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Inhibition of Sulfate Respiration by 1,8-Dihydroxyanthraquinone and Other Anthraquinone Derivatives

F. B. COOLING III, C. L. MALONEY, E. NAGEL, J. TABINOWSKI, and J. M. ODOM*

Central Research & Development Department, E. I. Du Pont De Nemours & Co., Wilmington, Delaware 19880

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Derivatives of 9,10-anthracenedione, or anthraquinone, were shown to inhibit respiratory sulfate reduction by pure cultures of sulfate-reducing bacteria, as well as by crude enrichment cultures. Structure-activity studies showed that an increasing degree of substitution of the anthraquinone nucleus resulted in increasing 50% inhibition (I_{50}) values for sulfate respiration. Addition of charged ring substituents also resulted in an increase in the I_{50} concentration. Experiments carried out with 1,8-dihydroxyanthraquinone demonstrated inhibition of hydrogen-dependent sulfate respiration but not hydrogen-dependent sulfite or thiosulfate respiration. Addition of pyruvate resulted in stimulation of sulfate-dependent hydrogen oxidation in the presence of the anthraquinone. These observations, together with a direct demonstration of uncoupling in French press vesicle preparations, suggest that the underlying mechanism of inhibition is uncoupling of ATP synthesis from electron transfer reactions. The low I_{50} values for inhibition (0.5 to 10 μM) and the relatively low general toxicity of anthraquinones suggest that these compounds may be useful for inhibition of sulfide generation in situations which are incompatible with the use of broadly toxic biocides.

Two quite distinct microbial processes are typically responsible for the production of hydrogen sulfide in nature and the industrial environment. The first of these is sulfate respiration, which is catalyzed by both eubacterial and archaebacterial representatives of the sulfate-reducing bacteria. Respiratory sulfate reduction may be the dominant respiratory process in anaerobic marine or even freshwater environments (20, 21). The second source of hydrogen sulfide derives from the release of sulfide from sulfur-containing organic compounds as a result of degradative processes carried out by a variety of physiological types. The latter process is a much smaller contributor to sulfide generation, in most environments, than sulfate respiration. These natural activities are important aspects of the microbial sulfur cycle and are integral to the mineralization of organic matter in many anaerobic ecosystems.

The occurrence of hydrogen sulfide in industrial situations is frequently found to be due to the action of sulfate-reducing bacteria. The problems caused by the activities of these organisms within industrial processes are well known (7). Sulfide-related corrosion of concrete or steel in wastewater treatment and corrosion and oil souring in secondary oil recovery are known to result in significant costs to these industries (12). Currently, control of sulfide generation in industrial situations is achieved with broad-spectrum biocides such as glutaraldehyde, chlorine, or hypochlorite, which are broadly chemically reactive, generally hazardous to personnel, and inconsistent with environmental trends and regulations. Furthermore, chemically reactive biocides may be rendered ineffective in many environments, necessitating high dosage levels and repetitive applications.

Respiratory sulfate reduction is mechanistically unique in the microbial world, and therefore more specific inhibition of this process may be feasible (4, 13, 15, 16). The key enzymes may be enzymologically distinct from analogous proteins in other species. Examples of these include the ATP sulfurylase, bisulfite reductase, adenosine-5′-phosphosulfate (APS) reductase, and c_3 cytochromes (16). Another distinctive feature of sulfate respiration is the stoichiometric ATP requirement for sulfate activation (15). Classically, the sulfate activation step, catalyzed by ATP sulfurylase, has been a target for inhibition by the sulfate analog molybdate, which is purported to be a specific inhibitor of sulfate reduction (14). In this example, sulfate respiration is particularly sensitive to formation of unstable molybdo-phospho anhydrides which result in depletion of ATP levels. Thus, sulfate respiration may be particularly sensitive to cellular energy charge.

The present report demonstrates that a group of structurally related derivatives of 9,10-anthracenedione, generically termed anthraquinones, inhibit the process of sulfate respiration at micromolar concentrations. Exemplary results have been obtained with 1,8-dihydroxyanthraquinone. The data presented suggest that the inhibition is due to uncoupling of electron transfer from ATP synthesis, and it is proposed that uncoupling is due to the redox-active nature of the anthraquinone. The uncoupling of ATP synthesis ultimately results in insufficient energy charge for further sulfate activation and sulfate reduction. A wide variety of anthraquinone derivatives are known, and these compounds are known to exhibit multitudinous biological effects, but this is the first report showing high sensitivity of a physiological process broadly associated with a large microbial group. The anthraquinones are generally nontoxic to plants and animals and therefore may represent relatively safe compounds for the environmental inhibition of sulfate respiration (11).

MATERIALS AND METHODS

Sources of bacteria and enrichments. *Desulfovibrio gigas* ATCC 19364, *D. desulfuricans* G100A and ATCC 27774, *D. salexigens* ATCC 14822, and *D. vulgaris* ATCC 29579 were all obtained from the laboratory of H. D. Peck (Department of Biochemistry, University of Georgia, Athens). *Thiobacillus denitrificans* ATCC 23642 and *Escherichia coli* ATCC 11775 were obtained from the American Type Culture Collection, Rockville, Md.

Sulfate-reducing enrichments (see Table 3) were prepared by collecting sediment samples at the surface or at a depth of approximately 7 cm from the surface at each designated site. Samples were classified accordingly as originating from an aerobic or surface zone (A) or a deeper anaerobic zone (AN or duplicate AN2). Four freshwater sites were sampled (VS, Valley Stream, N.Y.; VG, Valley

* Corresponding author. Mailing address: DuPont/GBC, Glasgow 300, Route 896, Newark, DE 19714. Phone: (302) 451-9947. Fax: (302) 451-9138. Electronic mail address: odomjm@esvax.dnet.dupont.com.

Garden State Park, Del.; WCC, White Clay Creek watershed, Avondale, Pa.; LUMS, Lums Pond State Park, Newark, Del.). One saltmarsh site (at Lewes, Del.) was sampled. Two different wastewater treatment facilities were sampled (WD, a Wilmington, Del., wastewater treatment facility; P, a Philadelphia wastewater treatment facility). Within the Philadelphia facility, samples were taken from various parts of the wastewater treatment and sludge disposal process and designated as follows: POC, old compost; PNC, new compost; P, primary settling tank; PS, primary settling tank sludge; PPD, postdigestor sludge; PF, particulate floated material; PSC, sludge cake.

The samples were mixed with BTZ-3 lactate-sulfate medium (10:1 medium-soil ratio) for a total volume of 20 ml in a 130-ml serum-stoppered Wheaton bottle. Modified BTZ-3 medium is a basal mineral salts medium without yeast extract, electron donors, or acceptors added (13). The anthraquinone was added as an acetone solution (4.1 mM) to the enrichments, and acetone was added to control enrichments.

Cultivation of bacteria. Pure cultures of sulfate-reducing bacteria were grown on BTZ-3 lactate-sulfate medium (13). *E. coli* was grown on modified BTZ-3 medium without sodium sulfate or sodium lactate but amended with 20 mM sodium succinate for aerobic growth. For anaerobic growth, *E. coli* was grown on BTZ-3 medium with 30 mM sodium fumarate under H_2-CO_2 (80/20, vol/vol). *T. denitrificans* was grown on BTZ-3 mineral medium amended with 20 mM sodium thiosulfate and 20 mM sodium nitrate. For studies of uncoupling effects or to investigate the effect of different anthraquinones (see below), *D. gigas* was grown at 30°C and harvested during the early stationary phase or after approximately 30 h of growth.

Anthraquinone activity assay for inhibition of sulfide production. The effectiveness of a particular anthraquinone for inhibition of sulfide production was determined by incubating the compound to be tested with a resting cell suspension of *D. gigas* for 3 h and then measuring the total sulfide formed in the incubation. For this purpose, a 500-ml lactate-sulfate culture of *D. gigas* was grown for 30 h. The cells were harvested by centrifugation at 8,000 rpm (Du Pont Sorvall GSA rotor) for 20 min, the pellet was resuspended in an excess of 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (pH 7; Sigma Chemical Co., St. Louis, Mo.)–2.5 mM sodium lactate–2.5 mM sodium sulfate (reaction mixture), and the centrifugation was repeated. The washed pellet was finally resuspended in 4 ml of the reaction mixture. For each assay, 200 μl of this cell suspension and 1 ml of the reaction mixture were combined in a 5-ml capacity Vacutainer tube (Becton-Dickinson and Company, Rutherford, N.J.) and gassed out with hydrogen for 20 min. The anthraquinone was then added by syringe injection of a 1,000-mg/liter stock solution (or acetone dilution) in acetone. The higher concentrations of some anthraquinones may exceed the actual solubility of the anthraquinone in water. After a 3-h incubation at 30°C, the reaction was stopped by addition of 100 μl of 1 N HCl and the acidified mixture was allowed to stand for 20 min. The total sulfide in the gas phase was then determined by the sulfide microdetermination method of Siegal (19). After removing an aliquot of gas and injecting the gas into 1.8 ml of pH 8 water, 200 μl of diphenylamine HCl-ferric chloride reagent was immediately injected. Color formation was allowed to proceed for 20 min, and the color due to methylene blue formation was read at 670 nm.

Respirometric assays of whole cell suspensions. Respiration by whole cells of sulfate-reducing bacteria was measured by determination of electron acceptor-dependent hydrogen uptake rates. The measurements were performed in Warburg flasks containing a reaction mixture composed of 200 μl of washed whole cells (approximately 8 mg of cell protein), 100 μl of 1 M potassium phosphate buffer (pH 7), 250 μl of 100 mM sodium sulfate, 1 to 20 μl of an anthraquinone derivative in acetone, and distilled water to 1 ml. The flask was purged for 15 min with 100% hydrogen and then closed. The reaction was initiated by tipping in the electron acceptor solution from the side arm. The incubation temperature was 30°C. The respirometer was a Gilson Submarine Respirometer (Gilson Medical Equipment, Manitowoc, Wis.).

Respirometric assays with membrane preparations and determination of phosphorylation efficiencies. French press vesicles of lactate-fumarate-grown *D. gigas* were prepared from a 1-liter culture of late-log-phase cells growing on the standard BTZ-3 lactate-sulfate medium adjusted to 30 mM sodium fumarate with 1 M sodium fumarate (pH 7). The cells were collected by centrifugation as already described, washed once with an excess of 50 mM HEPES buffer (pH 7), and then resuspended in 2 ml of the same buffer. Cell lysis was achieved with a single passage of a 2-ml suspension through a French pressure cell (SLM Aminco, Rochester, N.Y.) at 20,000 lb/in^2. The lysed cell suspension was then briefly centrifuged at 15,000 rpm for 4 min in an Eppendorf tabletop microcentrifuge to remove unbroken cells. The supernatant was then centrifuged at 108,000 × *g* for 45 min to sediment the cell membranes. The pellet was resuspended in an excess of cold buffer and recentrifuged. The washed pellet was resuspended in 1 ml of 50 mM HEPES buffer (pH 7).

Phosphorylation efficiency of the cell membrane fraction was determined by measuring hydrogen consumption and ATP production coupled to fumarate reduction. The reactions were performed with Warburg flasks with a reaction mixture containing 200 μl of 1 M HEPES buffer (pH 7), 200 μl of 100 mM ADP, 50 μl of 100 mM magnesium chloride, 50 μl of 1 M monobasic potassium phosphate, 30 μl of 1 M potassium fumarate (pH 7), and distilled water to 2 ml. The flasks were gassed for 15 min before the reaction was started by tipping in the potassium fumarate. Hydrogen was measured by standard manometric methods in a Gilson Submarine Respirometer.

Reactions were stopped by adding 200 μl of 20% trichloroacetic acid, and the mixture was neutralized by adding 1 N NaOH. For determination of ATP, Sigma Luciferin-Luciferase reagent was used. The neutralized sample was diluted 100-fold in Sigma glycine buffer, and 20 μl of the dilution was added to 280 μl of glycine buffer. A separate vial contained 100 μl of the Luciferin-Luciferase mixture. The sample (300 μl) was added to this vial, and light emission was read immediately with an Integrating Photomultiplier Model 3000 (SAI Technologies, San Diego, Calif.)

Sources of chemicals. The Luciferin-Luciferase kit and 2-heptyl-4-hydroxyquinoline-*N*-oxide were obtained from Sigma. The dye-binding assay used for protein determination was obtained from Bio-Rad Laboratories, Richmond, Calif. 9,10-Anthraquinone and all derivatives of this compound (except those mentioned below) were obtained from Aldrich Chemical Co., Milwaukee, Wis. Anthraquinone-1-sulfonic acid was obtained from Eastman Kodak Co., Rochester, N.Y. Anthraquinone-2-chloro-3-carboxylic acid and 1,2,7-trihydroxyanthraquinone were obtained from Pfaltz & Bauer Inc., Waterbury, Conn.

TABLE 1. Concentrations of various anthraquinones required for 50% inhibition of the rate of sulfide production by resting cells of *D. gigas*

Anthraquinone	I_{50} value (μM)
9,10-Anthraquinone	0.5
1-Chloroanthraquinone	1.6
2-Chloroanthraquinone	0.4
1-Fluoroanthraquinone	1.3
2-Bromoanthraquinone	0.7
1,8-Dichloroanthraquinone	14.0
1,5-Dichloroanthraquinone	>18.0
1,2,3,4-Tetrachloroanthraquinone	14.0
1,4,4,8-Tetrachloroanthraquinone	>14.0
1-Chloro-2-methylanthraquinone	8.0
1-Chloro-4-methylanthraquinone	15.0
1-Chloro-2-carboxylanthraquinone	>18.0
1-Chloro-5-carboxylanthraquinone	>18.0
1-Chloro-3-carboxylanthraquinone	14.0
1-Chloro-8-hydroxyanthraquinone	11.0
1-Chloro-8-aminoanthraquinone	8.0
2-Chloro-6-sulfonicanthraquinone	>15.0
2-Chloro-3-carboxylanthraquinone	8.0
1-Hydroxyanthraquinone	2.2
1,2-Dihydroxyanthraquinone	2.0
1,8-Dihydroxyanthraquinone	1.6
2,6-Dihydroxyanthraquinone	2.0
1,4-Dihydroxyanthraquinone	8.0
1,2,7-Trihydroxyanthraquinone	16.0
1,2,5,8-Tetrahydroxyanthraquinone	>18.0
1,4,5,8-Tetrahydroxyanthraquinone	>18.0
1,2,3,4,5,8-Hexahydroxyanthraquinone	>18.0
1-Aminoanthraquinone	18.0
1,2-Diaminoanthraquinone	4.0
1,4,5,8-Tetraaminoanthraquinone	>18.0
Anthraquinone-1-sulfonic	>15.0

RESULTS

Effects of anthraquinones on sulfide production: the structure-inhibition relationship. A variety of anthraquinone derivatives, as well as unsubstituted 9,10-anthraquinone, were tested for the ability to inhibit sulfide production by resting cells of *D. gigas*. Five types of anthraquinone compounds were tested. These included unsubstituted, halogenated, hydroxylated, amino-substituted, and mixed derivatives. The concentrations of these compounds which inhibited sulfide production by 50% (I_{50} values) are shown in Table 1. Unsubstituted or

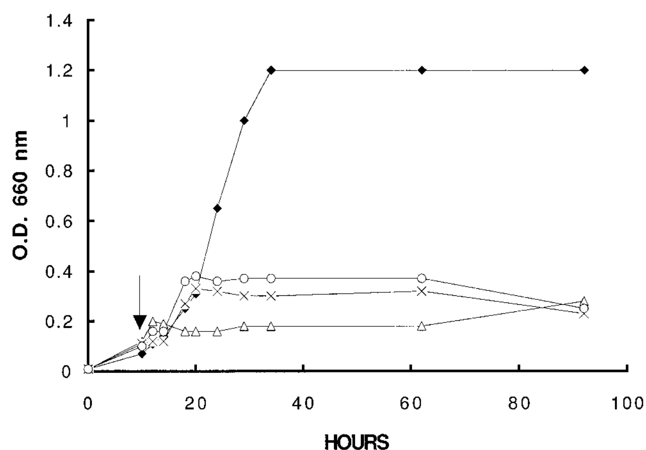

FIG. 1. Growth of *D. gigas* on lactate-sulfate medium in the presence of three different anthraquinones. The arrow shows the point at which acetone (◆), 1,8-dihydroxyanthraquinone (△), 1-chloroanthraquinone (×), and the water-soluble derivative 3-chloro-2-carboxyanthraquinone (○) were added to separate cultures of *D. gigas*. All anthraquinones were added at a final concentration of 12 μM. O.D., optical density.

TABLE 2. Effect of 1,8-dihydroxyanthraquinone on growth of sulfate reducers of the genus *Desulfovibrio* and two non-sulfate-reducing organisms, *E. coli* and *T. denitrificans*

Organism	Optical density at 660 nm	
	Control[a]	Anthraquinone[b]
Sulfate reducers		
"*Desulfovibrio multispirans*"	>1.5	0.28
Desulfovibrio desulfuricans G100A	>1.5	0.20
Desulfovibrio salexigens	>1.5	0.06
Desulfovibrio desulfuricans 27774	0.45	0.08
Desulfovibrio vulgaris	0.20	0.05
Non-sulfate-reducers		
Escherichia coli (aerobic)[c]	>1.5	>1.5
Escherichia coli (anaerobic)[d]	0.75	0.87
Thiobacillus denitrificans	>1.5	>1.5

[a] Acetone solvent was added as a control.
[b] 12 μM 1,8-dihydroxyanthraquinone.
[c] Aerobic growth with succinate as the sole carbon source.
[d] Anaerobic under H_2-CO_2 with fumarate as the electron acceptor.

singly substituted halogen derivatives were generally the most effective inhibitors, with I_{50} values at or below 1 μM (Table 1). Further substitution with any group, such as a methyl, halo, carboxy, or sulfonic group, resulted in decreased inhibitory activity. Mono- or dihydroxyanthraquinones were the next most active class of anthraquinones, with I_{50} values of 1.6 to 8 μM. An increase in the degree of hydroxylation of the anthraquinone nucleus resulted in loss of inhibitory activity. The only highly water-soluble derivative which exhibited inhibition was 2-chloro-3-carboxyanthraquinone. Other ionic species, such as 2-chloroanthraquinone-1-sulfonic acid or anthraquinone-1-sulfonic acid, were not inhibitory at 100 μM (the highest concentration tested).

Inhibition of growth of sulfate-reducing bacteria and sulfate-reducing enrichments. Growth on lactate as the sole carbon source is dependent on sulfate respiration, since there is insufficient energy in lactate fermentation for ATP synthesis and growth; therefore, inhibition of sulfate respiration should inhibit lactate-dependent growth. When 1,8-dihydroxy-, 1-chloro-, or 2-chloro-3-carboxyanthraquinone was added as a solution in acetone (for 1,8-dihydroxy and 1-chloro derivatives) to a *D. gigas* culture, growth ceased immediately with the hydroxylated compound whereas there was a lag of several hours before growth stopped with the chlorinated compounds (Fig. 1). The reason for a lag phase with the chlorinated compounds is unknown; however, these derivatives are typically less soluble than the hydroxylated anthraquinones, resulting in differential rates of exposure of the bacteria to the compounds. Cultures usually recovered after a period of days to weeks, with resumption of full growth. The bacteria from these recovered cultures retained the same sensitivity to the anthraquinone as bacteria which had never been exposed to an anthraquinone when subsequently exposed to the same anthraquinone.

To examine the possibility that anthraquinones generally inhibit respiratory activity, or perhaps only low-potential respirations, aerobic growth on succinate and hydrogen-dependent growth by fumarate respiration in *E. coli* were compared (Table 2). At a concentration of 12 μM, 1,8-dihydroxyanthraquinone had no effect on either aerobic or anaerobic growth of *E. coli*, nor was the anaerobic growth with nitrate of *T. denitrificans* affected. The aerobic growth of *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, and *Staphylococcus aureus* was also shown to be unaffected by 1,8-dihydroxyanthraquinone at concentrations as high as 100 μM (data not shown). In contrast to these results, all five strains of sulfate-reducing bacteria were completely inhibited by 12 μM 1,8-dihydroxyanthraquinone.

Lactate-sulfate enrichments of sulfate-reducing bacteria from functionally and geographically disparate environments were tested for sensitivity to 1,8-dihydroxyanthraquinone (Table 3). These environments included freshwater sediments, saltwater sediments, anaerobic digestor material, and samples from various stages of a wastewater treatment facility. Rates of sulfide generation varied widely among the enrichments. At a concentration of 12 μM, 1,8-dihydroxyanthraquinone induced significant inhibition in all of these except WD (derived from the Wilmington Anaerobic Digestor Facility). The WD enrichment was transferred through several subcultures in the presence of 20 μM 1,8-dihydroxyanthraquinone until the culture was dominated by a single organism. This organism appears to be a pyruvate-fermenting, desulfoviridin-positive vibrio typical of the genus *Desulfovibrio*. Sulfate respiration in all three samples from this particular location was only slightly inhibited by 1,8-dihydroxyanthraquinone, suggesting that the organism was dominant in these samples.

Effect of anthraquinone on respiratory pathways. The effect of 1,8-dihydroxyanthraquinone on specific respiratory and fermentative pathways was investigated as a first step in elucidating the mechanism of anthraquinone inhibition. Washed cells of *D. gigas* were assayed respirometrically for hydrogen utilization in the presence or absence of 12 μM 1,8-dihydroxyanthraquinone. Hydrogen-dependent respiration with sulfate, sulfite, thiosulfate, or fumarate was investigated (Table 4). The anthraquinone completely abolished sulfate-dependent hydrogen uptake, whereas the other respirations were either only partially affected or not affected at all. Fermentative hydrogen evolution from pyruvate was not affected. However, pyruvate-dependent hydrogen utilization was obtained, which suggests that pyruvate reactivated electron transfer from hydrogen to sulfate even in the presence of anthraquinone.

ATP may be generated either by membrane-bound electron transfers or by the phosphoroclastic reaction wherein pyruvate is fermented to hydrogen, CO_2, and acetate. The data show that sulfate-dependent hydrogen uptake is uniquely sensitive

TABLE 3. Effect of 12 μM 1,8-dihydroxyanthraquinone on sulfide production from crude enrichment cultures of sulfate-reducing bacteria

Enrichment type[a]	Micromoles of S^{2-}/day[b]	
	Control	Anthraquinone
Freshwater		
VS-A	49	0
VS-AN	12	0
VS-AN2	116	0
VF-A	24	0
VF-A2	64	0
VF-AN	67	3
WCC-A1	411	67
WCC-A2	388	49
WCC-AN1	345	80
WCC-AN2	288	37
LUMS-AN	142	6
LUMS-AN2	240	6
Anaerobic digestor		
WD-AN1	60	50
WD-AN2	150	146
WD-AN11	95	70
POC	17	0
PNC	217	17
P	114	8
PS	52	0
PPD	210	0
PF	120	0
PSC	168	0
Salt marsh		
SM-A	90	24
SM-AN1	50	0
SM-AN2	180	80

[a] See Materials and Methods for explanations of abbreviations.
[b] Average sulfide production rate over a 72-h time course.

to the anthraquinone. Sulfate respiration is also unique in having a stoichiometric requirement for ATP. The fact that pyruvate reactivates sulfate respiration supports a mechanism whereby anthraquinone causes a decrease in respiratory ATP synthesis which can be compensated for by fermentative or substrate level ATP generated via the phosphoroclastic reaction.

Uncoupling effect of anthraquinones on *D. gigas* membrane vesicles. Terminal reductases involved in sulfate reduction are soluble cytoplasmic enzymes, whereas hydrogen oxidation is typically localized on the periplasmic side of the cell membrane (16). Electrons must cross the cell membrane because of the separation of hydrogen-oxidizing reactions and sulfate-reducing reactions on the exterior and interior, respectively, of the cell (4, 8). However, in disrupted cells, these electron transfers to sulfur oxy-acids are destroyed because of dilution of soluble electron carriers and loss of electron transfer from the membrane to terminal reductases. In contrast, the hydrogen-fumarate and hydrogen-nitrite electron transfer couples are totally membrane bound and high rates of electron transfer through these pathways have been demonstrated by using French press vesicles (5). In our *D. gigas* French press membrane vesicle preparations, hydrogen-dependent reductions of fumarate were typically fivefold higher than those for nitrite. Therefore, we chose to study only electron transfer and phosphorylation associated with the hydrogen-fumarate couple.

TABLE 4. Respiration rates in whole cells of *D. gigas* in the presence and absence of 12 μM 1,8-dihydroxyanthraquinone

Electron transfer	H_2 uptake (nmol/min/mg)	
	Control	Anthraquinone
Hydrogen-sulfate	200	0
Hydrogen-sulfite	300	300
Hydrogen-thiosulfate	700	650
Hydrogen-fumarate	200	150
Pyruvate-$2H^+$	40	60
Pyruvate + H_2-sulfate	200	100

To determine whether anthraquinones can uncouple ATP synthesis from electron transfer to fumarate, *D. gigas* membrane vesicles were assayed in a Warburg flask for phosphorylation coupled to electron transfer. Three anthraquinones were tested: the sparingly soluble compound 1,8-dihydroxyanthraquinone, the water-soluble compound 2-chloro-3-carboxyanthraquinone, and the water-soluble compound anthraquinone-1-sulfonic acid. The sulfonic acid derivative is the only anthraquinone that did not inhibit sulfate reduction and thus serves as a control compound to validate a cause-and-effect relationship between inhibition of sulfide production and the effect of anthraquinone on phosphorylation and electron transfer from hydrogen to fumarate. Table 5 shows the effects of three anthraquinones on fumarate-dependent hydrogen uptake and phosphorylation by membrane vesicles of *D. gigas*. If ATP synthesis is uncoupled from electron transfer, hydrogen uptake should be stimulated, whereas phosphorylation should diminish as a function of anthraquinone concentration. This effect was, in fact, observed only for (the respiratory inhibitors) 1,8-dihydroxyanthraquinone and 2-chloro-3-carboxyanthraquinone, whereas anthraquinone-1-sulfonic acid, which is not a sulfide inhibitor, did not interfere with electron transfer and phosphorylation. P/2e ratios varied somewhat from preparation to preparation; however, with any particular preparation, P/2e ratios always declined with the sulfide-inhibiting anthraquinones.

2-Heptyl-8-hydroxyquinoline-*N*-oxide is known to block electron transfer in many bacterial respirations, including sulfate respiration (9). A 50 μM concentration of 2-heptyl-8-hydroxyquinoline-*N*-oxide was shown to reduce fumarate-dependent hydrogen uptake by French press vesicles from 700 to 0 nmol of H_2 per min per mg. Addition of 20 μM 1,8-dihydroxyanthraquinone partially restored the rate to 420 nmol of H_2 per min per mg of membrane particles. This experiment established that the anthraquinone can act as an artificial electron carrier from hydrogen to fumarate. It is not known whether the electron transfer occurred within the cell membrane or externally in the aqueous phase. However, on the basis of the solubility of anthraquinone and recoveries of anthraquinone from the membrane fraction of inhibited cells, it is possible that the anthraquinone cycled between oxidized and reduced forms within the lipid membrane of the cell.

DISCUSSION

This report shows that sulfate respiration in *D. gigas* and other sulfate-reducing bacteria, as well as sulfate-reducing enrichments, can be inhibited by many, but not all, anthraquinone derivatives. Sensitivity of *D. gigas* was shown to decrease with an increasing degree of substitution for hydroxylated and halogenated anthraquinone derivatives. Introduction of charged groups such as carboxyl or sulfonate groups on the anthraquinone ring generally increases water solubility but results in loss of inhibitory efficacy. On the basis of the correla-

TABLE 5. Hydrogen uptake, phosphorylation, and phosphorylation efficiency catalyzed by fumarate-respiring membrane vesicles of *D. gigas* with 25 mM sodium fumarate in the presence and absence of three anthraquinones[a]

Compound and concn (μM)	Amt of H_2 taken up (μmol)	Amt of ATP synthesized (μmol)	P/H_2 ratio
1,8-Dihydroxyanthraquinone			
0	7.8	4.8	0.62
2	9.1	4.8	0.53
8	11.0	3.5	0.32
41	12.5	2	0.16
102	12.3	2.0	0.16
2-Chloro-3-carboxyanthraquinone			
0	8.4	2	0.24
2	9.3	2	0.21
8	9	1.2	0.13
41	10.2	1.2	0.12
102	11.0	1.0	0.09
Anthraquinone-1-sulfonic acid			
0	8.9	2.4	0.27
2	8.8	3.1	0.35
8	8.6	3.1	0.36
41	9.2	3.6	0.39
102	9.8	2.6	0.27

[a] 180 min of incubation.

tion between declining P/2e ratios with increasing anthraquinone concentrations, the ability of 1,8-dihydroxyanthraquinone to overcome a 2-heptyl-8-hydroxyquinoline-*N*-oxide block in fumarate-respiring vesicles and the known redox properties of anthraquinones, we propose that the underlying mechanism of inhibition is uncoupling of electron transfer from ATP synthesis via anthraquinone-mediated electron transfer reactions.

Sulfate reduction requires an activation step involving conversion of sulfate and ATP by ATP sulfurylase to activated sulfate or APS. We propose that an uncoupling mechanism can account for anthraquinone inhibition of sulfate reduction, and this is in agreement with two lines of evidence. First, in whole cells, the sensitivity of sulfate reduction and the relative insensitivity of sulfite, thiosulfate, or fumarate respiration to anthraquinone suggests that either the activation of sulfate with ATP or the reduction of APS is the inhibited step or that the ATP required for sulfate activation is present at an insufficient concentration. Electron transfer to APS was not specifically investigated; however, ATP sulfurylase and APS reductase activities were found to be insensitive to 1,8-dihydroxyanthraquinone (data not shown). Restoration of sulfate-dependent hydrogen uptake by pyruvate is difficult to explain by any mechanism other than compensation of ATP shortage with fermentative ATP from the phosphoroclastic reaction. Secondly, in phosphorylating membrane vesicles, the uncoupling mechanism was directly demonstrated. Increasing anthraquinone concentrations correlated with increasing respiration rates and decreasing ATP synthesis. The P/H_2 ratios reported here compare favorably with the values reported by Barton et al. (5). All sulfate-reducing bacteria have a unique requirement for two ATP molecules per sulfate molecule reduced; therefore, it might be anticipated that a sulfate-reducing organism growing under purely respiratory conditions on sulfate might be very sensitive to any uncoupling of ATP synthesis (4, 15).

Anthraquinones are redox-active molecules with negative midpoint potentials for the fully oxidized-to-fully reduced transition which lies within the redox span of many anaerobic respirations (i.e., −400 to −100 mV) (6, 18). The absolute midpoint redox potential of a particular anthraquinone used in this work (in water) is difficult to assess, although it has been reported that the redox potential of hydroxylated anthraquinones (in dimethylformamide) becomes more positive with an increasing degree of substitution (18). This may underlie the structure-inhibition correlation we observed by rendering the highly substituted anthraquinones too positive in redox potential to be reoxidized and regenerated by cellular electron transfer reactions. Alternatively, the relationship may depend on relative partitioning of the anthraquinone between water and the cell membrane.

The results presented here, as well as previously existing literature, suggest that simple, unsubstituted anthraquinones are exceptionally toxic to sulfate respiration. Several reports have demonstrated that anthraquinones may interfere with microbial metabolism (1–3, 9, 10, 17). However, these reports do not suggest broad or general inhibition of a particular physiological process; instead, there appears to be sporadic and species-specific toxicity which does not correlate with any physiological process. Anthraquinone-based dyes, such as Cibacron blue, are known to inhibit certain microbial oxidoreductase reactions, but these compounds are structurally much more complex than those found effective against sulfate-reducing bacteria. Furthermore, simple anthraquinones completely lack activity against oxidoreductases (17). In contrast, in this study, only simple anthraquinones were found to be effective against all of the pure strains of sulfate-reducing bacteria tested. Anthraquinones with additional ring substituents were typically less active. Several anthraquinone dyes, including Cibacron blue, were tested for inhibition of sulfate reduction and found to be devoid of activity (data not shown).

The lack of inhibition of aerobic or anaerobic metabolism in *E. coli* suggests that there is some degree of specificity in the interaction between anthraquinone and *Desulfovibrio* species. The specificity of the inhibition for sulfate-reducing bacteria was not broadly investigated here; however, specificity is unlikely to be absolute if redox uncoupling of electron transfer is the sole mechanism of inhibition. One isolate from the WD enrichments was found to be insensitive to anthraquinone. This organism appears to be a typical, desulfoviridin-positive vibrio capable of growth by pyruvate fermentation (data not shown). The extent to which insensitive organisms are present in nature is not known; however, the WD isolate is the only example currently in isolation.

The effect of anthraquinones on methanogenesis appears to be more unpredictable. We have found toxicity of 1,8-dihydroxyanthraquinone toward methanogenesis in crude enrichments derived from a rumen or an anaerobic digestor. These anthraquinone-inhibited methanogenic enrichments also accumulate hydrogen. However, inhibition of methanogenesis has not been observed with pure cultures of *Methanobacterium formicicum* or *Methanosarcina barkeri* under H_2-CO_2 (80:20) (unpublished data). The fact that anthraquinone does not inhibit pyruvate-dependent hydrogen evolution by *D. gigas* is also consistent with the observation of hydrogen accumulation in the methanogenic enrichments if *Desulfovibrio* spp. participate in interspecies hydrogen transfer reactions. It is not clear whether the inhibition is due to direct or indirect effects of the compounds on methanogenic bacteria; however, this aspect clearly limits the use of anthraquinone as a specific inhibitor of sulfate reduction relative to methanogenic respiration.

Many simple anthraquinones are naturally occurring compounds with widespread occurrence among plants, fungi, and insects. All of the pure cultures of sulfate-reducing bacteria

tested and all but one of the crude enrichment cultures tested were sensitive to anthraquinone inhibition of sulfate respiration. The general lack of toxicity to plants and animals and the fact that anthraquinones are not considered to be broadly antimicrobial suggest that these compounds may be useful in the inhibition of sulfate respiration in environments where human health and safety and general toxicity are issues.

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