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REGULATION OF THE REDUCTION OF SULFITE AND THIOSULFATE BY FERREDOXIN, FLAVODOXIN AND CYTOCHROME cc'_3 IN EXTRACTS OF THE SULFATE REDUCER *DESULFOVIBRIO GIGAS*

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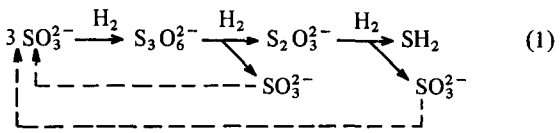
SUMMARY

Cytochrome cc'_3, flavodoxin and ferredoxin are able to stimulate the reduction of thiosulfate by molecular H_2 in an extract of the sulfate reducer *Desulfovibrio gigas*. Only flavodoxin and ferredoxin will stimulate the reduction of sulfite by the same extract, whereas cytochrome cc'_3 is specific for thiosulfate reduction. Sulfite accumulates during the early stage of thiosulfate reduction and is reduced only when thiosulfate has disappeared. The results are discussed in terms of the regulation, by these different electron carriers, of the electron flow from H_2 to the various sulfur compounds acting as terminal electron acceptors in sulfate-reducing bacteria.

INTRODUCTION

The dissimilatory pathway of sulfite reduction by the dissimilatory sulfate-reducing bacteria belonging to the genus Desulfovibrio is not entirely understood. Nevertheless, recent work has thrown some light on the possible intermediates involved between sulfite and the terminal product, sulfide[1,2]. Quite recently, the trithionate-forming enzyme of *Desulfovibrio gigas* has been isolated and identified as desulfoviridin[3]. This enzyme corresponds to the first dissimilatory reductase in the scheme proposed by KOBAYASHI *et al.*[2]:



(1)

The two other enzymes are the thiosulfate-forming enzyme and the thiosulfate reductase. These proteins have not yet been isolated from *D. gigas* but thiosulfate formation in *D. vulgaris* extracts has been reported by SUH AND AKAGI[1] and FINDLEY

AND AKAGI[4], also, thiosulfate reductase has been isolated from *D. desulfuricans*[5] and this enzyme has been purified from *D. vulgaris*[6].

The stoichiometry of the reduction of thiosulfate has been determined by ISHIMOTO *et al.*[7] as follows:

$$S_2O_3^{2-} + H_2 \rightleftarrows S^{2-} + SO_3^{2-} + 2\,H^+ \quad (2)$$

$$SO_3^{2-} + 3\,H_2 \rightleftarrows S^{2-} + 3\,H_2O \quad (3)$$

$$S_2O_3^{2-} + 4\,H_2 \rightleftarrows 2\,S^{2-} + 3\,H_2O + 2\,H^+ \quad (4)$$

If the reaction allowed the accumulation of sulfite, the ratio of H_2 absorbed to sulfide formed would be 1:1. If, on the contrary, all $S_2O_3^{2-}$ molecules were converted into sulfide, the same ratio would then be 2:1.

Cytochrome c_3 has been reported to act as an electron carrier in the reduction of sulfite and thiosulfate in *D. desulfuricans*[8,9] and, more recently, ferredoxin and flavodoxin have been implicated in the same reactions in *D. gigas*[10–12].

We would like to report here the stimulation of the reduction of thiosulfate by cytochrome cc'_3, as compared to flavodoxin and ferredoxin activity in the same reaction. It will also be shown that neither cytochrome c'_3 nor cc'_3 appears to be able to act as an efficient electron carrier in the reduction of sulfite, whereas flavodoxin and ferredoxin can couple this reaction.

MATERIAL AND METHODS

Preparation of extracts

The cultivation and harvesting of *D. gigas* have been described previously[13]. The problem was to obtain an extract devoid of electron carriers but still able to reduce thiosulfate and sulfite in the presence of an artificial electron carrier (methyl viologen). This was achieved by using the following procedure:

The bacteria (20 g wet wt.) were suspended in 25 ml of 0.05 M potassium phosphate buffer (pH 7.0) and extracted with a French pressure cell. The suspension was centrifuged for 20 min at 35 000 × *g* in a Servall refrigerated centrifuge and the pellet was discarded.

The supernatant was then centrifuged at 140 000 × *g* for 2 h in a Spinco centrifuge and the pellet was discarded. The supernatant was brought to 45% saturation with $(NH_4)_2SO_4$ and the mixture was stirred for 20 min at 4°. It was then centrifuged at 30 000 × *g* for 15 min and the clear supernatant solution was discarded. The pellet was resuspended in 0.1 M phosphate buffer (pH 7.0). The resulting suspension was dialysed against 1 l of 0.05 M phosphate buffer (pH 7.0) for 5 h. The resulting extract was devoid of ferredoxin, flavodoxin, cytochrome cc'_3 and cytochrome c'_3, but possessed hydrogenase, thiosulfate reductase and sulfite reductase activity, it was used as the enzyme extract.

Preparation of the electron carriers

The preparation of cytochrome c'_3, cytochrome cc'_3, flavodoxin and ferredoxin have already been described (see refs. 10–14) and have been used without modification. In each case, crystallizable material was used.

Enzyme assays

Thiosulfate and sulfite reductase activity was determined using the Warburg manometer.

The pH maxima for thiosulfate and sulfite reduction were determined in the enzyme extract using methyl viologen as electron carrier. They were found to be 7.6 for thiosulfate and 6.5 for sulfite. Since we wanted to compare the activity of the different electron carriers in the two reactions, a pH of 7.0 was used in both cases.

Each Warburg flask contained: phosphate buffer (pH 7.0), 150 μmoles; enzyme extract, 30 mg; and electron carrier and thiosulfate or sulfite as indicated in the figures or tables. The center well contained 0.1 ml of 10 M NaOH; the total volume was 3 ml and the temperature was 37°. After flushing with H_2 for 10 min the substrate was tipped into the main compartment and H_2 uptake was measured.

For H_2S determination 0.2 ml of 5 M HCl was introduced in the second side-arm of each flask and tipped into the main compartment to stop the reaction and allow the H_2S to be evolved. After a 10-min incubation, the NaOH solution was removed from the center well and analyzed for H_2S.

Methods of analysis

Protein content was estimated by the biuret method of GORNALL *et al.*[15]. The spectrophotometer method of FOGO AND POPOWSKI[16] was used for H_2S determination. Sulfite was determined according to the method of GRANT[17].

The concentration of enzymes and of electron carriers was adjusted in such a way that the H_2 uptake was slow enough to allow the different measurements and assays with maximum accuracy.

TABLE I

STIMULATION OF THIOSULFATE REDUCTION BY VARIOUS AMOUNTS OF CYTOCHROME cc_3'

The reaction mixture contained all the reactants, as indicated in MATERIALS AND METHODS. Each flask contained 25 μmoles of thiosulfate. The reactions were stopped 50 min after the introduction of thiosulfate. The amounts of cytochrome are expressed in nmoles.

	c_3'	cc_3'	H_2 *absorbed* (μmoles)	H_2S *produced* (μmoles)
Enzyme extract without thiosulfate	0	0	0	0.25
Enzyme extract + thiosulfate	0	0	2.0	1.7
	22.7	0	2.5	2.3
	0	2.85	5.9	5.2
	0	5.7	7.8	8.3
	0	10.2	10.0	10.3

RESULTS

The results showing the stimulation of thiosulfate reduction by cytochrome cc'_3 are presented in Table I. Thiosulfate reductase activity is greatly increased by cytochrome cc'_3, whereas cytochrome c'_3 has a very slight activity in the coupling of hydrogenase with thiosulfate reductase.

Fig. 1 shows the effect of the different carriers on the reduction of thiosulfate. It can be seen that the activation due to cytochrome c'_3 is very weak as compared

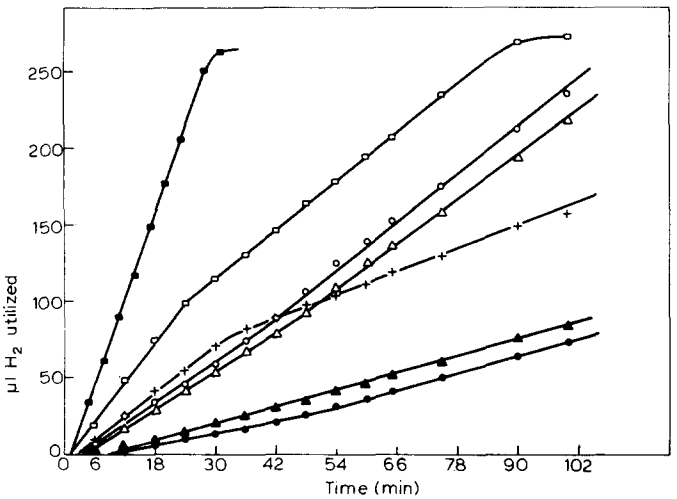


Fig. 1. Reduction of thiosulfate by H_2 in the presence of various electron carriers. The reaction mixture contained all the reactants as indicated in MATERIALS AND METHODS. Thiosulfate (3 µmoles) was used as substrate. ●—●, control without added electron carrier; ▲—▲, *plus* cytochrome c'_3, 10 nmoles; +—+, *plus* cytochrome cc'_3, 10 nmoles; △—△, *plus* ferredoxin, 100 nmoles; ○—○, *plus* flavodoxin, 100 nmoles; □—□, *plus* cytochrome cc'_3 (10 nmoles) *plus* flavodoxin (100 nmoles); ■—■, *plus* methyl viologen, 100 nmoles.

to the activation due to cytochrome cc'_3. If both ferredoxin and flavodoxin allow the reaction to proceed to completion, the same result is not obtained with cytochrome cc'_3 and the H_2 uptake reaches the endogenous value very quickly. Addition of cytochrome cc'_3 and flavodoxin has an additive effect on the rate of the reduction.

It is to be noted that when cytochrome cc'_3, flavodoxin and ferredoxin are used, a change in the rate of reduction occurs when 3.9 ± 0.5 µmoles of H_2 have been utilized. Since 3 µmoles of thiosulfate are present in each flask, it appears that the reaction first proceeds according to Reaction 2. If this were actually the case, then the ratio between the H_2 consumed and the H_2S formed should be equal to unity and sulfite would accumulate. In order to check this hypothesis, we have first followed the variation of the ratio H_2/H_2S during the several phases of the reaction, in the presence of cytochrome cc'_3. The results are reported in Table II: as expected, the ratio is first very close to 1, then it slowly approaches the value of 2, corresponding to

TABLE II

RATIO BETWEEN SULFIDE FORMED AND H_2 ABSORBED DURING THIOSULFATE REDUCTION IN PRESENCE OF CYTOCHROME cc'_3

The reaction mixture is described in MATERIALS AND METHODS. Thiosulfate (3 µmoles) was used as substrate; each flask contained 8.5 nmoles of cytochrome cc'_3.

Time (min)	*H_2 absorbed (µmoles)*	*H_2S formed (µmoles)*	*Ratio H_2/H_2S*
30	4.28	3.9	1.09
63	7.68	4.68	1.64
100	11.47	6.6	1.74
120	12.57	6.54	1.92

TABLE III

SULFITE ACCUMULATION DURING THIOSULFATE REDUCTION

The reaction mixture is reported in MATERIALS AND METHODS. Thiosulfate (3 μmoles) was used as substrate; the amounts of cytochrome c'_3 and cc'_3, flavodoxin and ferredoxin were the same as indicated in the legend to Fig. 1.

	H_2 absorbed (μmoles)		*H_2S formed (μmoles)*		*SO_3Na_2 formed (μmoles)*		*Ratio H_2/H_2S*	
	*A**	*B***	*A*	*B*	*A*	*B*	*A*	*B*
Enzyme extract (without added electron carrier)	0.95	2.24	0.7	1.48	0.24	0.56	1.35	1.51
Enzyme extract + cytochrome c'_3	1.02	2.68	0.85	1.62	0.15	0.68	1.20	1.65
Enzyme extract + cytochrome cc'_3	3.66	5.8	2.7	3.2	2.80	2.15	1.72	1.81
Enzyme extract + flavodoxin	4.06	7.76	2.4	4.2	1.35	0.8	1.68	1.84
Enzyme extract + ferredoxin	3.88	7.14	2.3	3.95	1.3	0.85	1.68	1.80
Enzyme extract + cytochrome cc'_3 + flavodoxin	4.55	10.4	2.9	4.85	2.08	0.40	1.56	2.14

* All the reactions were stopped 42 min after the introduction of thiosulfate, and H_2S and sulfite were estimated (*A*).

** All the reactions were stopped 75 min after the introduction of thiosulfate, and H_2S and sulfite were estimated (*B*).

Eqn. 4. The rate of this second part of the reaction is equal to the endogenous rate.

In a second set of experiments we measured the ratio H_2/H_2S, together with the sulfite present in the reaction mixture, in the presence of the various electron carriers

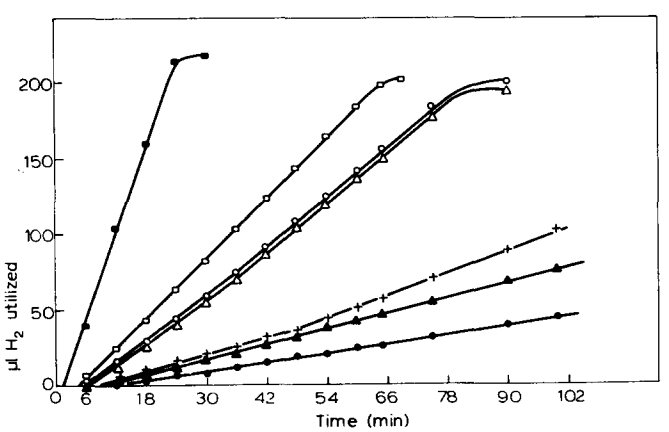


Fig. 2. Reduction of sulfite by H_2 in the presence of various electron carriers. The reaction mixture contained all the reactants as indicated in MATERIALS AND METHODS. Sulfite (4 μmoles) was used as substrate. ●—●, control without added electron carrier; ▲—▲, *plus* cytochrome c'_3, 10 nmoles; +—+, *plus* cytochrome cc'_3, 10 nmoles; △—△, *plus* ferredoxin, 100 nmoles; ○—○, *plus* flavodoxin, 100 nmoles; □—□, *plus* cytochrome cc'_3 (10 nmoles) *plus* flavodoxin (100 nmoles); ■—■, *plus* methyl viologen, 100 nmoles.

(Table III): this experiment was done in a set of Warburg flasks exactly as described for the experiments shown in Fig. 1. After 42 min, *i.e.* just after the change in the rate of H_2 uptake, significant amounts of sulfite are present, almost 3 μmoles with cytochrome cc'_3. For the endogenous flask and the flask containing only cytochrome c'_3, the H_2/H_2S ratios are still close to unity, whereas they have intermediate values between 1 and 2 in all the other flasks. After 75 min, the reaction is almost completed in the flask containing cytochrome cc'_3 *plus* flavodoxin (Fig. 1). The figures in Table III show that the ratio H_2/H_2S is equal to 2 and the sulfite has almost disappeared. Again, the other flasks give intermediate values ,but the amount of sulfite has decreased in all but the endogenous flask and the flask containing cytochrome c'_3, where sulfite is still accumulating.

Fig. 2 shows the effect of the various electron acceptors on the reduction of sulfite, where both cytochrome cc'_3 and cytochrome c'_3 have only a very weak effect on the stimulation of the reaction. As has already been reported[10,11], ferredoxin and flavodoxin are very active carriers for the reaction.

Table IV shows the amount of H_2S formed after 48 min of reaction, compared with the H_2 consumed. In all flasks the ratio H_2/H_2S is equal to 3, thus corresponding to the theoretical ratio of Eqn. 2.

Finally, it is to be noted that in both the reduction of thiosulfate and of sulfite, the artificial electron carrier, methyl viologen, is active.

TABLE IV

RATIO BETWEEN SULFIDE FORMED AND H_2 ABSORBED DURING SULFITE REDUCTION

The reaction mixture is reported in MATERIALS AND METHODS. Sulfite (4 μmoles) was used as substrate; The amounts of cytochrome c'_3 and cc'_3, flavodoxin and ferredoxin were the same as indicated in the legend to Fig. 2. All the reactions were stopped 48 min after the introduction of sulfite, and H_2S was estimated.

	H_2 absorbed (μmoles)	*H_2S formed (μmoles)*	*Ratio H_2/H_2S*
Enzyme extract (without added electron carrier)	0.84	0.3	2.80
Enzyme extract + cytochrome c'_3	1.4	0.45	3.11
Enzyme extract + cytochrome cc'_3	1.68	0.52	3.23
Enzyme extract + flavodoxin	4.82	1.54	3.12
Enzyme extract + ferredoxin	4.68	1.45	3.23
Enzyme extract + cytochrome cc'_3 + flavodoxin	6.42	2.08	3.08

DISCUSSION

Our results show that the reduction of thiosulfate in extracts of *D. gigas* can be stimulated by at least three different proteins, namely a non-heme iron protein, ferredoxin, a flavoprotein, flavodoxin, and a heme iron protein, cytochrome cc'_3. Cytochrome c'_3 does not appear to be a carrier for the reaction.

Both ferredoxin and flavodoxin are carriers for the reduction of sulfite, but cytochromes c'_3 and cytochrome cc'_3 show little stimulation of the reaction. Thus, cytochrome cc'_3 seems to be specific for the reduction of thiosulfate, whereas ferredoxin and flavodoxin show no specificity.

The concentration of flavodoxin and ferredoxin (100 nmoles) that brings about

the same stimulation as 10 nmoles of cytochrome cc'_3 deserves further consideration. First, the heme content of cytochrome cc'_3 is still questionable. We have been using the extinction coefficient derived from the data of DRUCKER *et al.*[18], as some of us described previously[19]. DRUCKER *et al.*[18] found that cytochrome c_3 has 3 hemes per mole, but some indirect evidence[20], as well as direct determination (T. MAYER, personnal communication), indicate that cytochrome c_3 from *D. vulgaris* contains, in fact, 4 moles of heme per molecule of protein. Thus, by analogy, cytochrome c'_3 would also have four hemes and cytochrome cc'_3 eight hemes (see ref. 14). Then cytochrome cc'_3 has, according to the data that one can compile from different authors, from four to eight hemes per mole. Of course, if this is the case, then the activity reported for the number of active centers (up to eight for cytochrome cc'_3, one for flavodoxin and ferredoxin) is indeed similar, as far as the reduction of thiosulfate is concerned. This is, however, if each heme is working individually, a fact that our experiments have not demonstrated.

It is also to be noted that the effect of adding both cytochrome cc'_3 and flavodoxin to the reaction mixture has only an additive effect on the rate of the reaction.

The transitory accumulation of sulfite during the reduction of thiosulfate has already been described by ISHOMITO *et al.*[7], together with the fact that a ratio of 1 mole of H_2 utilized to 1 mole of H_2S formed, is obtained in the first part of thiosulfate reduction. Such results seem to be in conflict with the conclusion of NAKATSUKASA AND AKAGI[21], which is that sulfite inhibits the thiosulfate reductase activity. It is to be noted that those results were obtained from a strain of *Desulfotomaculum nigrificans*, where the dissimilatory reduction of sulfur compounds appears to be very different from the pathway present in Desulfovibrio[22].

Of course, one also has to point out that our system is purely artificial. Our extract containing both sulfite and thiosulfate activity has been obtained from only one part of the total cell extract (soluble proteins), whereas some sulfite activity stays within the particles, and the ammonium sulfate precipitation that is needed to separate the reductase activities from the electron carriers also disturbs the system. Thus we could be using a system far from actual physiological conditions.

Another point to notice is the fact that the steps leading to the formation of sulfide from sulfite are very complex, as shown in Reaction 1. In *D. vulgaris*, in addition to the "dissimilatory" enzymes, a sulfite reductase that leads to the direct conversion of sulfite to sulfide, similar to the assimilatory type of sulfite reductases of other organisms, has been purified[23]. None of the carriers specific for each isolated enzyme is known yet and our results with cytochrome cc'_3 on the stimulation of sulfite or thiosulfate reduction is the first evidence that such a specificity does exist. It is to be noted that in *D. vulgaris*, SUH AND AKAGI[1] have shown that both cytochrome c_3 and ferredoxin are required for the formation of thiosulfate from sulfite.

In *D. gigas*, flavodoxin and ferredoxin are synthesized at the same time. In the medium normally utilized for its growth, the molar ratio of flavodoxin to ferredoxin in *D. gigas* is about 5 to 1. In the presence of a large excess of iron, the same ratio drops to unity. It is also impossible to repeat the experiments of KNIGHT AND HARDY[24] with *Clostridium pasteurianum*, since *D. gigas* will not grow on very low-iron media because the respiratory metabolism of the bacteria requires cytochrome synthesis. Thus, it is impossible to find conditions where only one of the two electron carriers, flavodoxin or ferredoxin, would be synthesized.

We have demonstrated in the present paper that a third molecule, cytochrome cc'_3, can replace the two non-heme electron carriers in the reduction of thiosulfate and that cytochrome c'_3 is not completely devoid of coupling activity (this is also true for cytochrome cc'_3 and c'_3 in the reduction of sulfite), thus posing the question of the significance of this abundance of electron carriers for the same activity.

Attempts to describe the electron carrier chain in Desulfovibrio must await the complete purification of hydrogenase, on the one hand, and of the terminal reductases on the other. It is quite possible that, in the intact cell, the reductases are distributed on different organelles, each having its own electron chain: disruption of the cells would then completely change the distribution of the electron carriers with respect to the terminal reductases and would lead to misleading activities.

However, it is possible to propose that the complexity of the electron carrying system of Desulfovibrio, as shown by our results, does in fact reflect the extremely intricate regulation mechanisms that have allowed the sulfate-reducing organisms to play such an important role in the biosphere from the earlier stages of the appearance of life on the earth[25].

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REFERENCES

1 B. Suh and J. M. Akagi, *J. Bacteriol.*, 99 (1969) 210.
2 K. Kobayashi, S. Tachibana and M. Ishimoto, *J. Biochem. Tokyo*, 65 (1969) 155.
3 J. P. Lee and H. D. Peck, Jr., *Bacteriol. Proc.*, (1971) 151.
4 J. E. Findley and J. M. Akagi, *Biochem. Biophys. Res. Commun.*, 36 (1969) 266.
5 M. Ishimoto and J. Koyama, *J. Biochem. Tokyo*, 44 (1957) 233.
6 R. H. Haschke and L. L. Campbell, *J. Bacteriol.*, 106 (1971) 603.
7 M. Ishimoto, Y. Kondo, T. Kameyama, T. Yagi and M. Shiraki, in *Proc. Int. Symp. on Enzyme Chemistry, Tokyo and Kyoto*, Maruzen, Tokyo, 1957, p. 212.
8 M. Ishimoto and J. Koyama, *Bull. Chem. Soc. Jap.*, 28 (1955) 231.
9 J. R. Postgate, *J. Gen. Microbiol.*, 14 (1956) 545.
10 J. Le Gall and N. Dragoni, *Biochem. Biophys. Res. Commun.*, 23 (1966) 145.
11 J. Le Gall and E. C. Hatchikian, *C.R. Acad. Sci. Paris*. 264 (1967) 2580.
12 L. J. Guarraia, E. J. Laishley, N. Forget and H. D. Peck, Jr., *Bacteriol. Proc.*, (1968) 133.
13 J. Le Gall, G. Mazza and N. Dragoni, *Biochim. Biophys. Acta*, 99 (1965) 385.
14 M. Bruschi, J. Le Gall, E. C. Hatchikian and M. Dubourdieu, *Bull. Soc. Fr. Physiol. Vég.*, 15 (1969) 381.
15 A. G. Gornall, G. J. Bardawill and M. M. David, *J. Biol. Chem.*, 177 (1949) 751.
16 J. K. Fogo and M. Popowsky, *Anal. Chem.*, 21 (1949) 732.
17 W. M. Grant, *Anal. Chem.*, 19 (1947) 345.
18 H. Drucker, E. B. Trousil, L. L. Campbell, G. H. Barlow and E. Margoliash, *Biochemistry*, 9 (1970) 1515.
19 J. Le Gall, M. Bruschi-Heriaud and D. V. Der Vartanian, *Biochim. Biophys. Acta*, 234 (1971) 499.
20 D. V. Der Vartanian and J. Le Gall, *Biochim. Biophys. Acta*, 243 (1971) 53.
21 W. Nakatsukasa and J. M. Akagi, *J. Bacteriol.*, 98 (1969) 429.
22 P. A. Trudinger, *J. Bacteriol.*, 104 (1970) 158.
23 J. P. Lee, J. Le Gall and H. D. Peck, Jr., *Fed. Proc.*, (1971) 875.
24 E. Knight, Jr. and R. W. F. Hardy, *J. Biol. Chem.*, 241 (1966) 2752.
25 H. D. Peck, Jr., *Some Evolutionary Aspects of Inorganic Sulfur Metabolism*, lecture presented at the University of Maryland, 1966.