flavoprotein of high reducing potential (E'_0 approximately -0.34 v.). Thioctic reductase is present also in spinach chloroplasts.

Note added in proof.—Since this paper was submitted for publication, Massey [*Biochim. Biophys. Acta*, **32**, 286, (1959)] has provided additional evidence that diaphorase is a component of the KG dehydrogenase complex.

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THE ATP-DEPENDENT REDUCTION OF SULFATE WITH HYDROGEN IN EXTRACTS OF DESULFOVIBRIO DESULFURICANS

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Communicated by Alexander Hollaender, March 6, 1959

Enzymatic systems that reduce sulfate seem to be widespread in nature since many organisms can satisfy their nutritional requirements for sulfur with sulfate. The ability to reduce sulfate is most highly developed in certain microorganisms, e.g., *Desulfovibrio desulfuricans*, in which inorganic sulfate functions in anaerobic respiration as the terminal electron acceptor.[1] *D. desulfuricans* can grow heterotrophically with sulfate and an organic electron donor or autotrophically with sulfate, hydrogen, and carbon dioxide.[2] Reaction (1) illustrates the reduction of sulfate to sulfide with molecular hydrogen.

$$SO_4^{--} + 4H_2 \rightarrow S^{--} + 4H_2O \qquad (1)$$

The change in free energy is sufficient for formation of at least four high-energy bonds. It is assumed that, at some point in the enzymatic sequence catalyzing reaction (1), the energy required for autotrophic growth is generated.

Although whole cells of *D. desulfuricans* utilize hydrogen in the presence of sulfate, the inability of cell-free extracts to reduce sulfate has been observed repeatedly by Postgate[1] and Ishimoto *et al.*[3] Sulfite, thiosulfate, and tetrathionate are reduced to hydrogen sulfide by cell-free extracts.[3] S^{35}-labeled sulfite has been isolated from reaction mixtures in which whole cells of *D. desulfuricans* reduced sulfate with hydrogen.[4] This observation, together with other data,[5] suggested that only free sulfite or a compound on the oxidation level of sulfite is an intermediate

in sulfate reduction by this organism. Whether the intermediates are free-sulfur compounds or derivatives of them, as has been postulated for nitrate reduction,[6] has not been determined.

D. desulfuricans is also unique among the nonphotosynthetic anaerobes in that the organism contains large amounts of a cytochrome identified as cytochrome c_3.[7] This cytochrome ($E_0' = -0.205$) functions as an electron carrier in the production of hydrogen from formate and couples sulfite and thiosulfate reductases with hydrogenase. It is also implicated in sulfate reduction, since reduced intracellular cytochrome c_3 is oxidized when sulfate is added to a suspension of whole cells.[1, 8]

In this communication some properties of a cell-free system that reduces inorganic sulfate with hydrogen will be described.

Materials and Methods.—Desulfovibrio desulfuricans was grown as described by Peck and Gest,[9] except that the alkaline pyrogallol seal was omitted and the 12-liter flasks were sealed with a sterile rubber stopper. Cell-free extracts were prepared by passing 30–40 g wet weight of cells suspended in 100 ml of 0.05 *M* tris(hydroxymethyl)aminomethane (Tris) buffer, pH 7.75, once through a French pressure cell (American Instrument Corporation). The extract was centrifuged at 30,000 × *g* for 30 minutes and then stored under hydrogen in an ice bath or at −20°C. Extracts prepared in this manner contained 50–60 mg of protein per milliliter.

Other preparations were made up as follows: (*a*) Cytochrome c_3—crude extract passed through Amberlite IRC-50 column[8] prepared as described by Margoliash;[10] column washed with distilled water and 0.02 *M* phosphate buffer, pH 6.0; cytochrome eluted with 0.25 *M* phosphate buffer (pH 6.0), boiled for 5 minutes, dialyzed overnight at 2°C against distilled water, and lyophilized. (*b*) Adenosine-5′-phosphosulfate—synthesized by method of Baddiley *et al.*[11] (*c*) Inorganic pyrophosphatase by method of Heppel.[12] ATP was purchased from Pabst Laboratories and methyl viologen from British Drug Houses, Ltd., Toronto. Sulfurylase (Fraction VII) was a gift of Dr. Phillips Robbins.

Determinations were made as follows: Cytochrome c_3 concentration, spectrophotometrically with a 5.4×10^4 molar extinction coefficient;[1] hydrogen utilization, in conventional Warburg apparatus at 30°C; inorganic phosphorus, by method of Fiske and SubbaRow;[13] pyrophosphate, by method of Flynn *et al.*;[14] sulfite, titrated with sodium thiosulfate;[15] protein, by method of Bucher.[16]

Results.—The reduction of sulfate by intact cells of *D. desulfuricans* is inhibited by selenate.[17] Ishimoto *et al.*[3] demonstrated that tungstate also will inhibit sulfate reduction. In both instances, however, the effect of these anions on sulfite reduction was small. Robbins and Lipmann[18] showed that the first step in the activation of sulfate by extracts of baker's yeast is the formation of adenosine-5′-phosphosulfate (APS) and pyrophosphate (PP) from ATP and sulfate as shown in reaction (2).

$$\text{ATP} + \text{SO}_4^{--} \rightarrow \text{APS} + \text{PP} \qquad (2)$$

Wilson and Bandurski[19] observed that the enzyme sulfurylase catalyzes a rapid liberation of inorganic phosphate in the presence of inorganic pyrophosphatase and Group VI anions (MoO_4^{--}, SeO_4^{--}, WO_4^{--}, and CrO_4^{--}). The selenate

and tungstate inhibition of sulfate reduction by whole cells of *D. desulfuricans* suggested that the first step in sulfate reduction might be the ATP-dependent activation of sulfate shown in reaction (2).

The effect of molybdate, tungstate, and chromate on phosphate liberation from ATP by a cell-free extract is shown in Table 1. Crude cell-free extracts exhibit

TABLE 1

EFFECT OF MOLYBDATE, TUNGSTATE, AND CHROMATE ON THE RELEASE OF INORGANIC PHOSPHATE FROM ATP IN CELL-FREE EXTRACTS

Reaction Mixture*	Activity, μmoles of P_i/hr/mg of protein
Complete	0.08
Minus Na_2SO_4	0.24
Minus ATP	0.0
Plus 10 μmoles of molybdate	3.10†
Plus 10 μmoles of tungstate	1.80†
Plus 10 μmoles of chromate	2.94†

* Complete system: 100 μmoles of Tris buffer pH 7.5, 2 μmoles of $MgCl_2$, 5 μmoles of ATP, 10 μmoles of cysteine, 10 μmoles of Na_2SO_4, 0.02 ml of pyrophosphatase. Total volume, 0.5 ml., temp., 30°C.
† Na_2SO_4 omitted.

a strong inorganic pyrophosphatase activity and also catalyze the liberation of inorganic phosphate from ATP when molybdate, tungstate, or chromate is included in the reaction mixture. An increase in the liberation of inorganic phosphate in the presence of sulfate was not observed in crude extracts. Liberation of inorganic phosphate was sulfate dependent after streptomycin precipitation and dialysis to remove endogenous sulfate.

Since these observations suggested that the extract could probably form APS, I attempted with these extracts to observe the utilization of hydrogen for sulfate reduction in the presence of ATP. Table 2 shows that hydrogen was consumed

TABLE 2

EFFECT OF ATP, MOLYBDATE, TUNGSTATE, AND CHROMATE ON SULFATE REDUCTION WITH HYDROGEN IN WHOLE CELLS AND CELL-FREE EXTRACTS

Reaction Mixture	Activity, μl of H_2/15 min	
	Whole Cells*	Cell-Free Extract†
Complete	175	86
Minus Na_2SO_4	0	0
Minus ATP	314	0
Plus 10 μmoles of molybdate	0‡	0
Plus 10 μmoles of tungstate	0‡	0
Plus 10 μmoles of chromate	0‡	0

* Complete system: 125 μmoles of phosphate buffer pH 7.15, 10 μmoles of $MgCl_2$, 40 μmoles of Na_2SO_4, 10 μmoles of ATP, 0.5 ml of a 20 per cent suspension of cells. Total volume, 2.0 ml in center well; 0.2 ml 20 per cent KOH in center well; temp., 30°C; gas phase, hydrogen.
† Complete system: 125 μmoles of phosphate buffer pH 7.75, 10 μmoles of $MgCl_2$, 10 μmoles of ATP, 40 μmoles of Na_2SO_4, 0.8 μmole of methyl viologen, 15 mg of protein. Total volume, 2.0 ml; 0.2 ml 20 per cent KOH in center well; temp., 30°C; gas phase, hydrogen.
‡ ATP omitted.

in the presence of both ATP and sulfate but not in the presence of ATP or sulfate alone. ATP was not required by the whole cells for sulfate reduction and actually seemed to inhibit the reduction. Tungstate, molybdate, and chromate completely inhibited sulfate reduction in whole cells and in cell-free extracts. The inhibition of sulfate reduction by molybdate, tungstate, and chromate and liberation of inorganic phosphate from ATP in the presence of these anions suggest that the

first step in sulfate reduction by molecular hydrogen requires the activation of sulfate by ATP with the formation of APS [reaction (2)].

The enzyme system is stable to freezing and also to storage at 2°C. After centrifugation at 120,000 × g for 1 hour, 50 per cent of the activity is lost. Addition of the particles, which show no sulfate-reducing activity by themselves, to the soluble extract completely restores the original activity. This stimulation seems to be the result of a portion of the factors, which are concerned in electron transport between hydrogenase and sulfate reductase, remaining on the particles.

Reduction of sulfite and thiosulfate[1, 8] by cell-free extracts requires cytochrome c_3. Cytochrome c_3 is also required for evolution of hydrogen from formate. Although reduced cytochrome c_3 is oxidized by sulfate in whole cells, treatment of such cells with detergent to render them permeable to cytochrome c_3 completely destroys this activity. But the treated preparations could still catalyze the oxidation of reduced cytochrome c_3 with sulfite or thiosulfate.[1] When cell-free extracts were passed through a column of Amberlite resin to remove cytochrome c_3,[8] the thiosulfate reductase and formic hydrogenlyase activities were lost. Addition of cytochrome c_3 completely restored both activities. As shown in Table 3, passage of the

TABLE 3

STIMULATION OF SULFATE REDUCTASE BY CYTOCHROME c_3 AND METHYL VIOLOGEN

Extract	Reaction Mixture*	Activity, μl of H_2/hr/mg of protein
Crude	Complete	5.2
Crude	Minus ATP	0.7
Amberlite treated	Complete	0.4
Amberlite treated	Plus 1 mg. of cytochrome c_3	6.0
Amberlite treated	Plus 0.8 μmole of methyl viologen	47.3

* Complete system: 300 μmoles of Tris buffer pH 7.5, 10 μmoles of $MgCl_2$, 40 μmoles of Na_2SO_4, 10 μmoles of ATP. Total volume, 2.0 ml; 0.2 ml of 20 per cent KOH in center well; temp., 30°C; gas phase, hydrogen.

extract over an Amberlite column also completely inactivates the sulfate reductase, but the activity can be restored by addition of purified cytochrome c_3. Addition of methyl viologen stimulates sulfate reduction eightfold over the activity of the crude extract. This indicates that electron transport is the limiting reaction. DPN and TPN could not substitute for methyl viologen in this reaction. Methyl viologen also facilitates the reduction of sulfite and thiosulfate with molecular hydrogen and presumably functions as an efficient electron carrier between hydrogenase and these reductases.[8]

While investigating the pH characteristics of the reaction, I observed something that has proved useful in identifying the products of sulfate reductase. As shown in Figure 1, at pH 7.75 the reaction proceeds rapidly for 15–20 minutes and then quickly stops. At pH 6.0, in the presence of identical amounts of ATP and sulfate, hydrogen consumption continues for 35–40 minutes, and the total hydrogen uptake is twice as great at this pH as at pH 7.75. The product of the reaction at pH 7.75 was tentatively identified as sulfite. It gave reactions characteristic of sulfite.[20] Titration of reaction mixtures with thiosulfate[3] indicated a 1:1 ratio between the amount of hydrogen utilized and the sulfite produced. At pH 6.0, the product of the reaction seems to be hydrogen sulfide. Probably because sulfite reductase is 6 times as active at pH 6.0 as at pH 7.75 hydrogen utilization is greater and

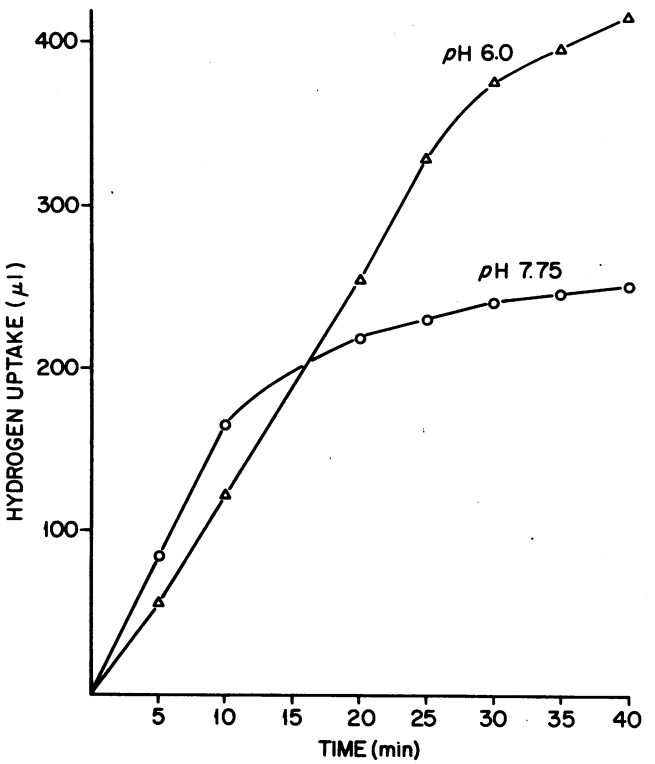

FIG. 1.—Effect of pH on the extent of sulfate reduction. The complete system contained 125 μmoles of phosphate buffer; 10 μmoles of $MgCl_2$; 40 μmoles of Na_2SO_4, 10 μmoles of ATP, 0.8 μmole of methyl viologen, 15 mg of protein. Total volume, 2.0 ml; 0.2 ml 20 per cent KOH in center well; temp., 30°C; gas phase, hydrogen.

hydrogen sulfide is produced. The inhibition of the over-all reduction of sulfate to hydrogen sulfide at pH 7.75 and the low activity of sulfite reductase at this pH suggest that free sulfite, not a bound sulfite, is an intermediate in the reduction of sulfate.

Sulfate activation for sulfate transfer as described by Robbins and Lipmann,[21] and Wilson and Bandurski[22] involves two enzymatic reactions. The first reaction is the formation of APS from ATP and sulfate [equation (2)]. The second is the phosphorylation of APS in the 3′ position to form 3′-phosphoadenosine-5′-phosphosulfate (PAPS) and ADP,[19] shown in equation (3).

$$\text{APS} + \text{ATP} \rightarrow \text{PAPS} + \text{ADP} \qquad (3)$$

In mammalian systems the sulfate of PAPS may be transferred to steroids, phenols and carbohydrates in the presence of the appropriate sulfokinase.[24]

The ATP requirement and the inhibition of sulfate reduction in extracts of *D. desulfuricans* by Group VI anions do not permit us to distinguish between APS and PAPS as the substrate for sulfate reductase. If the intermediate were APS, equal amounts of hydrogen and ATP should be utilized. Also, for each micromole of hydrogen utilized or sulfite produced, 2 μmoles of inorganic phosphate should be

liberated from ATP in the presence of inorganic pyrophosphatase. It should be noted here again that the crude extract contains a potent inorganic pyrophosphatase. If PAPS were the substrate for sulfate reductase and if the presence of myokinase is assumed, a maximum of 0.67 μmole of hydrogen should be consumed per micromole of added ATP. Results of an experiment in which known amounts of ATP were added in the presence of excess sulfate are shown in Figure 2. The amount of hydrogen consumed in the presence of 5, 10, and 15 μmoles of ATP is shown; in each case, 1 μmole of hydrogen is utilized per micromole of added ATP. The curve representing the course of hydrogen utilization with 5 μmoles of ATP should be particularly noted. In this instance, hydrogen consumption completely stopped after 5 μmoles of hydrogen was utilized. This indicates that there was little or no contribution by the product, sulfite, to the total hydrogen uptake. This result suggested that APS rather than PAPS was the substrate for sulfate reductase.

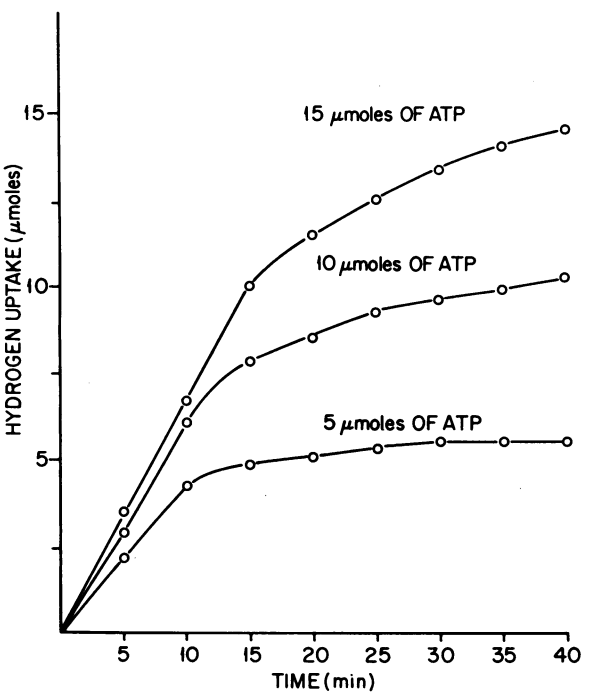

FIG. 2.—The relation between ATP concentration and hydrogen consumption. The complete system contained 125 μmoles of phosphate buffer pH 7.75, 10 μmoles of $MgCl_2$, 40 μmoles of Na_2SO_4, ATP as indicated, 0.8 μmole of methyl viologen, 15 mg of protein. Total volume, 2.0 ml; 0.2 ml 20 per cent KOH in center well; temp., 30°C; gas phase, hydrogen.

A partial balance for the over-all reduction is shown in Table 4. Hydrogen utilization was determined manometrically, sulfite formation by the incorporation of S^{35} in KOH after acidification of the reaction mixture, and ATP utilization by the liberation of inorganic phosphate. The only values subtracted are the zerotime figures. Therefore, 0.4 μmole should be subtracted from the values for hydrogen utilization to correct for the hydrogen uptake caused by the presence of 0.8 μmole of methyl viologen. The values for inorganic phosphate liberation should be regarded as maximum. Electrophoresis of reaction mixtures shows the presence of only AMP and ATP. Thus any 3′,5′-diphosphoadenosines that might have been formed had been hydrolyzed to AMP. The phosphorylation of APS to PAPS and subsequent hydrolysis of 3′,5′-diphosphoadenosine would be expected to increase the ratio of phosphate released to hydrogen utilized, or sulfite produced to a value greater than 2. The observed ratio of 2 is further evidence that APS, not PAPS, is the substrate for sulfate reductase.

When ATP and S^{35}-labeled sulfate were incubated together in crude extracts

TABLE 4

PARTIAL STOICHIOMETRY FOR THE REDUCTION OF SULFATE TO SULFITE WITH HYDROGEN*

Time, min.	μmoles of H_2 Consumed	μmoles of S^{35} Incorporated into KOH	μmoles of P_i Liberated
0	0	0	0
10	4.15	3.31	8.1
15	5.9	4.74	9.72
20	7.75	5.45	10.7

* Complete system: 150 μmoles of Tris buffer pH 8.0, 10 μmoles of $MgCl_2$, 12.5 μmoles of $Na_2S^{35}O_4$ (20,000 cpm/μmole), 20 μmoles of ATP, 0.8 μmole of methyl viologen, 15 mg of protein. Total volume, 2.0 ml; 0.2 ml 20 per cent KOH in center well; gas phase, hydrogen; reaction was stopped by the addition of 0.2 ml of 10 N H_2SO_4.

under helium or oxygen, $PAPS^{35}$ did not form. However, an S^{35}-labeled nucleotide was formed having the same mobility as APS^{35} prepared by incubation of ATP and S^{35}-labeled sulfate with sulfurylase (Fraction VII) and pyrophosphatase.[16] In enzymatic systems that form PAPS, S^{35}-labeled PAPS can be detected but labeled APS usually cannot. This results from the rapid phosphorylation of APS in the 3′ position to form PAPS.[23] Also, the extract utilizes hydrogen in the presence of crude preparations of APS[11] without additional ATP. Heating under acid conditions, which hydrolyzes the sulfate bond, completely destroys this activity.[24]

Discussion.—An ATP-dependent reduction of S^{35}-labeled sulfate to sulfite[25, 26] and to hydrogen sulfide[26] has been reported to occur in cell-free extracts of baker's yeast. In both cases, PAPS was suggested as the substrate for sulfate reductase.

Evidence that PAPS is not an intermediate in sulfate reduction by extracts of *D. desulfuricans* is presented. However, APS seems to be the first intermediate in sulfate reduction by this system, and the data presented are consistent with the idea that APS is the substrate for sulfate reductase. The product of the reduction, adenosine-5′-phosphosulfite, is unstable and probably immediately decomposes to AMP and free sulfite. This conclusion is based on Wilson and Bandurski's observation[19] that sulfite in the presence of sulfurylase catalyzes the liberation of inorganic phosphate from ATP but does not exchange P^{32}-labeled pyrophosphate into ATP.

The over-all reaction for reduction of sulfate with hydrogen can be represented as follows:

$$H_2 + \text{cytochrome } c_3\ (2Fe^{+++}) \rightarrow \text{reduced cytochrome } c_3\ (2Fe^{++}) + 2H^+$$

$$ATP + SO_4^{--} \rightarrow APS + PP$$

$$APS + \text{reduced cytochrome } c_3\ (2Fe^{++}) + 2H^+ \rightarrow AMP + SO_3^{--} + \text{oxidized cytochrome } c_3\ (2Fe^{+++}) + H_2O \quad (4)$$

The possibility remains, however, that APS itself is not the substrate for sulfate reductase, but rather transfers its sulfate to an unknown sulfate carrier on which the sulfate is then reduced to sulfite.

The extracts used in these experiments were prepared from heterotrophically grown cells. The presence or absence of this system in autotrophically grown cells should be investigated. Assuming for the moment that the system is present in autotrophically grown cells, at least three high-energy phosphate bonds must be generated during reduction of sulfate to hydrogen sulfide since two high-energy bonds are utilized in the activation of sulfate. The free-energy change in the

reduction of cytochrome c_3 with hydrogen is sufficient to permit generation of one high-energy phosphate. Since all the sulfate-reducing enzymes can oxidize cytochrome c_3, their function in anaerobic respiration seems to be analogous to that of cytochrome oxidase in aerobic respiration.

Summary.—A cell-free system from heterotrophically grown cells of *Desulfovibrio desulfuricans* reduces sulfate in the presence of ATP and molecular hydrogen and is completely inhibited by molybdate, tungstate, and chromate. Hydrogenase and the sulfate reductase are coupled by cytochrome c_3 or the dye, methyl viologen. At pH 7.75 the product of the reaction is sulfite, but at pH 6.0 sulfate appears to be reduced to hydrogen sulfide. The stoichiometry of the reaction indicates that 1 μmole of ATP is utilized for each micromole of hydrogen consumed and sulfite produced. These data and the absence of 3′-phosphoadenosine-5′-phosphosulfate formation indicate that adenosine-5′-phosphosulfate is the substrate for sulfate reductase.

I wish to express my appreciation to Dr. G. David Novelli for his advice and encouragement during the course of this investigation.

Note added in proof: Ishimoto has recently reported the ATP-dependent reduction of sulfate to sulfide in cell-free extracts of *D. desulfuricans*. *J. Biochem.* (*Tokyo*), **46**, 105 (1959).

* Operated by Union Carbide Corporation for the U. S. Atomic Energy Commission.

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