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A Thermodynamic Assessment of Possible Substrates for Sulphate-reducing Bacteria

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Abstract

A thermodynamic feasibility study was applied as a means of predicting suitable energy-yielding substrates for growth of sulphate-reducing microorganisms. The average free energy release per electron pair for a substrate–sulphate oxidoreduction may be more or less than the energy requirement for ATP synthesis from ADP and Pi. Substrates were divided into two groups on this thermodynamic basis and the division was shown to accord with previous experimental reports; those substrates which released an average of at least 8·4 kcal per electron pair (35·2 kJ per electron pair) were able to support growth whilst those releasing less than 8·4 kcal were unable to do so. It is proposed that the thermodynamic assessment could be applied to a wide range of possible substrates to predict the likelihood of their serving as sole substrates for growth of these organisms.

The literature concerning the use of hydrocarbons by sulphate reducers is confused and indefinite, but inclines toward the idea that use of long-chain hydrocarbons by these organisms is possible. In contrast, however, thermodynamic analysis showed that the highest energy release is from the short-chain alkynes.

Introduction

Implicit in the growth of any organism on a specific substrate is the fulfillment of the following requirements: (1) that the organism be permeable to the substrate so that the substrate reaches the necessary reaction sites; (2) that the organism has the enzymic capacity to degrade the substrate; (3) that the substrate degradation yields sufficient energy for growth; (4) that the organism possesses means of trapping energy derived from substrate degradation to utilize it for anabolic purposes.

Since the thermodynamics of an organism–substrate interaction (requirement 3) are independent of the physiology of the organism, any thermodynamic assessment should predict with certainty those interactions which are not possible; of those assessed as possible, the interactions which actually occur will be directly dependent on the organism's genotypic and phenotypic characteristics.

In the course of our investigations concerning microbial participation in such diverse processes as sulphate reduction, hydrocarbon oxidation, and leaching of mineral ores, it was recognized that thermodynamic assessment might explain certain environmental requirements for the development of some bacteria and could also provide a means of predicting the likelihood of an organism utilizing novel substrates. The existing literature did not satisfy our requirements and therefore effort was directed into formulating a suitable means of assessment. Baas-Becking *et al.* (1960) showed that, in the natural aqueous milieu described in terms of E_h (the redox

potential of a system or couple, with reference to a standard half cell) and pH, defined areas could be associated with the growth of specific groups of micro-organisms. Pourbaix (1966) showed how thermodynamic data could be used to construct equilibrium diagrams showing the domains of relative predominance of aqueous ions in terms of E_h and pH; from such a diagram it is possible to identify the most thermodynamically stable ionic form of a given chemical element at any given E_h and pH combination. It was upon this foundation that the means of assessment presented below was developed. Whilst the treatment in terms of equilibrium thermodynamics is not, in theory, strictly applicable to microbial environments, since the introduction of microorganisms into the system necessarily involves a departure from equilibrium, the assessment nevertheless appears to fulfill the requirements detailed above.

The assessments formulated were applied to known growth substrates of sulphate reducers and to some of the compounds reported in the literature as unable to support growth of these organisms. A high degree of correlation between the previously reported experimental data and the predictions of the thermodynamic assessment gave credence to the methods of assessment being applicable to prediction of other possible substrates such as hydrocarbons.

Application of the thermodynamic assessment to hydrocarbon–sulphate oxido-reduction predicted that anaerobic oxidation of methane or higher hydrocarbons [with the exceptions of acetylene (ethyne) and propyne] to CO_2 by sulphate could not serve as the sole energy source for microbial growth.

In formulating the method of assessment the following observations were taken into account.

Peck (1960) had proposed that sulphate reduction by hydrogen or lactate oxidation depended on oxidative phosphorylation for any net production of energy. Subsequently Peck (1966) demonstrated that phosphorylation coupled to electron transfer was dependent on hydrogen oxidation by sulphite or thiosulphate in extracts of *Desulfovibrio gigas* [see also p. 284 of Roy and Trudinger (1970) for a general review of this aspect].

The presence of four haem groups per molecule of the cytochrome c_3 of *D. vulgaris*, *D. gigas* and *D. desulfuricans* had been established (Ambler 1968; Ambler *et al.* 1969, 1971; Meyer *et al.* 1971; Yagi and Maruyama 1971). Der Vartanian and LeGall (1971) showed that cytochrome c_3 of *D. vulgaris*, containing four haem groups, was not readily reduced beyond its half-reduced state; therefore the difference between this state and the oxidized form represents a two-electron transfer. This provides some justification for expressing the free energy of redox reaction in terms of the average free energy per electron pair.

Peck (1966) suggested that the respiratory sequence of sulphate reducers contained only one active phosphorylation site, which would presumably catalyse the production of one mole of ATP from ADP and Pi. Thus for a single electron pair traversing the respiratory sequence, generation of one mole of ATP by oxidative phosphorylation would require an energy yield not less than that liberated by hydrolysis of one mole of ATP (assuming 100% efficiency). Burton (1958) suggests that, although ATP hydrolysis energy changes as high as $-12{\cdot}5$ kcal/mol* can be measured under likely physiological conditions, $-8{\cdot}4$ kcal/mol is the best estimate at pH 7·0 and 1 M

* 1 cal = 4·1868 J.

concentrations of ionic species. Hence, as a working hypothesis, we adopted the criterion that if a redox reaction liberates free energy per electron pair greater than 8·4 kcal, then this reaction is potentially capable of supporting growth. Conversely, if the energy release is less than 8·4 kcal per electron pair, the reaction is unable to support growth.

The primary thermodynamic data used in the assessment, namely the free energies of formation (Pourbaix 1966; Weast and Selby 1966; Klotz 1967; Mahler and Cordes 1967; Perry and Chilton 1973) and the calculated free energies of the half reactions considered in the applications below, are available as an Accessory Publication.*

Theory

A redox reaction

$$aA + bB \rightleftharpoons cC + dD \tag{1}$$

can be divided into two partial reactions:

$$aA \rightleftharpoons cC + ne^- , \tag{1a}$$

$$dD \rightleftharpoons bB + ne^- , \tag{1b}$$

and, in terms of electrical potentials, the two couples involved in the partial reactions can be represented by equations (2a) and (2b) respectively:

$$E_{h\,(a)} = E^{\circ}_{h\,(a)} + (RT/nF)\ln(\alpha_C^c/\alpha_A^a), \tag{2a}$$

$$E_{h\,(b)} = E^{\circ}_{h\,(b)} + (RT/nF)\ln(\alpha_B^b/\alpha_D^d), \tag{2b}$$

where E_h is the redox potential of a system or couple (with reference to a standard half cell), E°_h is the standard redox potential of a system or couple (at pH = 0), R is the universal gas constant, T is absolute temperature, n is the number of electrons transferred, F is Faraday's constant and α is the activity (of products or reactants).

In general terms, for a partial reaction representing an oxidation:

$$E_h = E^{\circ}_h + (RT/nF)\ln(\alpha_{\text{oxidized species}}/\alpha_{\text{reduced species}}), \tag{3}$$

and for a partial reaction representing a reduction, the two terms on the right hand side of equation (3) would both become negative.

In the case where x hydrogen ions are involved in a partial reaction, the equation for an oxidation (equation (3)) can be modified as follows:

$$E_h = E^{\circ}_h - \frac{0{\cdot}059\,x\,\text{pH}}{n} + \frac{RT}{nF}\ln\left(\frac{\alpha_{\text{oxidized species, except H}^+}}{\alpha_{\text{reduced species}}}\right). \tag{4}$$

The net free energy change for reaction (1) can be obtained by subtracting the free energy change for the partial reaction (1b) from that for the partial reaction (1a):

$$\Delta G = \Delta G_{(a)} - \Delta G_{(b)} \tag{5}$$

* The Accessory Publication is lodged with the Editor-in-Chief, Editorial and Publications Service, CSIRO, P.O. Box 89, East Melbourne, Vic. 3002. Copies are available upon request.

$$\Delta G = nF(E_{h\,(a)} - E_{h\,(b)}) \quad (6)$$

$$= nFE, \quad (7)$$

where E is the difference in redox potentials of the reaction couples.

Using equation (4), an E_h–pH diagram can be constructed for any ratio of oxidized to reduced species, and is most conveniently done for a ratio of one. Superimposition of two such diagrams amounts to combination of two partial reactions to represent a (total) redox reaction, and a region is delineated within which the stable product species occur. Moreover, the height of this region at any given pH within the limits of the diagram is equal to $E_{h\,(a)} - E_{h\,(b)}$ and proportional to the free energy released by the redox reaction at that pH (equation (6)).

It is postulated that an organism relying on a particular redox reaction as a source of energy would, of necessity, exist in a natural environment represented on the E_h–pH diagram by the region of mutual product stability of the partial reactions. The pH limits of the environment in which an organism will grow may be determined solely by the physiology of the organism but, since the free energy release of redox reactions will normally vary with pH, it is also likely that the energy release will be sufficient to support growth only within certain pH ranges; such thermodynamic constraints on growth will override any enzymic considerations. Moreover, for a redox reaction to serve as an energy source for growth of an organism, not only must the reaction release free energy, but the energy released must exceed certain minimum requirements equal in magnitude to the energy required to synthesize ATP from ADP and Pi.

Where equimolar quantities of reductant and oxidant are involved the organism would be required to synthesize at least one mole of ATP per mole of substrate utilized. However, since ATP generation results from the passage of pairs of electrons along a respiratory sequence it is probably more realistic to relate ATP production to the electrons transferred rather than to the substrate reacted. It was therefore assumed as part of the working hypothesis that each electron pair transferred would be equally responsible for the total free energy release of the reaction and would be required to have sufficient free energy available for synthesis of at least one mole of ATP for growth of the organism to occur.

A coupled substrate–sulphate oxidoreduction involves four electron pairs per molecule of sulphate. The criterion therefore employed to predict whether growth was thermodynamically feasible was that the oxidoreduction must yield at least 33·6 kcal (4 × 8·4). Whilst the free energy of the reaction is unlikely to be exactly equal for each electron pair, as is intimated in calculating the *average* free energy per electron pair, it is more unlikely that it will be totally concentrated in one electron pair; average free energy per electron pair is a compromise engendered by lack of data. This compromise is at least partially offset by the fact that the efficiency of conversion of substrate energy into the synthesis of ATP is usually much less than 100%; in at least one instance it is less than 25% (Stadtman 1966). The studies of Vosjan (1970) suggest that of the four electron pairs involved in the reduction of sulphate, either one or two will lead to ATP generation by oxidative phosphorylation.

By using an average free energy release per electron pair it is not implied that, should the energy release exceed 8·4 kcal per electron pair, each of the electron pairs will lead to generation of one mole of ATP, but only that at least one mole of ATP can be generated from the four electron pairs together. This concept of average

energy release per electron pair is used as an alternative to a rigorous treatment which accounts fully for the efficiency of energy conservation, a method impossible in this instance because of lack of data. Thus, in the instance of lactate partial oxidation, where the average free energy release is 10·5 kcal per electron pair, synthesis of one mole of ATP would result in an overall efficiency of 20% when sulphate is reduced to sulphide.

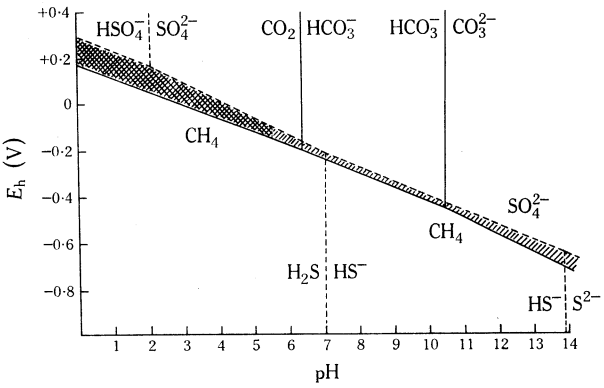

Fig. 1. E_h–pH diagram at 25°C [compiled from equation (4), for equal dissolved product and reactant concentrations] for the methane–water system (solid lines) and the sulphur–water system (broken lines). The region of mutual stability for methane oxidation products and sulphate reduction products is indicated by hatching. Where the hatching is crossed the difference in E_h is sufficient for generation of at least one mole of ATP per mole of sulphate reduced. At no pH is the difference in E_h sufficient for generation of one mole of ATP per electron pair.

Applications

(1) *Combination of Sulphate Reduction and Methane Oxidation*

Using equation (4) and published thermodynamic data (Pourbaix 1966) for standard free energies of formation of species involved in the redox reaction, E_h values were calculated for equal concentrations in aqueous solution of reduced and oxidized species of the SO_4^{2-}/S^{2-} couple and the CH_4/CO_2 couple; these values were plotted against the corresponding pH values. Thus the domains of relative predominance of the dissolved forms of each species were delimited by the oblique lines in Fig. 1. A region of mutual product stability, marked by hatching, represents the availability of free energy when methane is oxidized by sulphate in the reaction

$$2H^+ + SO_4^{2-} + CH_4 \rightleftharpoons H_2S + CO_2 + H_2O. \quad (7)$$

Whether H_2S exists as such or as HS^- or S^{2-} depends on the pH; similarly the occurrence of CO_2 as such or as HCO_3^- or CO_3^{2-} is also pH dependent. The domains of predominance of the forms of H_2S and of CO_2 are indicated by the vertical lines in the figure.

The depth of the oblique band at any particular pH indicates the amount of energy made available by the reaction. One may thus predict, in terms of E_h and pH, the thermodynamic feasibility of sulphate–methane oxidoreduction providing an energy source for growth of an organism which possessed the appropriate enzymes.

Comparison of the energy required for ATP synthesis with the free energy derived from the oxidoreduction shows that one mole of ATP can be synthesized per mole of sulphate reduced only at pH values below 5·5; this is indicated by cross-hatching in Fig. 1. It should be noted in this context that synthesis of ATP is pH dependent,

energy consumption increasing with increase in pH (see Mahler and Cordes 1967, equation 2–54, p. 26).

In this example, for the sake of simplicity, equal dissolved concentrations of oxidized and reduced species are assumed in constructing Fig. 1 [i.e. $SO_4^{2-} = H_2S$ (or HS^- or S^{2-}) and $CH_4 = CO_2$(or HCO_3^- or CO_3^{2-})]. It should be noted that each ten-fold decrease in the ratio of products to reactants in equation (7) would increase the available free energy (hatched band depth) by 1·4 kcal and increase the possibility of reaction; however such a situation would be limited by the accumulation of products unless these were removed. Thermodynamic efficiencies typical of biological systems would mean that energy for ATP synthesis would be available only at pH values less than approximately 2·0, an acidity tolerated by few known organisms.

When assessed on the criterion of production of a minimum of one mole of ATP from the average free energy released per electron pair (i.e. the total free energy change divided by four), the reaction is unable to generate sufficient energy for growth of an organism at any pH in the whole pH range.

Experiments conducted in this laboratory to isolate such an organism were, as theoretically predicted, unsuccessful. Furthermore, known sulphate-reducing microorganisms have been shown experimentally not to grow on methane as the sole carbon source (Sorokin 1957; Ryzhova and Ivanov 1961; Postgate 1969), and more recently, when attempts were made to grow *Desulfovibrio desulfuricans* on radioactive methane either as the sole carbon and energy source or in conjunction with lactate, no statistically significant incorporation of the label into the cells occurred (Davis and Yarbrough 1966).

(2) *Combination of Sulphate Reduction and Oxidation of a Range of Hydrocarbons*

The effect of chain length and the degree of unsaturation on the free energy available from hydrocarbon–sulphate oxidoreduction is conveniently illustrated at a fixed pH in Fig. 2. This figure, drawn for equimolar product and reactant concentrations at pH 7·0, does not necessarily represent the most favourable conditions, since the energy release varies with both pH and reactant/product ratios; it is, however, useful in illustrating general tendencies.

In Fig. 2 methane is seen to be the only hydrocarbon which is unable to provide sufficient energy for synthesis of one mole of ATP per mole of sulphate at pH 7·0. However, when cognizance is taken of the thermodynamic efficiency of typical bacterial systems, only the unsaturated hydrocarbons appear to be possible substrates. When the average energy available per electron pair (i.e. total free energy change divided by four) is compared with the energy required for synthesis of one mole of ATP, it is seen that only the lowest members of the alkyne series can now be considered possible substrates for sulphate reduction.

The negative results from the following two experiments may now be explicable in the following terms.

(i) In this laboratory known sulphate reducers were shown to be unable to grow when incubated with acetylene plus sulphate. Nor was it possible to isolate an organism able to couple acetylene oxidation and sulphate reduction in screening programs utilizing inocula from a variety of natural sources. These experiments employed a variety of conditions among which were incubation in a basal salts medium under an acetylene atmosphere, in basal salts plus acetate (a known carbon source for sulphate reducers) under an acetylene atmosphere, and in basal salts under an atmosphere

of acetylene and hydrogen (a known energy source for sulphate reducers); neither growth nor sulphate reduction was detected under any of the conditions employed. Thus it was concluded that acetylene could not serve as either a carbon or an energy source for sulphate reducers. These experiments do not establish whether the limitations preventing acetylene utilization are thermodynamic, enzymic or due to permeability barriers, but, on the basis of our criterion, lack of growth would be attributable to non-thermodynamic constraints (see also application 3). Higher members of the hydrocarbon series, on the other hand (with the exception of propyne), are excluded by the thermodynamic criterion.

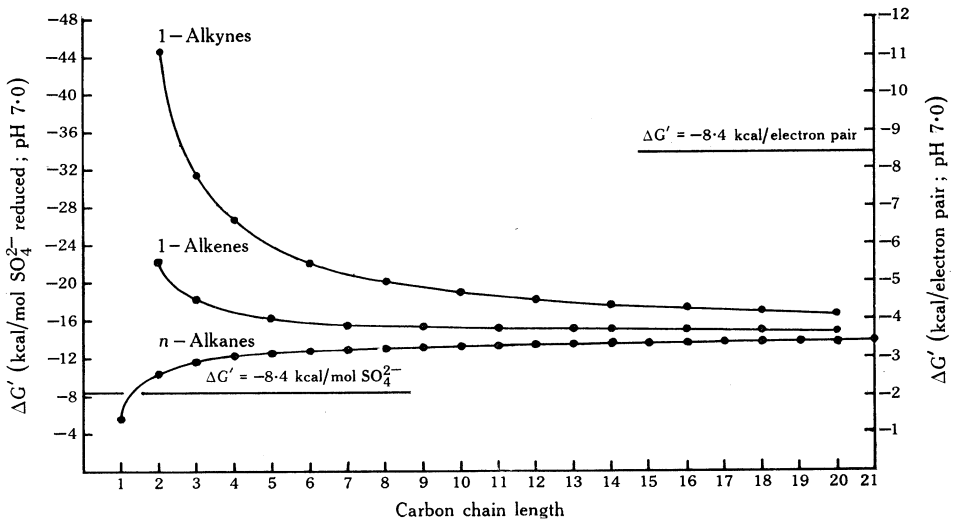

Fig. 2. Variation of the standard free energy of reaction ($\Delta G'$) at pH 7·0 with chain length and degree of unsaturation for coupled hydrocarbon oxidation and sulphate reduction according to the following reactions.

Alkanes:

$$C_nH_{2n+2} + \frac{3n+1}{4} SO_4^{2-} + \frac{3n+1}{2} H^+ \rightleftharpoons nCO_2 + (n+1)H_2O + \frac{3n+1}{4} H_2S.$$

Alkenes:

$$C_nH_{2n} + \frac{3n}{4} SO_4^{2-} + \frac{3n}{2} H^+ \rightleftharpoons nCO_2 + nH_2O + \frac{3n}{4} H_2S.$$

Alkynes:

$$C_nH_{2n-2} + \frac{3n-1}{4} SO_4^{2-} + \frac{3n-1}{2} H^+ \rightleftharpoons nCO_2 + (n-1)H_2O + \frac{3n-1}{4} H_2S.$$

The energy release is expressed on the basis of the number of moles of sulphate involved in the reaction (left-hand axis), and on the basis of the number of pairs of electrons involved in the reaction (right-hand axis).

(ii) It is doubtful whether statistically significant incorporation of the radioactive label into either CO_2 or cell biomass occurred when Davis and Yarbrough (1966) attempted to grow *D. desulfuricans* on labelled ethane in the absence of lactate. Since the thermodynamic assessment precludes the use of ethane as an energy source it is more likely that the organism utilizes ethane only by its co-metabolism in the presence of an energy-yielding substrate.

(3) *Total Oxidation of Ethane, Ethylene and Acetylene by Sulphate*

In the light of application 2, a more rigorous examination in terms of E_h–pH diagrams was prepared for ethane, ethylene and acetylene; these results are depicted in Figs 3*a*, 3*b* and 3*c* respectively.

Ethane, like methane, is unable to meet the criterion for production of one mole of ATP per electron pair at any pH in the whole pH range.

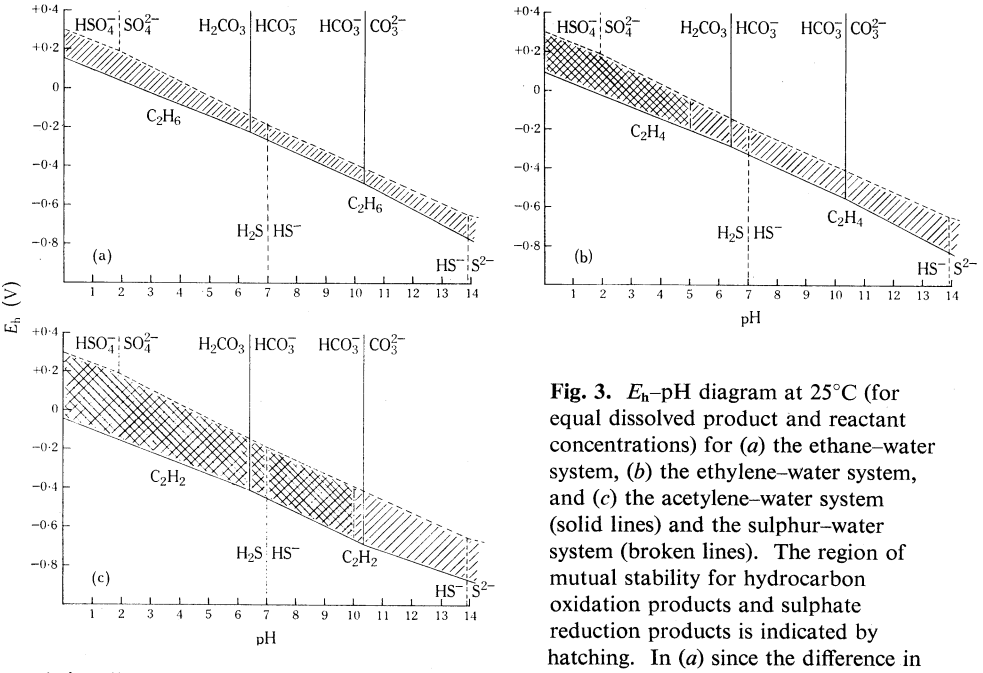

Fig. 3. E_h–pH diagram at 25°C (for equal dissolved product and reactant concentrations) for (*a*) the ethane–water system, (*b*) the ethylene–water system, and (*c*) the acetylene–water system (solid lines) and the sulphur–water system (broken lines). The region of mutual stability for hydrocarbon oxidation products and sulphate reduction products is indicated by hatching. In (*a*) since the difference in E_h is insufficient for generation of 1 mole of ATP per electron pair, at any pH, there is no region of cross-hatching. In (*b*) and (*c*), where the hatching is crossed the difference in E_h is sufficient for generation of at least 1 mole of ATP per electron pair.

In contrast, both ethylene and acetylene can meet the criterion below pH 5 and 10 respectively. Since neither growth nor sulphate reduction could be demonstrated experimentally with either of these compounds as the sole energy source, the limitations on their utilization must be other than thermodynamic. In the case of ethylene a possible explanation could be that the pH range in which a favourable energy release is achieved may to be too low for the development of sulphate reducers [Connell and Patrick (1968) report the lower pH limit for sulphate reducers as approximately 6·4, whilst Baas–Becking *et al.* (1960) give a value close to 4·0]; in the case of acetylene, constraints other than those imposed by thermodynamics and acid tolerance must be envisaged.

(4) *Combination of Sulphate Reduction and Oxidation of Complex Organic Compounds*

For equal dissolved concentrations of reactants and products, the free energy of reaction at pH 7·0 was calculated from the standard free energies of formation of the species involved in each of the redox reactions examined.

Table 1 presents two alternative stoichiometries for the oxidation of six common substrates (known to support the growth of sulphate reducers) by sulphate. Reaction 1a for the partial oxidation of lactate yields an average of 10·5 kcal per electron pair compared with a minimum requirement of 8·4 kcal per electron pair for ATP production, i.e. it is energetically favourable for the support of growth, judged by the criterion. Conversely, reaction 1b for the total oxidation of the same substrate (lactate) yields only 3·4 kcal per electron pair, and when compared with the criterion would be judged energetically unfavourable. However, whilst all known sulphate reducers are known to oxidize lactate (Campbell and Postgate 1965; Postgate and Campbell 1966) the experimentally established stoichiometry is that of reaction 1a (Senez 1954; Coleman 1959). Furthermore, it has been established experimentally that oxidation of pyruvate, malate and fumarate by sulphate reducers occurs according to reactions 1c, 1e and 1g respectively (Senez 1954; Grossman and Postgate 1955) and that in general the end product of carbon dissimilation (for substrates of more than two carbon atoms) is acetate (Grossman and Postgate 1955; Postgate 1965; LeGall and Postgate 1973). Experimental findings in the case of pyruvate, malate and fumarate therefore also agree with our criterion, which predicts that reactions 1c, 1e and 1g would be energetically favourable whilst the total oxidation of those substrates, represented in reactions 1d, 1f and 1h, would be judged unfavourable.

In circumstances where partial oxidation of a substrate (e.g. lactate to acetate; reaction 1a) is energetically favourable whereas total oxidation (e.g. lactate to CO_2; reaction 1b) is energetically unfavourable, the decreased energy yield of the total oxidation would result from the unfavourable energetics of oxidation of the intermediate (e.g. acetate to CO_2; reaction 3c). Teleologically then, one would expect that the organism would carry out only the more favourable partial oxidation and thus maximize the potential energy yields; the experimental data recorded in Table 1 are thus compatible with the teleological concept.

Consequently, whilst a favourable energy release for the total oxidation of a potential substrate indicates that there are no thermodynamic constraints on its use, an unfavourable energy release for total oxidation of the substrate does not preclude the possibility that partial oxidation of that substrate may be energetically favourable. For this reason the thermodynamics of partial oxidation of some hydrocarbons are examined in application 5 on p. 168.

Ethanol is readily oxidized by sulphate reducers (Mechalas and Rittenberg 1960; Sorokin 1966*a*) presumably according to the stoichiometry of reaction 1i rather than being completely oxidized in accordance with reaction 1j.

Reactions 1k and 1l show the partial and total oxidation respectively of glucose. Of the two only partial oxidation would meet the criterion. It appears that in general sulphate reducers do not metabolize carbohydrates (Coleman 1960; LeGall 1963; Campbell *et al.* 1966). The provisionally accepted classification of the genus *Desulfotomaculum* (Campbell and Postgate 1965) lists all three species as unable to utilize glucose, and in the classification of *Desulfovibrio* (Postgate and Campbell 1966) it is stated that *D. desulfuricans* does not form gas from carbohydrates. However, Postgate (1951, 1953) demonstrated growth of *D. vulgaris* (Hildenborough strain) on glucose, and when resting cells of *D. desulfuricans* were used the presence of enzymes of the Embden–Meyerhoff–Parnes pathway was reported (Anderson *et al.* 1958). Akagi and Jackson (1967) showed that growing cells of *Desulfotomaculum nigrificans* degraded glucose to acetate, ethanol and carbon dioxide, primarily via

Table 1. Reduction of sulphate by some common substrates which are characteristically only partially oxidized

No.	Reaction	Av. $\Delta G'$ (pH = 7) (kcal/electron pair)	Comparison with criterion[A]	Growth[B]	Reference	Notes[C]
1a	$2\ Lactate^- + SO_4^{2-} \rightleftharpoons 2\ acetate^- + 2H_2O + 2CO_2 + S^{2-}$	−10·5	S	+	Campbell and Postgate (1965), Postgate and Campbell (1966)	1
1b	$2\ Lactate^- + 3SO_4^{2-} + 2H^+ \rightleftharpoons 6H_2O + 6CO_2 + 3S^{2-}$	−3·4	U			
1c	$4\ Pyruvate^- + SO_4^{2-} \rightleftharpoons 4\ acetate^- + 4CO_2 + S^{2-}$	−26·5	S	+	Senez (1954), Campbell and Postgate (1965), Postgate and Campbell (1966)	2, 3
1d	$4\ Pyruvate^- + 5SO_4^{2-} + 4H^+ \rightleftharpoons 8H_2O + 12CO_2 + 5S^{2-}$	−4·8	U			
1e	$2\ Malate^{2-} + SO_4^{2-} + 2H^+ \rightleftharpoons 2\ acetate^- + 2H_2O + 4CO_2 + S^{2-}$	−14·8	S	+	LeGall and Postgate (1973)	2, 3
1f	$2\ Malate^{2-} + 3SO_4^{2-} + 4H^+ \rightleftharpoons 6H_2O + 8CO_2 + 3S^{2-}$	−4·5	U			
1g	$2\ Fumarate^{2-} + SO_4^{2-} + 2H^+ \rightleftharpoons 2\ acetate^- + 4CO_2 + S^{2-}$	−15·2	S	+	Grossman and Postgate (1955)	2, 3
1h	$2\ Fumarate^{2-} + 3SO_4^{2-} + 4H^+ \rightleftharpoons 4H_2O + 8CO_2 + 3S^{2-}$	−4·6	U			
1i	$2\ Ethanol + SO_4^{2-} \rightleftharpoons 2\ acetate^- + 2H_2O + 2H^+ + S^{2-}$	−10·2	S	+	Mechalas and Rittenberg (1960), Sorokin (1966*a*)	3
1j	$2\ Ethanol + 3SO_4^{2-} \rightleftharpoons 6H_2O + 4CO_2 + 3S^{2-}$	−3·0	U			
1k	$Glucose + SO_4^{2-} \rightleftharpoons 2\ acetate^- + 2H_2O + 2CO_2 + 2H^+ + S^{2-}$	−23·4	S	+	Postgate (1951, 1953), Akagi and Jackson (1967)	4
1l	$Glucose + 3SO_4^{2-} \rightleftharpoons 6H_2O + 6CO_2 + 3S^{2-}$	−7·4	U			

[A] S = satisfactory, U = unsatisfactory free energy release with respect to the criterion.

[B] +, Growth occurs on this substrate.

[C] 1. Experimentally established stoichiometry (Senez 1954; Coleman 1959).
2. Experimentally established stoichiometry (Grossman and Postgate 1955).
3. Acetate is the normal end product of substrates C_2 or greater.
4. Close agreement with the experimentally established stoichiometry.

the Embden–Meyerhoff–Parnas pathway (>90%) but also via the Entner–Doudoroff pathway. Acetate and carbon dioxide were produced in an approximately 1 : 1 ratio; the glucose : sulphate : sulphide ratio was approximately 1 : 1 : 1 and ethanol production varied but was low compared with the quantity of acetate produced. These results and the favourable energy release compared with the criterion both support the stoichiometry of reaction 1k for the partial oxidation of glucose. Akagi and Jackson (1967) did, however, note that little (<0·1%) labelled glucose was incorporated as cell carbon, suggesting that, like the substrates set out in Table 2, glucose acts as an energy but not a carbon source; this is in contrast to the other five substrates in Table 1 which apparently supply cell carbon as well as energy.

Table 2 illustrates the oxidation of five substrates which provide energy, but not cell carbon, for the growth of sulphate reducers. Total oxidation of formate (Postgate 1963; Sorokin 1966*a*; LeGall and Postgate 1973) and of oxalate (Postgate 1963) occurs and in both cases the energetics are favourable compared with the criterion. Stephenson and Strickland (1931) isolated a sulphate reducer able to couple sulphate reduction to the oxidation of molecular hydrogen. For some time many strains of sulphate reducers were held to be facultative autotrophs (Butlin and Adams 1947), but this was later disproved (see, for example, Postgate 1960), organic carbon being shown to be necessary for synthesis of cell components while hydrogen oxidation provided the energy. Iso-butanol and propanol were each shown to be able to provide energy for growth and sulphate reduction when they were oxidized to the respective carboxylic acids (Mechalas and Rittenberg 1960; Postgate 1965). It can be seen that oxidation of hydrogen (reaction 2c) and partial oxidation of iso-butanol and propanol to the corresponding carboxylic acids (reactions 2d and 2f respectively) are all energetically favourable compared with the criterion. However, reactions 2e and 2g for iso-butanol and propanol respectively reveal that total oxidation would be energetically unfeasible compared with the criterion.

Table 3 sets out data for compounds unable to support growth of sulphate reducers. When compared with the criterion, they are all judged energetically unfavourable. It has been reported that methanol does not support growth of sulphate reducers (Campbell *et al.* 1966) and reactions 3a and 3b reveal that energetically neither partial nor total oxidation of methanol could support growth. In the case of acetate, reaction 3c, it can be seen that the energy release is small and unfavourable. It does not support growth of sulphate reducers (see Table 1 of LeGall and Postgate 1973) and is a normal end product from oxidation by these bacteria of substrates of more than two carbon atoms (Postgate 1959). As illustrated in Table 1, partial oxidation of a substrate to acetate gives a higher energy yield per electron pair than the corresponding total oxidation. Acetate is, however, assimilated as a carbon source when an alternative energy source is available for the growth of sulphate reducers (Postgate 1960; Sorokin 1966*a*, 1966*b*, 1966*c*, 1966*d*).

In an elegant thermodynamic analysis McCarty (1972) points out that it is thermodynamically possible to couple oxidation of acetate with reduction of sulphate, and that the reduced energy yield compared with acetate oxidation using oxygen as a terminal electron acceptor does not inherently exclude sulphate reduction by acetate. In contrast, using our method of assessment, where a certain minimum free energy release must be attained before any oxidoreduction is considered able to support growth, the acetate–sulphate combination is excluded since it fails to meet this criterion.

Table 2. Sulphate reduction by substrates which serve as energy source, but not carbon source

No.	Reaction	Av. $\Delta G'$ (pH = 7) (kcal/electron pair)	Comparison with criterion[A]	Growth[B]	Reference	Notes[C]
2a	$4\ Formate^- + SO_4^{2-} + 4H^+ \rightleftharpoons 4H_2O + 4CO_2 + S^{2-}$	−11·5	S	+	Postgate (1963), Sorokin (1966*a*), LeGall and Postgate (1973)	
2b	$4\ Oxalate^{2-} + SO_4^{2-} + 8H^+ \rightleftharpoons 4H_2O + 8CO_2 + S^{2-}$	−14·9	S	+	Postgate (1963)	
2c	$4H_2 + SO_4^{2-} \rightleftharpoons 4H_2O + S^{2-}$	−11·1	S	+	Stephenson and Strickland (1931)	1
2d	$2\ iso\text{-}Butanol + SO_4^{2-} \rightleftharpoons 2\ iso\text{-}butyrate^- + 2H_2O + 2H^+ + S^{2-}$	−9·2	S	+	Mechalas and Rittenberg (1960), Postgate (1965)	1
2e	$iso\text{-}Butanol + 3SO_4^{2-} \rightleftharpoons 5H_2O + 4CO_2 + 3S^{2-}$	−1·9	U			
2f	$2\ Propanol + SO_4^{2-} \rightleftharpoons 2\ propionate^- + 2H_2O + 2H^+ + S^{2-}$	−9·2	S	+	Mechalas and Rittenberg (1960), Postgate (1965)	
2g	$4\ Propanol + 9SO_4^{2-} \rightleftharpoons 16H_2O + 12CO_2 + 9S^{2-}$	−2·4	U			

[A] S = satisfactory, U = unsatisfactory free energy release with respect to the criterion.
[B] +, Growth occurs on this substrate.
[C] 1. Experimentally established stoichiometry.

Table 3. Sulphate reduction by compounds unable to support growth

No.	Reaction	Av. $\Delta G'$ (pH = 7) (kcal/electron pair)	Comparison with criterion[A]	Growth[B]	Reference	Notes[C]
3a	$2\ \text{Methanol} + SO_4^{2-} \rightleftharpoons 2\ \text{formate}^- + 2H_2O + 2H^+ + S^{2-}$	−2·5	U	—	Campbell *et al.* (1966)	
3b	$4\ \text{Methanol} + 3SO_4^{2-} \rightleftharpoons 8H_2O + 4CO_2 + 3S^{2-}$	−5·5	U	—	Campbell *et al.* (1966)	
3c	$\text{Acetate}^- + SO_4^{2-} + H^+ \rightleftharpoons 2H_2O + 2CO_2 + S^{2-}$	+0·6	U	—	LeGall and Postgate (1973)	1
3d	$4\ \text{Benzene} + 7SO_4^{2-} + 4H_2O \rightleftharpoons 8\ \text{acetate}^- + 8CO_2 + 8H^+ + 7S^{2-}$	+1·7	U	—	Novelli and Zobell (1944)	
3e	$4\ \text{Benzene} + 15SO_4^{2-} \rightleftharpoons 12H_2O + 24CO_2 + 15S^{2-}$	−1·3	U	—	Novelli and Zobell (1944)	
3f	$4\ \text{Xylene} + 21SO_4^{2-} \rightleftharpoons 20H_2O + 32CO_2 + 21S^{2-}$	−0·5	U	—	Novelli and Zobell (1944)	
3g	$2\ \text{Cyclohexane} + 9SO_4^{2-} \rightleftharpoons 12H_2O + 12CO_2 + 9S^{2-}$	−0·8	U	—	Novelli and Zobell (1944)	
3h	$4\ \text{Propanoate}^- + 3SO_4^{2-} \rightleftharpoons 4\ \text{acetate}^- + 4H_2O + 4CO_2 + 3S^{2-}$	+0·4	U	—	Adams and Postgate (1959), Coleman (1960), Campbell *et al.* (1966)	2
3i	$4\ \text{Propanoate}^- + 7SO_4^{2-} + 4H^+ \rightleftharpoons 12H_2O + 12CO_2 + 7S^{2-}$	−0·4	U	—	Adams and Postgate (1959), Coleman (1960), Campbell *et al.* (1966)	2
3j	$2\ \text{Butanoate}^- + SO_4^{2-} \rightleftharpoons 4\ \text{acetate}^- + 2H^+ + S^{2-}$	−4·3	U	—	Adams and Postgate (1959), Coleman (1960), Campbell *et al.* (1966)	2
3k	$2\ \text{Butanoate}^- + 3SO_4^{2-} \rightleftharpoons 2\ \text{acetate}^- + 4H_2O + 4CO_2 + 3S^{2-}$	−1·0	U	—	Adams and Postgate (1959), Coleman (1960), Campbell *et al.* (1966)	2
3l	$2\ \text{Butanoate}^- + 5SO_4^{2-} + 2H^+ \rightleftharpoons 8H_2O + 8CO_2 + 5S^{2-}$	−0·4	U	—	Adams and Postgate (1959), Coleman (1960), Campbell *et al.* (1966)	2

[A] U = unsatisfactory free energy release with respect to the criterion.

[B] — This compound is unable to support growth.

[C] 1. Acetate is assimilated as a carbon source under some circumstances, but is not metabolized as an energy source (Sorokin 1966*a*, 1966*b*, 1966*c*, 1966*d*).

2. *D. rubentschikii* was reported to grow on these two substrates (Postgate 1959); the organism has been lost and has defied attempts at re-isolation (Selwyn and Postgate 1959).

Baars (1930, cited by Postgate 1959), claimed that both *Desulfovibrio rubentschikii* and its variety, *anomalous*, were able to oxidize butyrate and propionate for growth. These organisms were lost and have defied attempts at reisolation (Selwyn and Postgate 1959); consequently the existence of any sulphate reducers able to oxidize these substrates remains doubtful, since other known sulphate reducers are unable to grow on these substrates. Neither total nor partial oxidation of butyrate or propionate will yield sufficient energy to meet the criterion for ATP production.

Table 4. Malate and fumarate reduction in the absence of sulphate

No.	Reaction	Av. $\Delta G'$ (pH = 7) (kcal/electron pair)	Comparison with criterion[A]	Growth[B]	Reference
4a	$3\ Malate^{2-} + H^+ \rightleftharpoons acetate^- + 2\ succinate^{2-} + H_2O + 2CO_2$	−27·6	S	+	Miller *et al.* (1970)
4b	$3\ Fumarate^{2-} + 2H_2O + H^+ \rightleftharpoons acetate^- + 2\ succinate^{2-} + 2CO_2$	−28·9	S	+	Miller and Wakerley (1966)
4c	$Fumarate^{2-} + H_2 \rightleftharpoons succinate^{2-}$	−24·8	S	—[C]	Barton *et al.* (1970)
4d	$2\ Fumarate^{2-} + lactate^- + H_2O \rightleftharpoons 2\ succinate^{2-} + acetate^- + CO_2$	−24·2	S	—[C]	Barton and Peck (1971)

[A] S = satisfactory free energy release with respect to the criterion.
[B] +, Growth occurs on this substrate.
[C] This reaction has to date been demonstrated only in cell-free extracts.

As the term 'sulphate reducer' suggests, these bacteria normally couple substrate oxidation with the reduction of sulphate. However, in the absence of sulphate, both fumarate and malate may be dismuted by sulphate reducers, i.e. the single substrate undergoes both oxidation and reduction. In both cases the predominant reduction product is succinate and the oxidation products are acetate and carbon dioxide. In the case of fumarate dismutation traces of malate are also formed (Miller and Wakerley 1966) and conversely, during malate dismutation, traces of fumarate can be detected (Miller *et al.* 1970). Reactions 4a and 4b represent the dismutation of malate and fumarate respectively; in the former case the stoichiometry agrees with that found experimentally (Grossman and Postgate 1955). In both cases, the energy yield greatly exceeds the criterion.

The known dismutation of malate and fumarate by sulphate reducers, where the substrate is both oxidized and reduced, raises the question of reduction of such substances by other hydrogen donors. Recently it was demonstrated that reduction of fumarate by both hydrogen (Barton *et al.* 1970) and lactate (Barton and Peck 1971) was coupled to oxidative phosphorylation in cell-free extracts of *D. gigas*. These reactions (i.e. 4c and 4d) both have a favourable energy release compared with the criterion.

(5) *Partial Oxidation of Hydrocarbons by Sulphate*

In application 4 it was shown that, where a partial oxidation (e.g. lactate to acetate) of a substrate was energetically favourable, the corresponding total oxidation might not be. Consequently the conclusions reached in applications 1 and 2 were only tentative until the partial oxidation reactions were assessed.

Table 5 includes data for the partial oxidation of hydrocarbons in the C_1–C_4 range (reactions 5a–5l); in no instance is an energy yield meeting the criterion

achieved. The range of hydrocarbons considered is necessarily restricted due to difficulties in obtaining certain thermodynamic data. Nevertheless, inspection of the data does reveal trends: (i) in direct contrast to lactate, for example, the free energy release for oxidation of any of the hydrocarbons examined is greater for total oxidation than for the partial oxidations formulated; whilst total oxidation to CO_2 and H_2O yields a net free energy release, partial oxidations are in fact net consumers of free energy; and (ii) like the total oxidations depicted in Fig. 2, the free energy change for a given extent of oxidation tends to become asymptotic, and for hydrocarbons greater than C_4 the free energy change would not reach the value required by the criterion.

Table 5. Partial oxidations of hydrocarbons

No.	Reaction	Av. $\Delta G'$ (pH = 7) (kcal/electron pair)	Comparison with criterion[A]
5a	4 Methane + $SO_4^{2-} \rightleftharpoons$ 4 methanol + S^{2-}	+20·3	U
5b	2 Methane + $SO_4^{2-} \rightleftharpoons$ 2 formaldehyde + $2H_2O$ + S^{2-}	+12·1	U
5c	4 Methane + $3SO_4^{2-} \rightleftharpoons$ 4 formic acid + $4H_2O$ + $3S^{2-}$	+6·6	U
5d	4 Ethane + $SO_4^{2-} \rightleftharpoons$ 4 ethanol + S^{2-}	+15·9	U
5e	2 Ethane + $SO_4^{2-} \rightleftharpoons$ 2 acetaldehyde + $2H_2O$ + S^{2-}	+9·7	U
5f	4 Ethane + $3SO_4^{2-} \rightleftharpoons$ 4 acetic acid + $4H_2O$ + $3S^{2-}$	+2·4	U
5g	4 Propane + $SO_4^{2-} \rightleftharpoons$ 4 propanol + S^{2-}	+15·6	U
5h	2 Propane + $SO_4^{2-} \rightleftharpoons$ 2 propanal + $2H_2O$ + S^{2-}	+7·3	U
5i	4 Propane + $3SO_4^{2-} \rightleftharpoons$ 4 propanoic acid + $4H_2O$ + $3S^{2-}$	+2·3	U
5j	4 Butane + $SO_4^{2-} \rightleftharpoons$ 4 butanol + S^{2-}	+13·2	U
5k	2 Butane + $SO_4^{2-} \rightleftharpoons$ 2 butanal + $2H_2O$ + S^{2-}	?	?
5l	4 Butane + $3SO_4^{2-} \rightleftharpoons$ 4 butanoic acid + $4H_2O$ + $3S^{2-}$	+1·5	U

[A] U = Unsatisfactory free energy release with respect to the criterion.

As discussed in applications 1 and 2, the best claims for growth of sulphate reducers on hydrocarbons are those of Davis and Yarbrough (1966) where incorporation of radioactive carbon from labelled methane and ethane is claimed. Examination of their published results suggests, however, that labelling, compared with sterile controls, occurred only when lactate, an energetically favourable substrate, accompanied ethane and did not occur from methane alone nor from methane plus lactate. The statistical significance was dubious in all instances.

Conclusions

Eleven of the complex organic substrates reported to support the growth of sulphate reducers were examined and all were found to meet the thermodynamic criterion that an average of at least 8·4 kcal must be released per electron pair when sulphate is reduced to sulphide by the substrate. Furthermore, in all of the eight cases where both partial and total oxidation of the substrate could be conceived, total oxidation did not meet the criterion whereas partial oxidation, a feature of the metabolism of sulphate reducers, did.

On the other hand seven complex organic compounds unable to meet the criterion have been reported as unable to support growth of sulphate reducers, except those belonging to the *Desulfovibrio rubentschikii* group, the existence of which is in dispute.

In fact, in all cases examined, including malate and fumarate utilization in the absence of sulphate, the thermodynamic assessment and the experimental data were in accord, with the single exception referred to above.

In certain instances (e.g. lactate utilization, reaction 1a in Table 1) an apparent efficiency of energy conservation approaching 80% is suggested; when it is realised, however, that only one (or two) of the four electron pairs involved in the reduction of sulphate to sulphide need eventually lead to ATP synthesis, then the overall efficiency would be only 20% (40%) or less.

In view of the correlation between the published data and the predictions, thermodynamic assessments were made of hydrocarbon–sulphate interactions with a fair degree of confidence. From the thermodynamic assessment it is deduced that methane oxidation by sulphate would yield insufficient energy for this process to be the sole energy source for growth of sulphate-reducing bacteria; this accords with reported experimental evidence discussed in application 1.

The assessment predicts that if growth is to occur as a result of total oxidation of hydrocarbons by sulphate (application 2), then the low-molecular-weight alkynes would be the most energetically favourable members. In contrast to the complex organic compounds, hydrocarbons yield more energy from their total oxidation than from their partial oxidation. Attempts to grow known sulphate-reducing organisms, and attempts to isolate an organism by screening, with acetylene–sulphate oxidoreduction as the sole energy source, have failed. The apparent failure of organisms to utilize the thermodynamically favourable acetylene–sulphate oxidoreduction as a source of energy for growth suggests that limitations other than thermodynamic constraints apply. This is reinforced by the fact that known sulphate reducers could not utilize acetylene as a carbon source when a separate energy source was available.

However, experimental evidence both for and against the concept of growth of sulphate reducers on long-chain hydrocarbons has been reported. In view of the correlation between theory and experiment discussed above it is suggested that the theory supports those findings which deny that growth can occur by utilization of such substrates as the sole energy source. It is possible, however, that by a process of co-metabolism, thermodynamically unfavourable substrates such as the long-chain hydrocarbons could be metabolized at the expense of an energy-yielding substrate.

The assessment has been limited to an examination of oxidoreduction reactions with a view to predicting whether they could conceivably supply the energy requirements for growth of sulphate-reducing microorganisms. It is considered that the degree of correlation between the data and the criterion warrants its emphasis here, and its consideration in future studies concerning the energetics of ATP generation.

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