4 dimethylloxazolidine 3,4,4-trimethyl oxazolidine	6	0	0	0	-	-

^aWhole cells.^bLysed cells.

TABLE 3. The effect of isothiazolones on lactate metabolism in lysed cells

Compound	10^{-3} M	ppm	% Inhibition of Methylene Blue Reduction
2-mercapto benzothiazole	0.03	1.1	0
	0.3	11	32
	2.8	109	47
	5.6	218	83
Thiabendazole, 2-(4'-thiazolyl) benzimidazole	0.3	46.5	0
	1.3	233	0
	2.6	465	25
4-Isothiazolin-3-Ones			
4-isothiazolin-3-one	1.5	109	0
	3.0	217	33
2-methyl	1.0	109	20
	2.0	217	27.5
2-(n-butyl)	0.9	64	0
	2.9	215	0
	8.9	652	28
2-(n-octyl)	0.2	21	4
	1.1	105	8
	2.1	209	27
2-benzyl	1.3	232	0
	2.6	464	14
2-cyclohexyl	0.7	109	17
	1.4	217	12
	2.7	434	36
4-5 dichloro-2-methyl	0.6	109	15
	1.3	217	11
	2.6	434	30
4-5 dichloro-2-(n-octyl)	0.4	109	5
	0.8	217	23
4-5 dichloro-2-cyclohexyl	0.5	109	0
	1.0	217	23
5-chloro-2-methyl	0.37	43.4	0
	1.1	130	20
	3.7	434	29

TABLE 4. The effect of isothiazolones on partially purified LDH

Compound	10^{-3} M	ppm	% Inhibition of Methylene Blue Reduction
2-mercapto benzothiazole	0.3	11	0
	2.8	109	39
	5.6	218	61
4-Isothiazolin-3-Ones			
4-isothiazolin-3-one	7.6	551	9
2-methyl ^(A)	2.0	217	0
2-(n-butyl)	13.4	980	0
2-(n-octyl)	2.1	209	0
2-benzyl	2.6	464	0
2-cyclohexyl	2.7	434	0
	6.0	980	0
4-5 dichloro-2-methyl	0.6	109	0
	2.6	434	8
	5.7	980	13
5-chloro-2-methyl ^(B)	3.7	434	10
	9.1	1,085	22
1,2 benzoisothiazolin	1	91	0
	10	913	33
(A) + (B) commercial mix	1	125	18
	10	1,250	35

minutes after the mixing of the biocides with the enzyme preparations. It is quite possible that there would be more substantial inhibition with longer contact time. Nevertheless, even within the short period of the reaction time, a definite metabolic site has been established for an important group of industrial antimicrobial agents.

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