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Biochemistry of Dissimilatory Sulphate Reduction [and Discussion]

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Biochemistry of dissimilatory sulphate reduction

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Extensive information is available on the enzymology of respiratory sulphate reduction and the structure of electron transfer proteins isolated from the sulphate-reducing bacteria; however, it has not yet been possible to delineate satisfactorily the function of these electron transfer proteins in terms of the enzymes involved in respiratory sulphate reduction. New information about differences in pyrophosphate metabolism by *Desulfovibrio* and *Desulfotomaculum*, cellular localizations of electron transfer proteins and enzymes, and the concepts of vectorial electron transfer plus hydrogen cycling suggest that previous data on the function of electron transfer proteins must be re-evaluated and new experimental approaches designed before the problem is resolved. New information on the enzymology of lactate dehydrogenase, pyruvate dehydrogenase, adenylyl sulphate reductase, bisulphite reductase and hydrogenase is presented and discussed in the context of enzyme localization and specifically for electron transfer proteins. The function of cytochrome c_3 (M_r = 13000) in the mechanism of the periplasmic hydrogenase and the role of the new [3Fe–3S] non-haem iron centres in electron transfer is emphasized.

'*Once we were out in nature . . . we talked about our personal preferences and tastes and discovered we were both fond of the same bacteria*' (Woody Allen, *Side effects*).

1. Introduction

The sulphate-reducing bacteria have been traditionally considered to consist of a small group of highly specialized anaerobic bacteria with similar physiological and bioenergetic systems (Postgate 1979). More recent evidence indicates that the sulphate-reducing bacteria encompass a much larger variety of bacteria than previously suspected, at least seven genera (Pfennig & Widdel 1981), and have a number of growth modes other than sulphate reduction. Thus some but not all species of these bacteria can grow fermentatively (Postgate 1952), utilize fumarate (Grossman & Postgate 1955) or nitrate (Steenkamp & Peck 1981) as terminal electron acceptors, obtain energy for growth from inorganic pyrophosphate (PP_i) (Liu *et al.* 1982) and grow in consortia with other micro-organisms under photosynthetic conditions (Biebl & Pfennig 1977) and by interspecies hydrogen transfer (Bryant *et al.* 1977). In the latter case, the sulphate-reducing bacteria serve as important members of microbial consortia responsible for the degradation of complex organic materials to methane and carbon dioxide. As a result of these associations, the sulphate-reducing bacteria are not restricted to high-sulphate situations such as hot springs and marine environments but are universally found in anaerobic environments.

The bioenergetics of respiratory sulphate reduction have been shown to be quite different in the two most extensively studied genera of these bacteria, *Desulfovibrio* and *Desulfotomaculum* (Liu & Peck 1981*a*). The major enzymes, ATP sulphurylase (Akagi & Campbell 1962), adenylyl sulphate (APS) reductase (Bramlett & Peck 1975) and bisulphite reductase (Lee *et al.* 1973*a*) and intermediates bisulphite, APS and PP_i are shown in figure 1 for the reduction of sulphate with H_2:

$$4H_2 + SO_4^{2-} \longrightarrow S^{2-} + 4H_2O. \quad (1)$$

The bisulphite reductases, desulfoviridin (Lee *et al.* 1973*a*), desulforubidin (Lee *et al.* 1973*b*) and P_{582} (Trudinger 1979) appear to be analogous with regard to mechanism and function although not identical with regard to optical and e.r.p. properties (Liu *et al.* 1979). Several lines of evidence suggest that the 'trithionate pathway' (Akagi 1981) is not generally functional in these bacteria, and this evidence will be discussed in detail in a later section. The major

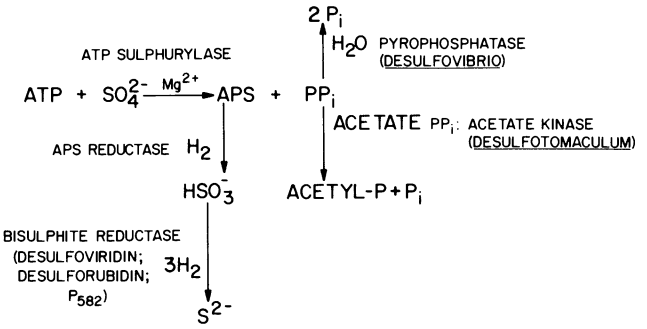

FIGURE 1. Respiratory sulphate reduction.

differences between the two genera of sulphate-reducing bacteria reside in the metabolism of PP_i, which is formed from ATP by ATP sulphurylase. High levels of inorganic pyrophosphatase (PPase) are only found in *Desulfovibrio* species (Akagi & Campbell 1963), and it is postulated that its function is to 'pull' the reaction catalysed by ATP sulphurylase in the thermodynamical unfavourable direction of APS formation (Wilson & Bandurski 1958). In contrast, members of the genus *Desulfotomaculum* metabolize the PP_i formed during sulphate activation by means of the enzyme PP_i: acetate kinase rather than by PPase,

$$PP_i + \text{acetate} \longrightarrow \text{acetyl phosphate} + P_i \qquad (2)$$

(Reeves & Guthrie 1975), and regenerate a molecule of ATP from acetyl phosphate and ADP by means of acetate kinase:

$$\text{acetyl phosphate} + \text{ADP} \longrightarrow \text{acetate} + \text{ATP}. \qquad (3)$$

The presence of these two enzymes allows desulfotomacula to conserve the energy of the pyrophosphate bond and changes the energy requirement for respiratory sulphate reduction from two high-energy phosphates in *Desulfovibrio* to one high-energy phosphate in *Desulfotomaculum*. This is of particular significance for the comparative physiology and bioenergetics of the two genera, as one can conclude that desulfotomacula have the potential, during growth on lactate plus sulphate, for producing one net high-energy phosphate per sulphate ion reduced by substrate phosphorylation, as shown in figure 2. It is thus not necessary for desulfotomacula to carry out electron transfer-coupled phosphorylation during growth with organic substrates and sulphate. On the other hand, *Desulfovibrio* require two high-energy phosphates per sulphate reduced and, during growth on lactate plus sulphate, must carry out electron-transfer phosphorylation (Barton *et al.* 1972) to obtain net ATP for growth (Peck 1962), as shown in figure 3. This idea has been confirmed by a study of the relative growth yields of *D. vulgaris*† and *Dt. orientis*† on lactate–sulphate media as well as by enzymological studies. The utilization of

† We shall use the abbreviations '*D.*' for *Desulfovibrio* and '*Dt.*' for *Desulfotomaculum* in this contribution.

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PP_i as a source of energy for the growth of *Dt. nigrificans*, *Dt. ruminis* and *Dt. orientis* but not for the growth of *Desulfovibrio* is also consistent with these observations (Liu *et al.* 1982). Major differences between these two genera were not entirely unexpected because immunological analysis of extracts show little cross-reactivity between the genera (Postgate 1979).

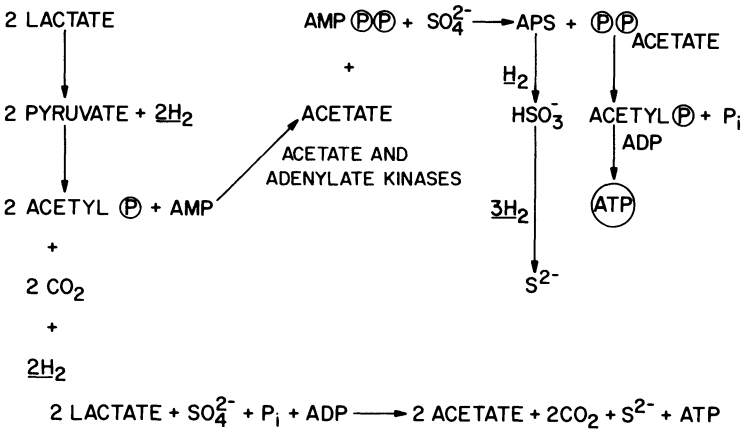

FIGURE 2. Bioenergetics of the growth of *Desulfotomaculum* on lactate plus sulphate.

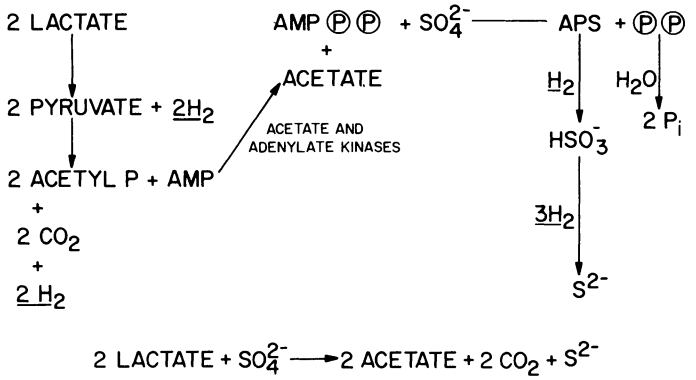

FIGURE 3. Bioenergetics of the growth of *Desulfovibrio* on lactate plus sulphate.

Conceptually, these conclusions allow one to distinguish the bioenergetics of respiratory sulphate reduction of *Desulfovibrio* from that of *Desulfotomaculum* and consider each as a separate physiological problem. The presence of a periplasmic hydrogenase of high specific activity and multiple *c*-type cytochromes in *Desulfovibrio* can no longer be considered as irrelevant for respiratory sulphate reduction because they are not found in *Desulfotomaculum* (Wood 1978).

From observations of H_2 production during growth (Tsuji & Yagi 1980; Traore *et al.* 1981), enzyme localization (Badziong & Thauer 1980; Odom & Peck 1981*a*) and vectorial electron transfer (Kroger 1978; Badziong & Thauer 1980), a chemiosmotic hydrogen cycle has been proposed as a mechanism by which *Desulfovibrio* produces the ATP required for growth on lactate plus sulphate (Odom & Peck 1981*b*). The scheme, shown in figure 4, involves the formation of molecular hydrogen, a permeant molecule, from lactate and pyruvate in the cytoplasm or on the cytoplasmic surface of the cell membrane and the rapid diffusion of H_2 across the cytoplasmic membrane. On the external surface of the membrane, the H_2 is oxidized

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by the periplasmic hydrogenase (Bell *et al.* 1974), which requires cytochrome c_3, and the electrons produced from this oxidation are transferred back across the membrane leaving the protons at the external surface of the membrane. The electrons are used in the cytoplasm for the reduction of sulphate to sulphide, which results ideally in the consumption of eight protons. The net effect is the transfer of eight protons across the cytoplasmic membrane without involving a

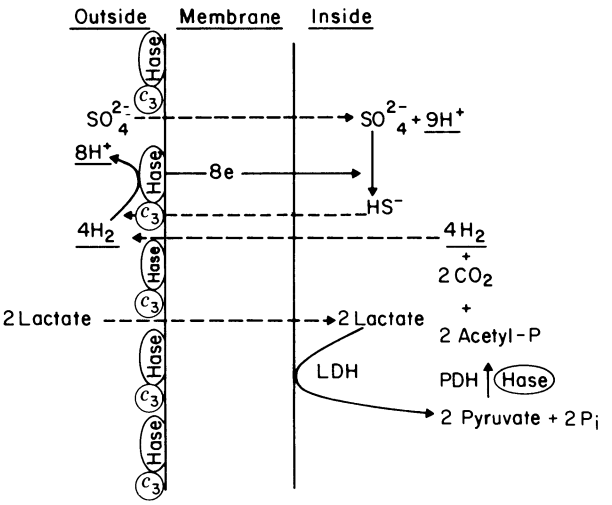

FIGURE 4. Cycling of hydrogen during growth on lactate plus sulphate. LDH, lactate dehydrogenase; Hase, hydrogenase; c_3, cytochrome c_3 (M_r = 130000).

typical Mitchell loop in which proton translocation is directly coupled to electron transfer. The proton gradient would then drive the synthesis of ATP in the conventional fashion via a reversible ATPase (Guarraia & Peck 1971). The scheme incorporates the ideas of Badziong & Thauer (1978) about the bioenergetics of growth on H_2 plus sulphate, and is supported by studies on enzyme localization (Badziong & Thauer 1980; Odom & Peck 1981*a*), the rapid production of protons coupled to the oxidation of H_2 with sulphite (C. L. Liu & H. D. Peck Jr, unpublished) and the restoration of lactate oxidation coupled to sulphate reduction in spheroplasts of *D. gigas* by the addition of pure hydrogenase and cytochrome c_3 (M_r = 13000). The scheme is also consistent with the growth yield studies of Badziong & Thauer (1978) if one assumes a proton requirement for the transport of sulphate or lactate or both into the cell. From the physiological aspect, the scheme can be used to understand H_2 production during growth on lactate plus sulphate (Tsuji & Yagi 1980; Hatchikian *et al.* 1976; Traore *et al.* 1981) and the curious ability of *Desulfovibrio* to function either as a H_2-producing (Bryant *et al.* 1977) or H_2-utilizing bacterium (Boone & Bryant 1980) in model microbial systems involving interspecies H_2 transfer.

The sulphate-reducing bactera belonging to the genus *Desulfovibrio* contain a splendid group of electron transfer proteins of low molecular mass as well as a number of unique reductases and dehydrogenases of higher molecular mass. A large amount of information is available concerning their structures and oxidation–reduction mechanisms; however, information concerning their function, particularly for the electron carriers, has been contradictory and inconclusive. This lack of information is due in part to the fact that not all the electron carrier proteins are uniformly distributed among *Desulfovibrio* (table 1) and to the contradictory

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results coming from cell-free reconstitution experiments. LeGall & Postgate (1973) proposed that the difficulties in the latter case have arisen from the disruption of cellular structures that localized or compartmentalized many of the electron carriers. The detailed information available about the structures of the electron transfer proteins has recently been reviewed in detail (LeGall *et al.* 1979, 1982) and will not be extensively reviewed here. Instead, we shall discuss in a speculative fashion the impact of the distribution of electron-transfer proteins in various species of *Desulfovibrio* and the hydrogen cycling mechanism on our ideas of the function of these electron transfer proteins and the electron donor and acceptor specificities of the dehydrogenases and reductases involved in respiratory sulphate reduction.

TABLE 1. DISTRIBUTION OF ELECTRON CARRIER PROTEINS IN *DESULFOVIBRIO*

	ferredoxins							cytochromes		
organism	$[Fe_4S_4]$	$2[Fe_4S_4]$	$[Fe_3S_3]$	Rb	Fl	Dr	MoFe/S	c_{553} or mono-haem	c_3 (13000)	c_3 (26000)
D. vulgaris (Hildenborough)	+	?	?	+	+	?	?	+	+	+
D. vulgaris (Miyazaki)	?	?	?	?	+	?	?	+	+	−
D. vulgaris (Marburg)	+	?	?	?	−	?	?	?	+	?
D. gigas	+	−	+	+	+	+	+	−	+	+
D. desulfuricans (Norway 4)	+	?	?	+	−	?	+	+	+	−
D. desulfuricans (27774)	?	?	?	+	+	?	?	?	+	?
D. desulfuricans (El Algheila 4)	+	?	?	?	+	?	?	?	+	?
D. africanus	2	+	?	+	−	?	+	?	+	?
D. salexigens	+	2	?	+	+	?	+	+	+	+

Rb, rubredoxin; Fl, flavodoxin, Dr, desulforedoxin, MoFe/S, molybdenum–iron–sulphur protein.

A specific requirement for the H_2-cycling mechanism is that reducing equivalents from organic substrates leading to H_2 production in the cytoplasm must not be diverted in the cytoplasm to the sulphate-reducing enzymes. This separation of electron transfer might be achieved by compartmentation, electron carrier specificity or regulation or a combination of these factors. It should always be kept in mind that micro-organisms in the genus *Desulfovibrio* may not be closely related, as evidenced by the primary structures of cytochromes c_3 (M_r = 13000) (LeGall *et al.* 1982) as well as their tertiary structures (Higushi *et al.* 1981) and that generalizations concerning the function of electron transfer proteins may not be valid for every species of *Desulfovibrio*.

2. PERIPLASMIC OR EXTERNAL ENZYMES AND ELECTRON TRANSFER PROTEINS

(*a*) *Hydrogenases*

Periplasmic or external hydrogenases have been conclusively demonstrated in *D. desulfuricans* (ATCC 27774) (Steenkamp & Peck 1981), *D. vulgaris* (Marburg) (Badziong & Thauer 1980), *D. vulgaris* (Hildenborough) (van der Westen *et al.* 1978) and *D. gigas* (Bell *et al.* 1974). Evidence for the external location of the hydrogenase of *D. desulfuricans* consist of whole-cell and extract

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assays coupled with the demonstration of a half-life for the change in pH accompanying H_2 oxidation with benzyl viologen, which is ten times faster than the corresponding half-life of the proton gradient generated by proton translocation with nitrite. In the other three cases, the external location of hydrogenase was demonstrated by the selective removal of hydrogenase from spheroplasts or intact cells. The external hydrogenases from the latter two micro-organisms have been purified to apparent homogeneity employing these selective elution procedures and are different with respect to molecular mass, subunit structure, specific activity and e.p.r. spectra. The purified hydrogenase from *D. vulgaris* (Mayhew *et al.* 1978) is composed of a single polypeptide chain, molecular mass 52000 Da, contains 12 iron and 12 sulphide atoms per molecule and exhibits a $g = 2.02$ e.p.r. in the oxidized state characteristic of 'high-potential type' iron–sulphur centres and the newly described three-iron–three-sulphur centres (Emptage *et al.* 1980). The reduced hydrogenase shows a low-intensity $g = 1.94$ e.p.r. signal (Van Dijk *et al.* 1982). The enzyme has a relatively high specific activity (*ca.* 3200) and is bidirectional. The hydrogenase from *D. gigas* has a molecular mass of 89000 Da and contains two subunits of molecular masses 62000 and 26000 Da (Hatchikian *et al.* 1978). It also contains 12 iron and 12 sulphide atoms per molecule but, in addition to a $g = 2.02$ e.p.r. signal in the oxidized state, exhibits a signal with g values at 2.31, 2.20 and 2.0 (LeGall *et al.* 1982*b*). The e.p.r. spectrum of the hydrogen-reduced enzyme consists of multiple species with a major set of g values at 2.17, 2.08 and 2.04. Based on the observations of Lancaster (1980, 1982) and the presence of one gram atom of nickel per mole of enzyme, it has been proposed that these new e.p.r. signals originate from the presence of a redox-sensitive Ni^{III} and that it is an important functional unit of this hydrogenase. This hydrogenase is also bidirectional but has a much lower specific activity (*ca.* 180) than the hydrogenase from *D. vulgaris*. In spite of these differences, which may in part be technical, the unique cellular localization of these hydrogenases and the fact that they both require cytochrome c_3 for activity (Bell *et al.* 1978; Yagi *et al.* 1968) with other electron transfer proteins suggest that these hydrogenases serve analogous functions in the metabolism of these two species of *Desulfovibrio*.

With regard to the localization of hydrogenases from other species of *Desulfovibrio*, *D. vulgaris* (Myazaki) has been shown to contain one soluble and two membrane-bound hydrogenases (Yagi *et al.* 1978). The latter hydrogenases have been solubilized and have molecular masses of 89000 and 180000 Da. The smaller hydrogenase is identical to that observed in the soluble extract, the both have been purified. They contain non-haem iron, exhibit specific activities of around 200 and require cytochrome c_3 for the reduction of other electron transfer proteins. It is curious that Tris–EDTA extraction of intact cells selectively elutes the high molecular mass hydrogenase rather than the low molecular mass hydrogenase as in *D. vulgaris* (Hildenborough). *D. desulfuricans* (Norway) contains a membrane-bound hydrogenase but it has not yet been extensively studied. Because of the high activities of hydrogenase observed with intact cells in the presence of non-permeant electron acceptors such as benzyl viologen, it seems reasonable to speculate that a significant fraction of total hydrogenase activity will be found to be localized on the external aspect of the cytoplasmic membrane in all species of *Desulfovibrio*; however, specific localization experiments must be done before this speculation can be accepted as completely valid.

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(*b*) *Formate dehydrogenase*

Formate dehydrogenase activity has been demonstrated to be periplasmic in *D. gigas* and *D. desulfuricans* (ATCC 27774). With spheroplasts of *D. gigas*, a significant fraction (30 %) of the dehydrogenase activity can be selectively eluted by treatment with EDTA (Odom & Peck 1981*a*). In *D. desulfuricans*, the specific activity of formate dehydrogenase is the same with intact cells and with extracts, again indicating an external localization. This conclusion is also supported by a rapid rate of proton production by intact cells in the presence of formate plus benzyl viologen (Steenkamp & Peck 1981). The formate dehydrogenase has only been partly purified (Yagi 1969, 1979; Riederer-Henderson & Peck 1970) and no definitive evidence is available about its physical properties; however, some information is available about its electron donor specificity. The dehydrogenase from *D. vulgaris* utilizes the periplasmic monohaem cytochrome c_{553} as its immediate electron acceptor but will not reduce cytochrome c_3 (M_r = 13000). In contrast, the partly purified formate dehydrogenase from *D. gigas* reduces cytochrome c_3 (M_r = 13000) but not the monohaem cytochrome. The association between *c*-type cytochromes and formate dehydrogenase is clearly indicated by the observation that, with *D. gigas*, growth with formate and sulphate leads to an increase in *c*-type cytochromes; however, the cytochromes were not specifically identified (Odom & Peck 1981*a*). As cytochrome c_{553} does not occur in *D. gigas* it appears that cytochrome c_3 (M_r = 13000) functions as a cofactor for formate dehydrogenase as well as hydrogenase in this bacterium. This localization of formate dehydrogenase is similar to that reported for the formate dehydrogenase of *Wolinella succinogenes* (formerly *Vibrio succinogenes* (Kroger 1978), where it is postulated to be involved in the generation of a proton gradient by vectorial electron transfer during growth on formate and fumarate. The existing evidence on the localization of formate dehydrogenase in *Desulfovibrio* suggests a similar function in these organisms.

(*c*) *Nitrite reductase*

Although growth with nitrate is rare among desulfovibrios, a new type of nitrite reductase has recently been isolated from *D. desulfuricans* (ATCC 27774) grown on lactate plus nitrate. It has a molecular mass of 65000 Da and contains six *c*-type haems per molecule (Liu & Peck 1981*b*). The reductase is largely membrane-bound and appears to be closely associated with hydrogenase (Steenkamp & Peck 1980). Proton translocation studies with H_2 and nitrite suggest that the proton-binding and nitrite-binding sites are located on the external aspects of the cytoplasmic membrane but the binding site for benzyl viologen appears to be internal. Cells grown on lactate plus sulphate contain lowered levels of the reductase (D. J. Steenkamp & H. D. Peck, Jr, unpublished) and nitrite reductase activity coupled to ATP formation has been found in *D. gigas*, which does not grow on nitrate (L. L. Barton, M. Odom, J. LeGall & H. D. Peck, Jr, unpublished). This activity is membrane-bound and thus does not reflect the nitrite reducing activity of bisulphite reductase (Lee *et al.* 1973*a*), which is located exclusively in the cytoplasm. Intact cells of *D. gigas* catalyse a rapid scalar utilization of protons coupled to the oxidation of H_2 with nitrite, as is observed with nitrite-grown cells of *D. desulfuricans*; however, it has not been conclusively demonstrated that the enzyme responsible for nitrite reduction in *D. gigas* is the hexahaem reductase found in *D. desulfuricans*. From studies with *D. gigas* and *D. desulfuricans* it is suggested that the benzyl viologen-binding site is localized on the interior

aspects of the cytoplasmic membrane and the reductase has been tentatively localized as transmembraneous (Odom & Peck 1981 *a*).

(*d*) *Cytochrome* c_3 (M_r = 13000)

The first evidence for the periplasmic location of this cytochrome was the report of LeGall *et al.* (1965) indicating that a substantial amount of the cytochrome c_3 of *D. gigas* could be removed by washing intact cells and that the remaining cytochrome was largely membrane-bound. More recently, these initial observations have been confirmed by Badziong & Thauer (1980) for *D. vulgaris* (Marburg) and Mayhew *et al.* (1978) for *D. vulgaris* (Hildenborough). In the former report, only 8% of the cytochrome c_3 was found in the cytoplasm but in the latter report no information about cytoplasmic cytochrome c_3 was presented, however, the authors clearly indicated that the cytochrome c_3 is periplasmic and present in a 4.5:1 ratio with hydrogenase. Other workers (Odom & Peck 1981 *a*) have found substantial amounts of *c*-type cytochromes in the cytoplasm and on the membrane; however, this situation may well result from the incomplete removal of cytochromes from the external aspect of the cytoplasmic membrane, or the presence of another cytochrome c_3 (M_r = 26000, earlier called cytochrome cc_3) in the cytoplasm. As pointed out by Thauer & Badziong (1980), it is difficult to rationalize the presence of cytochrome c_3 in both the cytoplasm and periplasm if one takes into consideration the modifications of primary structure that occur with known mechanisms for the transport of proteins across membranes and we have concluded that in all probability cytochrome c_3 (M_r = 13000) is exclusively localized on the outer aspect of the cytoplasmic membrane.

Cytochrome c_3 (M_r = 13000) is a cofactor for the hydrogenase of *Desulfovibrio* in that it is required for the reduction of ferredoxin, flavodoxin and rubredoxin by hydrogenase plus H_2 (Yagi *et al.* 1968; Bell *et al.* 1978). N.m.r. and e.p.r. data suggest a specific interaction between cytochrome c_3 and the high-potential ferredoxin II purified from *D. gigas* (Xavier *et al.* 1979; Moura *et al.* 1980*a*). Cytochrome c_3 has also been reported to stimulate the exchange reaction catalysed by hydrogenase; however, this is not a universal observation and may be due to the removal of traces of O_2 by the cytochrome because the reduced form is readily auto-oxidizable (Berlier *et al.* 1982). A third function of cytochrome c_3 is that of a sulphur reductase in species of *Desulfovibrio* that can grow on organic substrates plus colloidal sulphur (Fauque *et al.* 1979). With the use of inverted membrane vesicle preparations it has been possible to demonstrate electron transfer-coupled phosphorylation with this simple system (Fauque *et al.* 1980). Although the phosphorylation may not be of physiological significance, these data strongly suggest that the membrane-bound cytochrome c_3 (M_r = 13000) is accessible to the colloidal sulphur from either side of the cytoplasmic membrane. Cytochrome c_3 has also been observed to stimulate a number of reactions in extracts of *Desulfovibrio* involving either the production or utilization of H_2; however, the cytochrome was never shown to be the immediate electron acceptor or donor in these reactions. The data now available indicate that these stimulations reflect the effect of cytochrome c_3 on hydrogenase activity rather than specific dehydrogenases or reductases. Finally, during the purification of hydrogenase, there is always a close association with cytochrome c_3 and we suggest that the cytochrome may be required to bind hydrogenase to the cytoplasmic membrane as well as the transfer of electrons across the membrane. An explanation is still lacking for the presence of four *c*-type haems in this molecule, their sophisticated oxidation–reduction properties (LeGall *et al.* 1982) and multiple oxidation–reduction potentials (DerVartanian *et al.* 1978).

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(*e*) *Cytochrome* c_{553}

Cytochrome c_{553} was first detected in *D. vulgaris* Hildenborough (LeGall & Bruschi 1968): it is an auto-oxidizable monohaem cytochrome found in most of the species of *Desulfovibrio* except *D. gigas* (table 1) and is related to the cytochrome *c* family (Almassy & Dickerson 1978). In *Desulfovibrio vulgaris* (Hildenborough) it has been reported to be present in a ratio of three cytochrome molecules per molecule of hydrogenase (Mayhew *et al.* 1978) in the periplasmic proteins. As mentioned earlier, cytochrome c_{553} is reduced by partly purified preparations of formate dehydrogenase (Yagi 1969), and the localization of both proteins in the periplasmic area suggests the cytochrome c_{553} is the immediate electron donor for the formate dehydrogenase

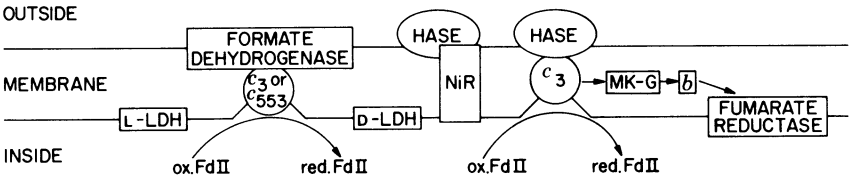

FIGURE 5. Localization of electron transfer components, dehydrogenases and reductases relative to the cytoplasmic membrane. L–LDH, L-lactate dehydrogenase; D-LDH, D-lactate dehydrogenase; c_3, cytochrome c_3 (M_r = 13000); c_{553}, cytochrome c_{553}; NiR, nitrite reductase; *b*, cytochrome *b*; MK-6, menaquinone-6; Fd II, ferredoxin II, Hase, hydrogenase.

although one must be aware of possible artefacts when dealing with relatively high-potential cytochromes. A soluble D-lactate dehydrogenase has recently been found in extracts of *D. vulgaris* (Miyazaki) and *D. vulgaris* (Hildenborough) which reduces cytochrome c_{553} (Ogata *et al.* 1981); however, the low specific activity of the enzyme with the cytochrome and the localization of the proteins (lactate dehydrogenase inside; cytochrome c_{553} outside) suggest that the cytochrome may not be the physiological electron acceptor for the D-lactate dehydrogenase. We therefore propose (figure 5) that in most species of *Desulfovibrio*, cytochrome c_{553} serves to bind the formate dehydrogenase to the membrane and channel electrons across the membrane; however, in *D. gigas* the cytochrome c_3 (M_r = 13000) functions with both hydrogenase and formate dehydrogenase.

(*f*) *Membrane organization*

Our present idea about the organization of proteins around the membrane of *Desulfovibrio* is presented in figure 5. Formate dehydrogenase, hydrogenase, cytochrome c_3 (M_r = 13000) and cytochrome c_{553} are operationally periplasmic proteins; however, cytochrome c_3 can reduce colloidal sulphur on the cytoplasmic aspect of the membrane and must be assumed to transfer electrons to cytoplasmically located electron acceptors such as ferredoxin II. Nitrite reductase has been postulated to be transmembranous and closely associated with hydrogenase as previously discussed. Cytochrome *b* and menaquinone-6 have been clearly demonstrated to be membrane-bound (Badziong & Thauer 1980; Hatchikian & LeGall 1972) and the increased amounts of cytochrome *b* observed during growth with fumarate suggests that the cytochrome *b* is associated with fumarate reductase (Hatchikian & LeGall 1972; Odom & Peck 1981); however, in *D. vulgaris* (Marburg), which lacks a fumarate reductase but does synthesize cytochrome *b*. Electron flow is proposed to proceed through *c*-type cytochromes to cytochrome *b*, based on steady-state studies of the redox states of the cytochromes (J. M. Odom & H. D.

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Peck, Jr, unpublished). The localization of the lactate dehydrogenases will be discussed in § 3*a*. In conclusion, the available data are consistent and support the idea of energy coupling based on the concept of vectorial electron transfer, as proposed by Kroger(1978) and Badziong & Thauer (1980).

3. Cytoplasmic electron transfer

(*a*) D-*Lactate and* L-*lactate dehydrogenases*

Lactate dehydrogenase activities have been shown to be internal and located on the membrane fraction of *D. desulfuricans* (27774), indicating that these activities are localized on the cytoplasmic aspect of the cytoplasmic membrane (Steenkamp & Peck 1981). The presence of a

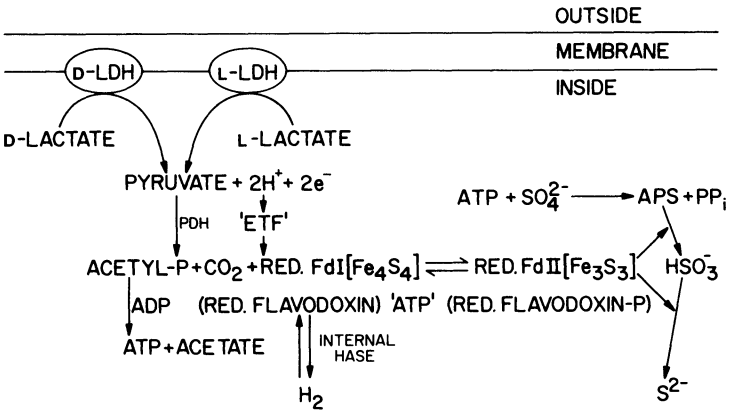

FIGURE 6. Cytoplasmic electron transfer. D-LDH, D-lactate dehydrogenase; L-LDH, L-lactate dehydrogenase; ETF, electron transfer flavoprotein; PDH, pyruvate dehydrogenase; Hase, hydrogenase; Fd, ferredoxin; Fl, flavodoxin.

pyridine nucleotide-independent D-lactate dehydrogenase has recently been reported (Ogata *et al.* 1981; Czechowski & Rossmore 1980), from the use of artificial electron acceptors in extracts of *D. vulgaris* (Miyazaki), *D. vulgaris* (Hildenborough) and *D. desulfuricans*. The activity was found in both the soluble and membrane fractions. In extracts of *D. desulfuricans* (27774) we have found the presence of both D- and L-lactate dehydrogenases, which can be clearly differentiated on the basis of their sensitivities to sulphydryl reagents such as iodoacetate. Thus the presence of sulphydryl-containing compounds such as β-mercaptoethanol stabilizes the L-lactate dehydrogenase but inactivates the D-lactate dehydrogenase which requires Zn^{2+} for activity. Intact cells of *Desulfovibrio* oxidize D,L-lactate to completion (Ogata *et al.* 1981), and these observations indicate that the utilization of D,L-lactate by *D. desulfuricans* involves two specific dehydrogenases. An L-lactate dehydrogenase has been found in extracts of *D. gigas*, which is also pyridine nucleotide-independent and membrane-bound (L. L. Barton, T. Farooqui & H. D. Peck, Jr, unpublished); however, no evidence for the presence of a D-lactate dehydrogenase was obtained. Instead, extracts of *D. gigas* appear to utilize D-lactate by means of a racemase and L-lactate dehydrogenase.

The scheme of H_2 cycling presented in figure 4 indicates that H_2 is produced from the oxidation of lactate to pyruvate; however, this is a thermodynamically unfavourable reaction, in that $E^{\circ\prime}$ of the lactate–pyruvate couple is -190 mV while that of the H^+/H_2 couple is -450 mV. Although *Desulfovibrio* do not exhibit significant growth on lactate in the absence of sulphate,

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in the presence of a H_2-utilizing methanogenic bacterium, lactate is converted to H_2 and used for the formation of methane in interspecies H_2 transfer (Bryant *et al.* 1977). The electron-transfer proteins involved in the formation of H_2 from lactate have not yet been investigated, but it is proposed in figure 6 by analogy with the D-lactate dehydrogenase of *Megasphaera elsdenii* (Brockman & Wood 1975) that an electron transfer flavoprotein (ETF) is the electron acceptor for both dehydrogenases. The electrons are then shown to be transferred to ferredoxin containing the four-iron centre and finally to hydrogenase.

(*b*) *Pyruvate dehydrogenase*

All species of *Desulfovibrio* contain a pyruvate phosphoroclastic activity which converts pyruvate to acetyl phosphate, CO_2 and H_2. The system is cytoplasmic (Odom & Peck 1981 *a*), requires thiamine pyrophosphate, coenzyme A and Mg^{2+} for activity and is stimulated by the presence of ATP (Yates 1967). This pyruvate dehydrogenase has not been purified from any of the sulphate-reducing bacteria and consequently has not been extensively studied; however, it appears to be similar to the clostridial pyruvate dehydrogenase, which is a non-haem iron protein and uses ferredoxin as its immediate electron acceptor (Uyeda & Rabinowitz 1971). Both the pyruvate–CO_2 exchange (Suh & Akagi 1966) and the evolution of H_2 from puruvate (Akagi 1967) have been shown to require cytochrome c_3 (M_r = 13000) and ferredoxin for maximum activity. An explanation for this result would be that the stimulation of the reactions by cytochrome c_3 (M_r = 13000) is due to the mixing of the external hydrogenase and cytochrome c_3 with the internal hydrogenase and ferredoxin. The crude extract would therefore not reflect the physiological situation. In *D. gigas* the stimulation by ferredoxin is complicated by the fact that the ferredoxin used in these and other studies may have been a mixture of ferredoxins containing both four-iron and three-iron centres (Huynh *et al.* 1980). It has been demonstrated that only the ferredoxin containing the four-iron centre is active in coupling the pyruvate dehydrogenase with hydrogenase in extracts devoid of ferredoxin and flavodoxin (Moura *et al.* 1978). In figure 6 the four-iron ferredoxin is shown as both the electron donor for hydrogenase and the electron acceptor for pyruvate dehydrogenase and ETF.

(*c*) *Ferredoxins*

The ferredoxin from *D. gigas* was the first such protein to be shown to contain four iron atoms and four sulphide residues per molecule (Laishley *et al.* 1969), and this observation has been confirmed by the amino acid sequence (Travis *et al.* 1971; Bruschi 1979). The ferredoxin is active in the phosphoroclastic reaction (Hatchikian & LeGall 1970) and in the reduction of sulphite (LeGall & Dragoni 1966), APS (L. J. Guarraia and H. D. Peck, Jr, unpublished) and possibly thiosulphate (Hatchikian *et al.* 1972) to sulphide. Ferredoxin has not been definitively localized in the sulphate reducing bacteria; however, its presence in cell washes has not been reported and we have not found significant quantities in the periplasmic proteins. We agree with Thauer & Badziong (1981) that these non-haem iron proteins are most probably restricted to the cytoplasm; however, it has been reported that *Rhodospirillum rubrum* excretes a ferredoxin as well as hydrogenase (Hiura *et al.* 1981).

More recently, ferredoxin from *D. gigas* has been shown to exist in two forms, ferredoxin I and ferredoxin II, which can be separated by chromatographic procedures. Both forms of ferredoxin contain the same basic monomeric unit but the ferredoxin I was shown to be a trimer and the ferredoxin II a tetramer (Bruschi *et al.* 1976). A third form of ferredoxin was also

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observed but this now appears to be a mixture of ferredoxin I and ferredoxin II. The ferredoxin I is a typical ferredoxin in that it is diamagnetic in its oxidized state and has an E°′ of −440 mV; on the other hand, the ferredoxin II is a paramagnetic in its oxidized form and has an E°′ of −130 mV, approximately 300 mV more than the trimeric form (Cammack *et al.* 1977). The ferredoxin has now been demonstrated to contain a novel three-iron–three-sulphur (Huynh *et al.* 1980), similar to that found in aconitase (Kent *et al.* 1981) and ferredoxin from *Azotobacter vinelandii* (Emptage *et al.* 1980). The ferredoxin II containing the three-iron centre is not active in catalysing the production of H_2 from pyruvate in the phosphoroclastic reaction; however, ferredoxin II is active in stimulating the oxidation of H_2 in the presence of sulphite (Moura *et al.* 1978). Presumably this reaction involves cytochrome c_3 and a specific interaction between cytochrome c_3 and ferredoxin II has been noted. It should be cautioned that the stimulation of activity was observed with crude preparations and it is possible that additional electron transfer proteins are required for H_2–sulphite couple. Conversely, ferredoxin I containing mainly the four-iron centre is less efficient in catalysing the oxidation of H_2 with sulphite but is fully active in the phosphoroclastic reaction. A most interesting and exciting observation concerns the chemical and biological interconversions of the three- and four-iron centres. When ferredoxin II is incubated with extract plus pyruvate, after a 30 min lag, there is some production of H_2. This activity is accompanied by the appearance of an e.p.r. signal at $g = 1.94$ characteristic of four-iron centre and the result has been interpreted to indicate that the three iron centre was converted to a four-iron centre, i.e. ferredoxin II to ferredoxin I (J. J. G. Moura, A. V. Xavier & J. LeGall, Jr, unpublished). By employing Mössbauer and e.p.r. spectroscopy, the chemical interconversions of ferredoxin I and ferredoxin II have been observed (Moura *et al.* 1982; Kent *et al.* 1982*a*). Incubation of the apoprotein from ferredoxin II in the presences of excess iron and sulphide produced a ferredoxin containing a four-iron centre which was similar to ferredoxin I and could be converted to a three-iron centre by treatment with ferricyanide. Incubation with stoichiometric amounts of iron and sulphide leads to a reconstitution of the three-iron centre. The ease with which these interconversions occur raises the question as to whether the three-iron centres have physiological significance or simply result from the oxidation of four-iron centres. Although the conversion of a three-iron centre to a four-iron centre accompanying the reductive activation of beef-heart aconitase (Kent *et al.* 1982*b*) does not necessarily support a physiological role for three-iron centres, the changes in specificity accompanying the conversion of ferredoxin I to ferredoxin II does support the idea that three-iron centres have physiological importance. Because of the requirement of H_2 cycling for the separation of electron transfer leading to H_2 production on the one hand and H_2 utilization on the other, we propose in figure 6 that the interconversion of ferredoxins I and II plays a central role in regulating this electron transfer. Thus, in different modes of growth it is expected that different concentrations of the two ferredoxins will be observed. At the extreme, those strains capable of growth on H_2 and sulphate would be expected to have largely ferredoxin II whereas those growing fermentatively on pyruvate or by interspecies H_2 transfer would be expected to contain largely ferredoxin I. Under natural conditions where the bacteria are presented with an array of different substrates the interconversion of these two ferredoxins may provide a mechanism for the regulation of electron transfer. The biophysical studies required for the characterization of ferredoxin have only been completed with the ferredoxins from *D. gigas*; however, the presence of multiple forms of ferredoxin in *D. desulfuricans* Norway 4 (Bruschi *et al.*

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1977) and in *D. africanus* (Hatchikian *et al.* 1979; Hatchikian & Bruschi 1981) suggests that the foregoing considerations or some modification of them apply to the regulation of electron transfer in other members of *Desulfovibrio*.

(*d*) *Flavodoxin*

The flavodoxin of *D. vulgaris* has been extensively studied, and both its primary (Dubourdieu *et al.* 1973) and tertiary structures (Watenpaugh *et al.* 1972) have been established. It has been reported to be absent from the periplasmic proteins and is assumed to be a cytoplasmic protein

TABLE 2. THE EFFECT OF FERREDOXIN AND FLAVODOXIN ON PHOSPHORYLATION COUPLED TO THE OXIDATION OF HYDROGEN WITH SULPHITE BY EXTRACTS OF *DESULFOVIBRIO GIGAS*

(L. L. Barton & H. D. Peck, Jr, unpublished data.)

		activity† μmol/20 min	
soluble protein	additions	H_2 oxidized	P_i esterified
crude	none	16.6	1.9
treated‡	none	0	0
treated	ferredoxin§ (0.09 mg)	12.5	0.6
treated	ferredoxin§ (0.18 mg)	15.0	1.6
treated	flavodoxin (0.1 mg)	8.5	0.2
treated	flavodoxin (0.2 mg)	11.8	0.1
treated	ferredoxin§ (0.09 mg) plus flavodoxin (0.1 mg)	11.5	0.8

† Preparation of extracts and assay as described by Peck (1965).
‡ Crude extracts treated with DEAE-cellulose to remove endogenous ferredoxin and flavodoxin.
§ Ferredoxin was an undefined mixture of ferredoxin I and II.

(Mayhew *et al.* 1978). Its spectrum of biological activities is very similar to that of ferredoxin in that it replaces ferredoxin in both H_2-producing reactions and in H_2-utilizing reactions. A similarity in function is also indicated by the reciprocal relation that exists between iron and the biosynthesis of flavodoxin and ferredoxin (Knight & Hardy 1966); however, this relation has not been established for the sulphate-reducing bacteria (LeGall *et al.* 1979). There also exists an important biochemical similarity between flavodoxin and ferredoxin in that flavodoxin has two stable oxidation–reduction states; the semiquinone–hydroquinone form ($E^{\circ\prime} = -440$ mV) and the semiquinone–quinone form ($E^{\circ\prime} = -150$ mV). This should be compared with the potentials of ferredoxin I ($E^{\circ\prime} = -440$ mV) and ferredoxin II ($E^{\circ\prime} = -130$ mV), and this oxidation–reduction behaviour of the two electron-transfer proteins is entirely consistent with the observed biological interchangeability. The possible importance of the two oxidation–reduction states of flavodoxin in biological systems is indicated by the report of Petitdemange *et al.* (1979) concerning the substitution of flavodoxin for ferredoxin in the oxidation of NADH by NADH–ferredoxin oxidoreductase. Flavodoxin is reduced to the blue neutral flavin semiquinone in the presence of NADH and extract from *C. tyrobutyricum*; however, the fully reduced form of flavodoxin is produced in the presence of acetyl-CoA. Edmondson & James (1979) have reported the presence of an acid-stable covalently bound phosphate in flavodoxin from *Azotobacter vinelandii* and have found the flavodoxin of *D. gigas* to contain tightly bound acid-labile

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phosphate (D. Edmondson, personal communication). By analogy with the ferredoxins, we propose that there exist two biologically active forms of flavodoxin: one form, indicated in figure 6 as a non-phosphorylated flavodoxin, that only exhibits activity in the phosphoclastic reaction, and a second form, indicated in figure 6 as a phosphorylated flavodoxin, that has activity in the phosphoclastic reaction and as electron donor for the reduction of APS and bisulphite. Neither form of flavodoxin reacts with membrane-bound cytochrome c_3. This would allow the bacteria to circumvent the H_2-cycling mechanism when substrates are oxidized that provide sufficient ATP for growth by substrate-level phosphorylation. An example of this type of growth may be with pyruvate plus sulphate where growth yields are lower than expected (Magee *et al.* 1978). Other considerations offer rationalizations for a number of unexplained observations concerning flavodoxin. Barton & Peck (1970) reported that electron transfer between hydrogenase and sulphite reductase of *D. gigas* could be restored by the addition of either ferredoxin or flavodoxin; however, electron-transfer phosphorylation was only reconstituted by the addition of ferredoxin, as shown in table 2. As indicated in figure 6, ferredoxin II is postulated to couple sulphite reduction with vectorial electron transfer, whereas flavodoxin could restore electron transfer by coupling bisulphite reductase with soluble periplasmic hydrogenase and cytochrome c_3 (M_r = 13000). *D. desulfuricans* (Norway) has been shown to lack flavodoxin (LeGall *et al.* 1979); however, all *Desulfovibrio* species that have been investigated possess ferredoxins. According to the scheme in figure 6, flavodoxin is not essential for growth on lactate plus sulphate.

(*e*) *Internal hydrogenase*

The first evidence for the existence of multiple forms of hydrogenase in a large number of microorganisms, including *D. desulfuricans*, was provided by Ackrell *et al.* (1966), but the significance of these observations was obscured by the demonstration that the multiple bands of hydrogenase were produced by adding purified hydrogenase to crude proteins of *C. pasteurianum* (Nakos & Mortenson 1971). More recently the existence of multiple hydrogenases has been shown in a number of different physiological types of bacteria, for example *C. pasteurianum* (Chen & Blanchard 1978), *Rhodospirillum rubrum* (Hiura *et al.* 1979), *Megasphera elsdenii* (Mayhew *et al.* 1978) and *Alcaligenes eutrophus* (Schink 1978).

Two hydrogenases, termed hydrogenase II (M_r = 66000) and hydrogenase I (M_r = 180000) have been isolated from *D. vulgaris* (Miyazaki) by Yagi *et al.* (1978); however, no differences in the catalytic properties of the two hydrogenases were noted. Hydrogenase I contains three subunits and can be converted to hydrogenase II by treatment with trypsin. Hydrogenase II has a single subunit and is unaltered by incubation with trypsin. In contrast to the results of Mayhew *et al.* (1978), the high molecular mass hydrogenase I appears to be periplasmic and the lower molecular mass hydrogenase II cytoplasmic. The presence of both membrane-bound and periplasmic hydrogenases has been reported in *D. desulfuricans* (Martin *et al.* 1980) and data have been presented to suggest that the two hydrogenases are different proteins. Further work is required to resolve this interesting problem.

A second approach to the problem of a second or cytoplasmic hydrogenase has been to investigate hydrogenase activities of whole cells or spheroplasts with impermeant electron donors or acceptors and to compare these results with hydrogenase activities found with extract or detergent-treated cells. Postgate (1965) showed that treatment of intact cells of *D. vulgaris* with detergent altered the electron-acceptor specificity of hydrogenase as determined with artificial electron acceptors. More recently, differences in the specific activity of hydrogenase have been

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observed between intact cells or spheroplasts and extracts, depending on growth conditions (Peck & Odom 1981; Odom & Peck 1981 *b*). By using four different experimental variations (sulphate limitation in a chemostat growth phase, different substrates and growth by inter-species H_2 transfer), consistent ratios of 2.0 for extract to intact cell hydrogenase activities with benzyl viologen have been observed with *D. vulgaris* and *D. gigas*. For the sake of comparison it should be noted that *Dt. ruminis* exhibits only a membrane-bound internal hydrogenase. The existing data strongly suggest that *Desulfovibrio* species have at least one external and one internal hydrogenase; however, definitive evidence for the existence of two hydrogenase must await purification and characterization of the specific enzymes.

(*f*) *APS reductase*

APS reductase is a cytoplasmic enzyme constituting 2–3 % of the soluble protein, and no evidence exists to indicate any association with the cytoplasmic membrane. It is a non-haem iron flavoprotein and its chemical and physical properties are presented in table 3 (Bramlett & Peck 1975). Cytochrome c_3 (M_r = 13000), ferredoxin and flavodoxin stimulate the reduction of sulphate in crude extracts but have not been demonstrated to function as immediate electron donors for the reductase. Reduced methyl viologen, oxygen, ferricyanide and cytochrome *c* have been used for the assay of enzymatic activity and a scheme (figure 7) has been proposed for the mechanism of action of APS reductase (Peck & Bramlett 1982). The function of the non-haem iron is of interest as it can be largely removed without effecting enzymatic activities with oxygen, cytochrome *c* or reduced methyl viologen. Enzymic activity with ferricyanide is destroyed; however, it is highly unlikely that the physiological function of the non-haem iron centres is the reduction of ferricyanide. In all probability the non-haem iron clusters represent the site at which the natural electron donor(s) interact with APS reductase. Demonstrating the natural electron donor may prove to be exceptionally difficult as we do not know whether the non-haem iron clusters of the purified enzyme are intact and capable of interacting with the natural electron donor.

(*g*) *Bisulphite reductase*

Two different bisulphite reductases are found in *Desulfovibrio* species, desulfoviridin (Lee & Peck 1971) and desulforubidin (Lee *et al.* 1973 *b*), and their physical and chemical properties are presented in table 4 (C.-L. Liu & H. D. Peck, Jr, unpublished). The two reductases differ with regard to their major absorption peaks (desulfoviridin, 580 and 628 nm; desulforubidin, 545 and 580 nm), e.p.r. spectra (Murphy *et al.* 1973) and behaviour of their sirohaem moieties. Although e.p.r. spectroscopy indicates the presence of a haem group in each reductase, desulforubidin yields a sirohaem upon denaturation, whereas desulfoviridin yields a siroporphyrin (Murphy & Siegel 1973), which is responsible for the characteristic red fluorescence observed in alkali generally diagnostic of *Desulfovibrio* species (Postgate 1959). In spite of these significant differences, the two bisulphite reductases are very similar with regard to kinetic properties (Lee *et al.* 1973 *b*; Jones & Skyring 1975), products (trithionate, thiosulphite and sulphide), sirohaem and siroporphyrin content, non-haem iron, molecular mass and subunit structure (table 4). Both reductases occur in the cytoplasm, up to 7 % of the soluble protein, and there is no physical evidence to indicate that the reductases are membrane-associated. However, Drake & Akagi (1978) have proposed that the reductases are membrane-associated to account for a shift in products observed in the presence of membranes. The presence of 12 non-haem

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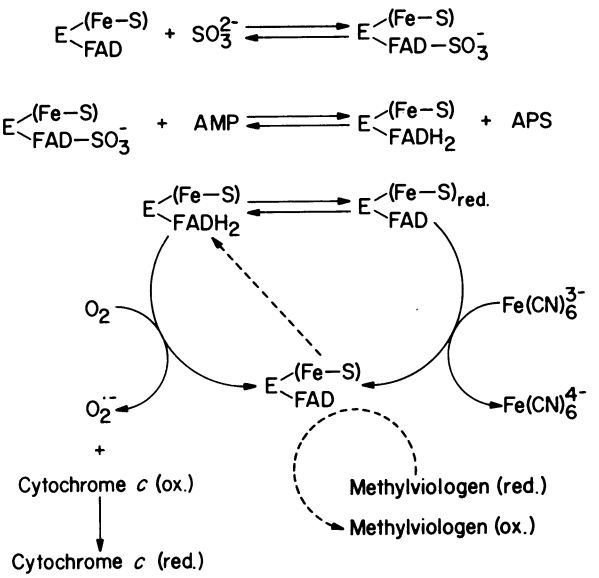

FIGURE 7. Mechanism of APS reductase.

TABLE 3. CHEMICAL AND PHYSICAL PROPERTIES OF APS REDUCTASE

monomeric M_r (Tris–maleate buffer or 10 mM AMP)	218500
$S_{20.w}$	9.8
subunit M_r	72000, 20000
dimeric M_r (phosphate buffer)	439000
$S_{20.w}$	16.2
moles iron/220000 Da	12.7
Moles sulphide/220000 Da	12.3
moles FAD/220000 Da	1.1

TABLE 4. PHYSICAL AND CHEMICAL PROPERTIES OF BISULPHITE REDUCTASE FROM *DESULFOVIBRIO*

	desulfoviridin, *D. gigas*	desulforubidin, *D. desulfuricans* Norway
M_r	200860	225000
absorption peaks/nm	374, 390, 408, 580, 628	392, 545, 580
non-haem Fe	14.6–16	14.8
acid-labile sulphide	14.0	14.7
total iron	16.5	16.6
sirohaem	—	+
haem number	—	2.0
siroporphyrin	2.0	—
dithionite	—	+
pyridine	—	+
CO	—	+
subunits	45000	45000
	50000	50000
structure	$\alpha_2\beta_2$	$\alpha_2\beta_2$

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iron atoms in both APS reductase and the bisulphite reductases suggest the possibility that both types of reductases share a common electron donor, as shown in figure 6, and ferredoxin and flavodoxin have been shown to stimulate the reduction of sulphite with hydrogen (Hatchikian *et al.* 1972) as well as APS. More recently, it has been shown that ferredoxin I is not very efficient in coupling hydrogenase and bisulphite reductase in extracts of *D. gigas* but ferredoxin II is fully active in the same system (Moura *et al.* 1978). It is proposed in figure 6 that ferredoxin II will also be found to be an electron donor for APS reductase. The non-haem iron clusters do exhibit differences by e.p.r. spectroscopy; APS reductase has an e.p.r. signal at $g = 2.0$ in the oxidized state and at $g = 1.94$ in the reduced state (Peck & Bramlett 1982), whereas desulforubidin is 'e.p.r.-silent' in both the oxidized and reduced states (C.-L. Liu, D. V. DerVartanian, J. M. Anderson & H. D. Peck, Jr, unpublished). This latter observation is in contrast to other reports of e.p.r.-active non-haem iron clusters (Liu *et al.* 1979; Hall *et al.* 1979) that may be due to the presence of contaminating non-haem iron proteins or partial denaturation of the bisulphite reductases.

As Akagi (1981) has comprehensively reviewed the development of the 'trithionate pathway' as the mechanism for respiratory sulphite reduction, we shall only summarize the evidence for and against the existence of the pathway. It is firmly established that desulfoviridin, desulforubidin, and P_{582} from *Dt. nigrificans* (Akagi & Adams 1973) irreversibly produce trithionate and thiosulphate, the proportions depending on assay conditions. Because of the irreversible formation of trithionate and thiosulphate, the pathway requires the presence of specific reductases for trithionate and thiosulphate. A specific thiosulphate reductase has been found (Ishimoto *et al.* 1955) and purified from extracts of the several sulphate-reducing bacteria (Haschke & Campbell 1971; Hatchikian 1975). The demonstration of a specific trithionate reductase has been more elusive but Drake & Akagi (1977*a*) have described an enzyme that forms thiosulphate from trithionate and sulphite by a mechanism analogous to the cyanolysis of trithionate, with the resulting thiosulphate molecule derived from added sulphite and the sulphane atom of the trithionate. Physiological evidence in support of the trithionate pathway is lacking; however, there is evidence to suggest that the pathway does not operate in intact cells. Chambers & Trudinger (1975) demonstrated with intact cells of *D. desulfuricans* and *D. gigas* grown on lactate plus sulphate that trithionate and thiosulphate do not accumulate as free intermediates. They also showed that the labelling pattern of thiosulphate in the presence of $^{35}SO_4^{2-}$ was not consistent with the operation of the trithionate pathway. In our laboratory, we have observed rapid proton production coupled to the reduction of sulphite with hydrogen, but no proton production was observed with either thiosulphate or trithionate although they were reduced by the intact cells. If thiosulphate and trithionate are intermediates in the reduction of sulphite, one would expect that proton production would be coupled to their reduction with hydrogen (C.-L. Liu & H. D. Peck, Jr, unpublished). From the enzymological point of view, the six-electron reduction of sulphite to sulphide is observed when the concentration of sulphite is low and that of the electron donor, reduced methyl viologen, high (Kobayashi *et al.* 1972). We have investigated the electrochemical properties of desulforubidin (C.-L. Liu, J. M. Anderson & H. D. Peck, Jr, unpublished) and found that the reduced reductase is oxidized by sulphite and produces 0.8 μmol of sulphide per μmol of enzyme, indicating a six-electron reduction. Another major problem for the trithionate pathway is the absence of a classical trithionate reductase (Akagi 1981). Trithionate reduction occurs in crude extracts but the activity appears to be due to a non-enzymatic cleavage of trithionate by a thiol to form sulphite and thiosulphate, which

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are further metabolized by thiosulphate reductase and bisulphite reductase (Odom 1978). We have been able to confirm the presence of the thiosulphate-forming enzyme in *D. vulgaris* (Drake & Akagi 1977*a*); however, it has not been possible to find this activity in extracts of *D. gigas*, suggesting that it is not uniformly distributed among *Desulfovibrio* species. Without question, the formation of trithionate and thiosulphate has important implications for the mechanism of sulphite reduction (Kobayashi *et al.* 1974; Drake & Akagi 1977*b*); however, the available evidence suggests that they are not free intermediates in respiratory sulphate reduction. Both thiosulphate and trithionate are able to support the growth of *Desulfovibrio*, but we suggest that these substrates are only involved in energy coupling when converted to sulphite.

(*h*) *Thiosulphate reductase*

Thiosulphate reductase has been purified from *D. gigas* (Hatchikian 1975) and *D. vulgaris* (Haschke & Campbell 1971) and differs significantly from APS reductase and the bisulphite reductase. It has a similar molecular mass (about 200000 Da), but does not contain flavin, haem or non-haem iron and uses cytochrome c_3 (M_r = 26000) as its electron donor (Hatchikian *et al.* 1969). It is usually found with the soluble proteins; however, when extracts are prepared at pH 6.0, a significant amount of the reductase is membrane-associated (Odom 1978). These considerations plus the fact that it has not been possible to observe the formation of a proton gradient during the reduction of thiosulphate with hydrogen (C.-L. Liu & H. D. Peck, Jr, unpublished) suggest that thiosulphate reduction *per se* is not coupled to the generation of energy.

4. Conclusion

The idea of hydrogen cycling as a mechanism for energy coupling in *Desulfovibrio* offers a conceptual framework within which a number of unique physiological and biochemical observations concerning these bacteria can be rationalized. For example, hydrogen production during growth (Hatchikian *et al.* 1976) can be explained by the efficiency of hydrogen recovery by the periplasmic hydrogenase, which is readily lost from cells (van der Westen *et al.* 1980). It is also proposed that the inhibition of growth by sparging with an inert gas is due to the loss of hydrogen (Tsuji & Yagi 1980) and that inhibition of growth on lactate plus sulphate by hydrogen results from the inhibition of the internal hydrogenase by hydrogen (Khosrovi *et al.* 1971; Tsuji & Yai 1980). Payne & Grant (1982) have reported that the growth of *D. gigas* but not *Dt. ruminis* is inhibited by acetylene, an inhibitor of hydrogenase (Smith *et al.* 1976). These results may be rationalized by the fact that hydrogen cycling is essential for the growth of *Desulfovibrio* but not *Desulfotomaculum*. The relevance of hydrogen cycling for interspecies hydrogen transfer has been discussed in a previous section. At present, we have no concise explanation for the production of H_2 by *D. vulgaris* growing under condition of restricted sulphate concentrations (Traoré *et al.* 1981).

From the enzymological point of view the concept of hydrogen cycling offers an explanation for the cellular localization of various electron transfer components, dehydrogenases and reductases and the necessity to separate electron transfer sequences leading to hydrogen production from those leading to its utilization focuses attention on the regulation of electron transfer, either by means of multiple electron carriers or modifications such as occur with ferredoxin I and ferredoxin II. Members of *Desulfovibrio* also contain a number of interesting and characterized electron-transfer components and proteins whose function is unknown. These include

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rubredoxin (Bruschi & LeGall 1972), a molybdenum-containing iron–sulphur protein (Moura *et al.* 1976), desulforedoxin (Moura *et al.* 1977) and a cobalt-containing protein (Moura *et al.* 1980*b*). Within the context of hydrogen cycling it may be possible to define functions for these proteins. The frequent occurrence of multiple forms of hydrogenase and electron transfer proteins suggest that hydrogen cycling may prove to be of general importance as a mechanism of energy coupling for the growth of other bacteria under anaerobic conditions. Suggestive evidence for this role of hydrogen cycling can be found for *Acetobacterium woodii* (Winter & Wolfe 1980) and rumen bacteria (Henderson 1980).

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PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Discussion

J. VAN BEEUMEN (*Rijksuniversiteit Gent, Belgium*). Cytochrome c_7 from *Desulfuromonas acetoxidans* is known to be homologous to the *Desulfovibrio* cytochromes c_3. In contrast to the latter, which contain four haem groups, the cytochrome c_7 contains only one covalently bound haem. In relation to the function of these haems, has it been tested whether cytochrome c_7 can substitute for cytochrome c_3 in the hydrogenase reaction?

J. LEGALL. Cytochrome c_7, which actually contains three covalently bound haems, is reduced by hydrogenases from *Desulfovibrio*; however, we do not know whether it will replace cytochrome c_3 in the reduction of flavodoxin, ferredoxin and rubredoxin.

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