ISOTOPE EFFECTS

IN

THE REDUCTION OF SULPHUR COMPOUNDS

ISOTOPE EFFECTS IN THE CHEMICAL AND BACTERIAL REDUCTION OF SULPHUR COMPOUNDS

by

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SCOPE AND CONTENTS:

Equilibrium exchange constants were calculated for exchange of sulphite with other sulphur compounds. The equilibrium constant for sulphur isotope exchange between sulphate in solution and solid calcium sulphate was calculated and measured experimentally. In the chemical reduction of sulphate to sulphide $S^{32}O_4^=$ reacted 2.5% faster than $S^{34}O_4^=$, in agreement with the calculated kinetic isotope effect for the step sulphate to sulphite. The isotope effect in the reduction of sulphate by Desulphovibrio desulphuricans was found to vary from 0.0 to 2.5%. The results were interpreted on the basis of a mechanism involving two consecutive steps, pick-up of sulphate and reduction of sulphate to sulphite, competing for control of the rate. The isotope effect in bacterial reduction of sulphite was studied briefly.

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INTRODUCTION

Following the discovery of isotopes about 1910 (1,2) there was considerable speculation concerning the possibility of differences in the chemical reactivity of the various isotopes of an element. The first theoretical approach to the problem was made by Lindemann (3) in 1919 when he applied the methods of statistical mechanics to a calculation of the differences in the vapour pressures of the isotopes of lead. He showed that considerable differences should exist if the half-quanta of zero-point energy were not present; at that time the status of the zero-point energy was still in question. Since experiments showed no detectable differences in the vapour pressures Lindemann concluded that the half-quanta must exist. As it turns out Lindemann was correct in his conclusions regarding the zero-point energy; the reason he found no differences in vapour pressure was that at the high atomic weight of lead the differences are too small to be detected experimentally.

With the discovery of heavy hydrogen in 1932 and the earlier discoveries of the rare heavy isotopes of carbon (4), nitrogen (5), and oxygen (6) in 1928-1930 the problem of the chemical differences of isotopes was attacked once more since there is a large percentage mass difference for the isotopes of these light elements and differences in the chemical properties of these isotopes were expected. Urey, Brickwedde and

Murphy (7,8) calculated that H_2 and HD should have large differences in vapour pressures and succeeded in concentrating deuterium sufficiently by fractional distillation to first permit its detection.

Equilibrium Isotope Effects

Following the discovery of deuterium, Urey and Rittenberg (9) calculated by the methods of statistical mechanics the equilibrium constants for the exchange reactions of H_2 and D_2 as well as for the hydrogen and deuterium halides. In every case they obtained values differing from unity, indicating that the isotopes of hydrogen were not chemically identical. These results were soon confirmed experimentally by the same authors (10). In the following years a fund of data, both theoretical and experimental, was built up substantiating Urey's observations on the isotopes of hydrogen.

Encouraged by the results with hydrogen Urey and Greiff (11), in 1935, calculated equilibrium constants for possible exchanges of lithium, carbon, nitrogen, oxygen, chlorine, and bromine isotopes. The calculated equilibrium constants differed slightly from unity, indicating that the isotopes differed significantly in their chemical properties. For example, the equilibrium constant for the exchange reaction

$$\tfrac{1}{2}\, CO_2{}^{16} + H_2O^{18} = \tfrac{1}{2}\, CO_2{}^{18} + H_2O^{16}$$

was calculated to be 1.044 at 0° C. This was determined experimentally by Weber, Wahl, and Urey (12) and found to be 1.046 at 0° C.

As mass spectrometers were developed to measure relative isotope abundances more accurately, many of these exchange reactions were studied experimentally with results consistently agreeing with theory. Urey has summarized the earlier results in an address to the Chemical Society in 1947 (13). Besides the theoretical importance attached to isotope exchange reactions, such reactions have been extensively studied with a view to the separation of isotopes. Although the separation is usually very small in a simple exchange, large separations are possible by counter-current extracting columns. The separated isotopes have many important applications in all fields of science.

Kinetic Isotope Effects

It follows that if the equilibrium constants of isotope exchange reactions differ from unity, the isotopic compounds of an element must react at different rates. This type of an isotope effect occurring in a unidirectional reaction has become known as a kinetic isotope effect.

For the hydrogen isotopes such kinetic effects were observed from the beginning. One of the earliest methods of concentrating deuterium was the electrolysis of water where deuterium is evolved at a significantly slower rate than protium (14). In 1934 Bonhoeffer, Bach, and Fajans (15) showed that H_2 reacted 3.3 times faster with bromine than did D_2. Similar results were found for the reaction of the hydrogen isotopes with chlorine (16,17). Geroe and Schmidt

(18) showed that deuterides were more stable than hydrides, while Wynne-Jones (19) determined the rate of ionization of protium as ten times that of deuterium. The early results on the chemical differences of the hydrogen isotopes have been summarized by Urey and Teal (20). Eidinoff (21) has published more recently a review on the fractionation effects in the chemical and biological reactions of the hydrogen isotopes.

Similar kinetic effects for isotopes other than hydrogen were not known until the late 1940's. In 1948 Stevenson and co-workers (22) reported that the electron-impact dissociation of propane-1-C^{13} in a mass spectrometer resulted in a 12% greater frequency of rupture of a $C^{12}-C^{12}$ bond than a $C^{12}-C^{13}$ bond. The same group then investigated (23) the isotope effect in the thermal cracking of the same compound and found in this case an 8% more frequent rupture of the $C^{12}-C^{12}$ bond than the $C^{12}-C^{13}$ bond.

In 1949 Yankwich and Calvin (24) reported that in the thermal decomposition of malonic acid labelled with C^{14} in a carboxyl position, the probability of rupture of a $C^{12}-C^{12}$ bond was 12% greater than the rupture of a $C^{12}-C^{14}$ bond. The magnitude of this effect seemed extremely large and the experiment was repeated independently by Bigeleisen and Friedman (25), and Lindsay, Bourns, and Thode (26) using malonic acid of normal C^{13} content and measuring the $C^{12}-C^{13}$ isotope effect mass spectrometrically. These workers obtained values of 2.0% and 2.5% respectively, in reasonable agreement

with Bigeleisen's theoretical value of 2.0% (27). It was expected from theory (28) that the effect for isotopes of the same element should be approximately proportional to ΔM. Therefore, the C^{14} isotope effect should have been only twice that found for C^{13}. Considerable controversy arose in the chemical literature on the ratio of C^{14} and C^{13} isotope effects in this and in other reactions (29,30).

The C^{14} effects were measured usually by counting techniques, with a reproducibility of only one to two percent at the best of times, while the C^{13} effects were measured mass spectrometrically with much greater reproducibility. Yankwich, Promislow, and Nystrom (31) have redetermined recently the C^{14} and C^{13} isotope effects in the malonic acid decomposition using mass spectrometry for both determinations. They have reported a C^{14} isotope effect of 5.5% compared to 2.9% for C^{13}. The ratio of the two effects is 1.91, a figure in excellent agreement with theory (28). It would appear that the controversy has now been settled; the original difficulties arose in the errors in counting enriched C^{14} samples.

Since one will observe a kinetic isotope effect in a unidirectional chemical reaction only if the rate-controlling step involves some change in the bonding of the element in question, the study of isotope fractionation has considerable application in the field of reaction mechanisms. Further, the

size of the isotope effect is determined, in part, by the nature of the activated complex with the result that, in theory at least, a study of isotope effects should provide some information on the structure of the complex. For these reasons kinetic isotope effects have been studied increasingly over the past seven years. The study of isotope fractionation in the reactions of organic compounds has been especially fruitful in determining reaction mechanisms. Several authors have recently reviewed kinetic isotope effects (29,30,32).

Variations in Isotopic Abundances in Nature

Closely related to the differences observed in the chemical properties of isotopes is the question of the constancy of the natural isotopic composition of the lighter elements. If, as we have seen, isotopes are fractionated in equilibrium and unidirectional processes in the laboratory, they should be fractionated in nature by similar processes occurring under natural conditions. These natural variations have been studied extensively since about 1935, with the hope that the results will aid in the explanation of biological, geological, and chemical phenomena in nature. In certain cases experiments have been carried out to determine the mechanism by which the isotopes are fractionated in nature.

One of the first elements whose isotopic variation in nature was studied was oxygen. Dole and co-workers (33-39), employing sensitive methods of determining water

densities, have studied extensively the natural variations in the abundance of the oxygen isotopes O^{16} and O^{18} in air, water, and minerals. They found variations up to four percent in the O^{18} content of these substances. Thode and Smith (40), using mass spectrometric analysis, have confirmed these results. More recently Baertschi and Silverman (41,43) have shown that the O^{18} content of sedimentary rock is greater than that of igneous rock.

Dole and co-workers found that atmospheric oxygen was enriched by some 3‰ in O^{18} compared to water. Since carbon dioxide is known to be enriched in O^{18} by about 3.9‰ in the $CO_2 - H_2O$ exchange it was suggested (44) that atmospheric oxygen might arise from carbon dioxide by means of photosynthesis. However, several groups (38,45,46) showed by tracer experiments that the isotopic content of photosynthetic oxygen was the same as the water used by the plant.

Dole, Hawkings, and Barker (47) carried out experiments to determine whether soil bacteria might cause the enrichment of the atmospheric oxygen by preferential removal of O^{16}. The slight fractionation found, while in the expected direction, was much too small to explain the enrichment of the atmosphere. Dole and Lane (48) recently have made an extensive study of the respiration of a wide variety of plants and animals, and have shown that the enrichment of O^{18} in the atmosphere can be accounted for

by the preferential removal of C^{16} by living organisms during respiration.

Considerable work has been done on the abundance of the stable carbon isotopes C^{12} and C^{13} in nature. Nier and co-workers (49,50) found variations up to 5% in C^{12}/C^{13} ratios, with limestones and inorganic materials enriched in C^{13}, and carbon of organic origin depleted. Mars (51) and Trofimov (52) have confirmed the earlier work of Nier. Wickman (53,54) has studied the carbon isotope content of a large number of plants and has found variations characteristic of the environment in which the plants were grown. Craig (55) has published more recently a comprehensive survey of carbon isotopes in both organic and inorganic materials, with results substantiating as a whole the results of earlier workers.

Buchanan, Nakao, and Edwards (56) have studied the distribution of carbon isotopes in the various biological systems of balanced aquaria, sealed for as long as three years. They found plants depleted in C^{13} and carbonates such as snail shells enriched in C^{13}; however, the fractionation was not as large as that found in nature. Taylor (57) has reported that the carbon dioxide produced by bacterial oxidation of organic acids is depleted in C^{13} by 1-1.5%. With the exception of this work, very little has been done to study the natural fractionation systems in the laboratory.

Sulphur Isotopes in Nature: In this laboratory Thode and co-workers have made an extensive study of the variations of the S^{32}/S^{34} ratio in nature (58-63). The results so far obtained are summarised in Figure 1. The δ value given at the left of Figure 1 is the per mil enrichment in S^{34} compared to meteorites as the standard, and is defined as follows:

$$\delta = \frac{(S^{34}/S^{32})\ \text{sample} - (S^{34}/S^{32})\ \text{standard}}{(S^{34}/S^{32})\ \text{standard}} \times 1000$$

Although considerable work still must be done to explain some of the finer details of the variations, a definite over-all pattern can be seen. In general, sulphates are enriched while sedimentary sulphides are depleted in S^{34}, compared to meteorites which have been found to be amazingly constant (59,64). Sulphides of igneous origin are reasonably constant in isotopic content and close to meteoritic sulphur. The results of Figure 1 have been confirmed by several other groups (65-67).

Thode, Macnamara, and Fleming (62) have found that if the S^{32}/S^{34} ratios of sedimentary sulphates and sulphides are plotted against their geological age the ratios converge on a value close to that of meteorites, at an age of about 800 million years. This suggests that all sulphur at one time had an isotopic abundance close to that of meteorites, but in the past 800 million years the isotopes have been fractionated at an ever increasing rate by some naturally

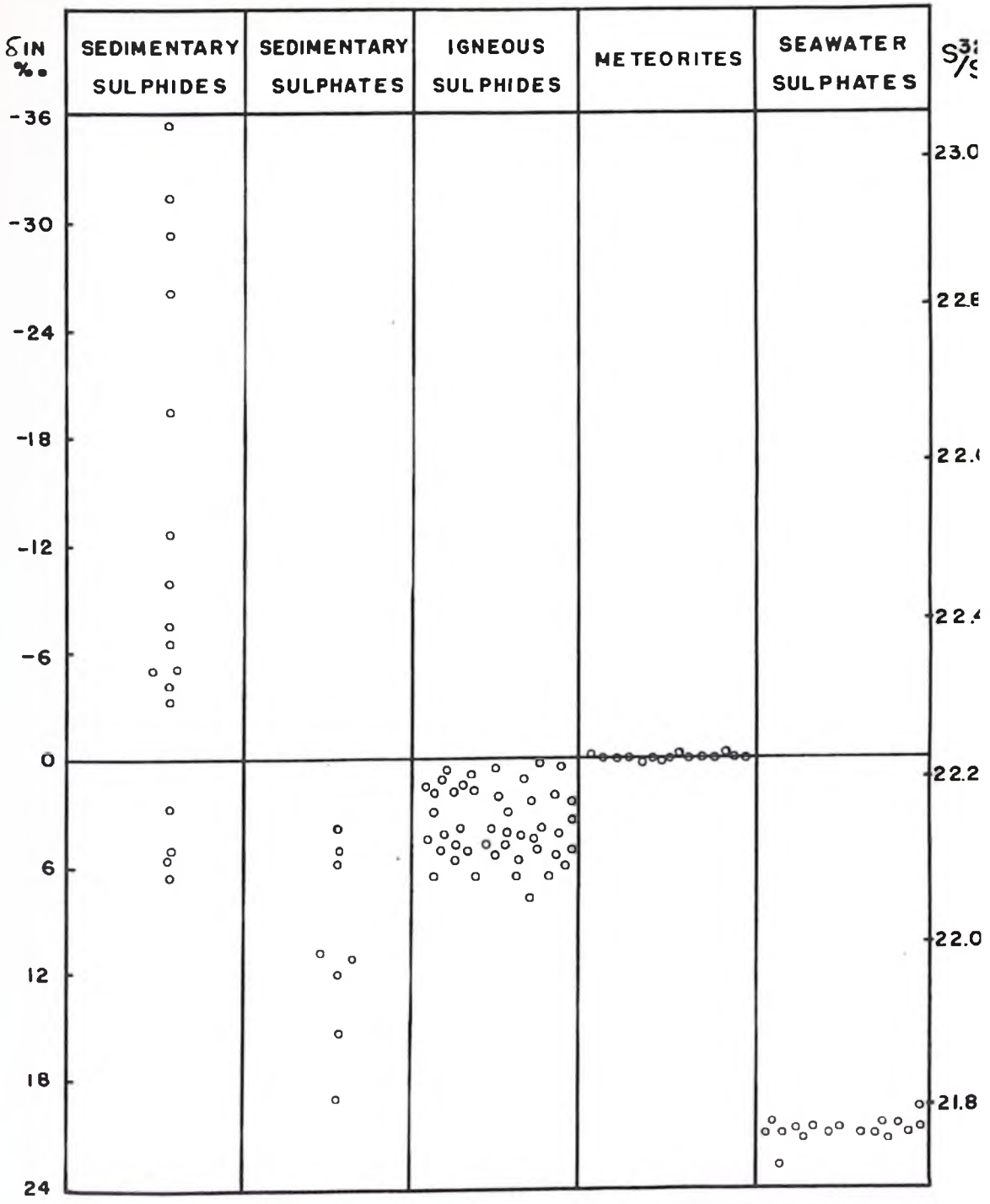


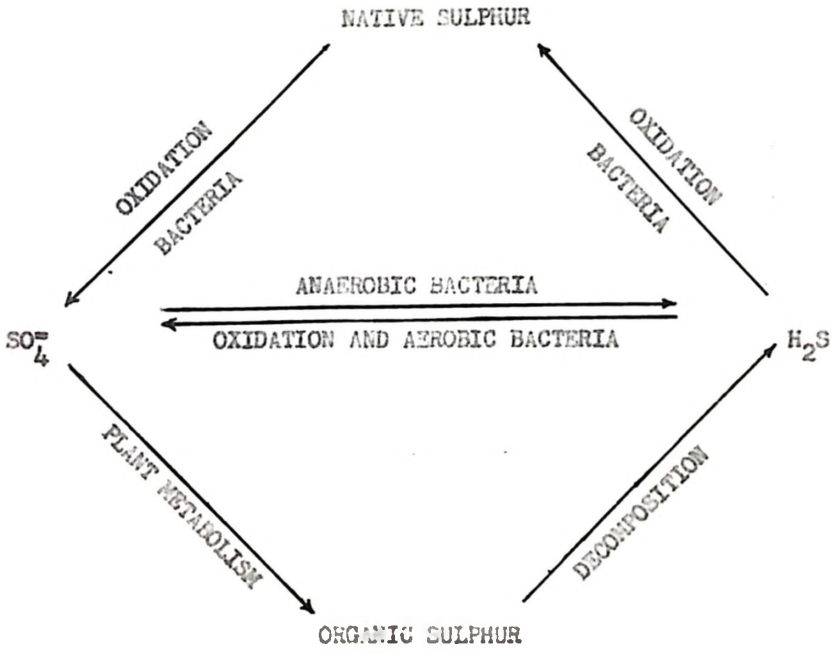
FIG. I SULPHUR ISOTOPE DISTRIBUTION IN NATURE

occurring geological or biological process.

The total spread found between sulphides and sulphates is about 8%; reasonably close to the equilibrium constant for the exchange reaction

$$H_2 S^{34} + S^{32} O_4^{=} \rightleftharpoons H_2 S^{32} + S^{34} O_4^{=}$$

calculated by Tudge and Thode (68) to be 1.074 at 25°C. (This constant has been recalculated in this thesis as 1.070 - See Table III). Since it is known (69) that this exchange does not take place in a chemical system, it has been suggested that the biological sulphur cycle, outlined below, could provide a mechanism for the fractionation of the sulphur isotopes in nature. Considerable evidence for this has been accumulated both from nature and from laboratory controlled experiments.



Concrete evidence for the fractionation of sulphur isotopes in the biological sulphur cycle has come from a study of sulphate and sulphur samples from the sulphur-producing lakes in the Cyrenaican district of Africa. Butlin and Postgate (70,71) have reported that these lakes abound both in sulphate-reducing bacteria and in photosynthetic hydrogen sulphide oxidizing bacteria. Each year a layer of elemental sulphur is formed on the bottom of the lakes by bacterial oxidation of the hydrogen sulphide produced from bacterial reduction of sulphate.

As presented in Table I analysis of the sulphur and sulphate samples showed that the sulphur is depleted in S^{34} by 1.5 to 3.2‰ compared to the source sulphate, present in the lake as a saturated solution of calcium sulphate. These results definitely show that the sulphur isotopes are fractionated by naturally occurring biological processes.

TABLE I

Isotope Fractionation in Cyrenaican Lakes

S^{32}/S^{34}		fractionation	reference
$SO_4^=$	S^o	$S^o/SO_4^=$	
21.68	22.57	1.032	61
22.190	22.516	1.015	This work

Further evidence that the biological sulphur cycle could fractionate the sulphur isotopes was supplied by a study of the sulphur wells of Texas and Louisiana by Thode, Wanless, and Wallouch (63). They found, on analyzing sulphide, sulphur, and sulphate deposits from ten of these wells, that the sulphur was depleted in S^{34} by about 4‰ with respect to the sulphate, while the sulphide was depleted by a further 1‰. Examination of the carbon isotopes in the surrounding limestone by these authors and by Taylor (57) showed the carbonate to be of organic origin. They thus postulated that the sulphur and sulphide arose by bacterial reduction of sulphate. These bacteria can utilize organic carbon compounds as a source of energy, oxidizing them in the process to carbon dioxide. These conclusions were strengthened by Miller's isolation of sulphate-reducing bacteria from core samples of the wells (72) and, as will be discussed in detail below, by laboratory experiments which showed that these sulphate-reducing bacteria do fractionate the isotopes.

Certain parts of the sulphur cycle have been, and still are, under investigation in this laboratory. Ishii (73) studied the sulphate metabolism of algae and found that the organic sulphur compounds had an isotopic ratio identical with that of the nutrient sulphate. This was explained on the assumption that the rate of pick-up of the sulphate by the cells was the rate-controlling step. McElcheran (74)

carried out a preliminary study of bacteria, both sulphur oxidizing and sulphate reducing species. Of special interest to this thesis was his study of the sulphate-reducing bacteria Desulphovibrio desulphuricans. Thode, McElcheran, and Kleerekoper (76) found that when these bacteria were grown in pure culture under laboratory controlled conditions the hydrogen sulphide produced at 25°C was depleted in S^{34} by about 1‰ compared to the nutrient sulphate.

Wallouch (77) made a more extensive study of the fractionation obtained in the reduction of sulphate by these bacteria. Working with continuous culture techniques he found that a gradual lowering of the temperature from 35° to 0°C over a period of months resulted in an increase in the fractionation from 0.9‰ to a maximum of 1.8‰ at 6°C followed by a decrease to 1.5‰ at 0°C (Figure 2).

It might be well at this point to quote Postgate (75) regarding the nomenclature of these bacteria. "The classification and hence the nomenclature of the sulphate-reducing bacteria is in a state of chaos. At present we are obliged to call them Desulphovibrio desulphuricans, although the French prefer Sporovibrio desulphuricans, and many workers still call them Vibrio desulphuricans; their discoverer Beijerinck called them Spirillum desulphuricans." For purposes of brevity we shall henceforth refer to them as D. desulphuricans.

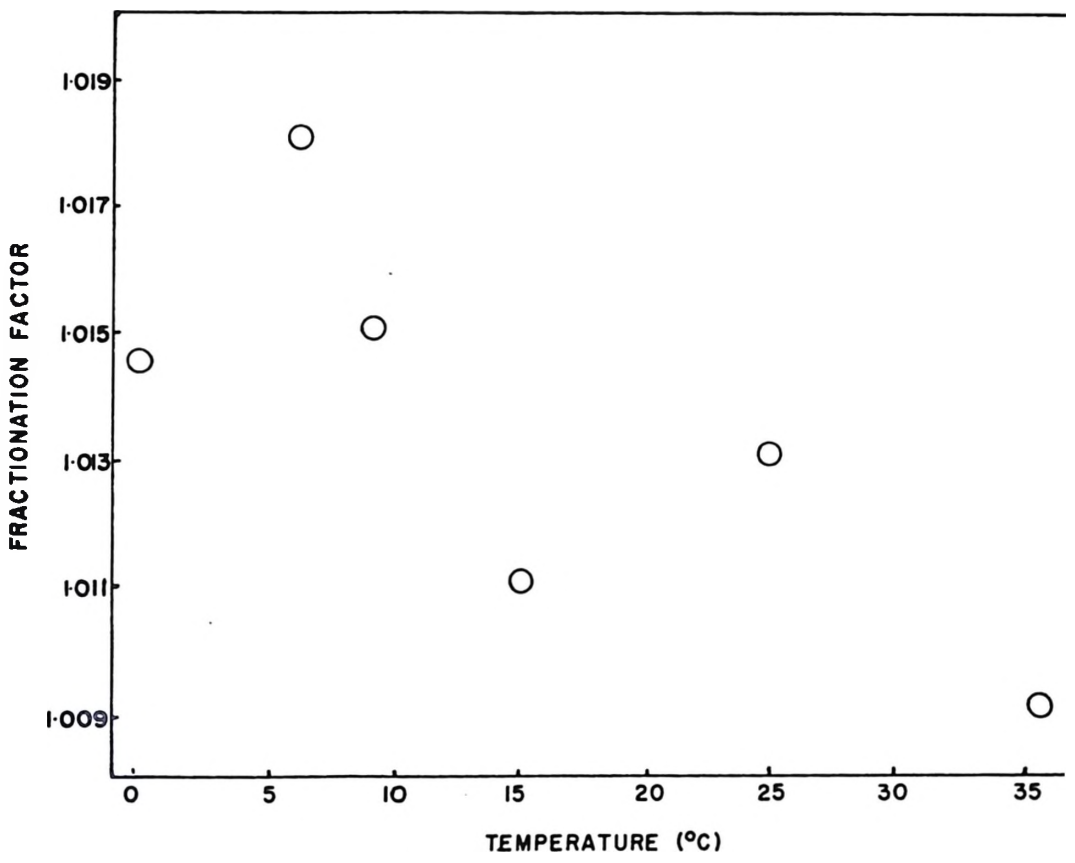


FIG. 2 FRACTIONATION IN BACTERIAL REDUCTION OF SULPHA

(FROM WALLOUCH REFERENCE 77)

The temperature coefficient of the fractionation factor found by Wallouch (Figure 2) is much bigger than that normally found in a unidirectional or an equilibrium isotope effect; it must be caused by some unknown factors inherent in the mechanism of the reduction. It therefore appeared interesting to study the bacterial reduction of sulphate to determine the factors which controlled the observed fractionation . Preliminary results obtained by this author, using the same techniques, showed that the bacterial reduction could be carried out to give no depletion of S^{34} in the hydrogen sulphide produced.

The work to be reported in this thesis is for the most part a study of the reduction of sulphur compounds by the bacteria D. desulphuricans in an attempt to determine the maximum fractionation of isotopes obtainable by these bacteria and also to elucidate, at least in part, the mechanism of the reduction process. The results to be presented here are the first reported extensive study of the factors which control the fractionation of isotopes in reactions as complex as occur in the metabolism of living organisms, and as such should be of considerable importance not only to those studying fractionation of isotopes in nature, but also to those studying metabolic processes in general.

THEORY

Theory of Isotope Exchange Reactions

As previously mentioned, Urey and Greiff (11) were the first to apply statistical mechanics to the calculation of equilibrium constants for isotope exchange r actions. Urey (13) since has developed a relatively simple relation making it possible to calculate equilibrium constants from a knowledge of the vibrational frequencies of the isotopic molecules. The theory to be presented here follows the Urey treatment.

The general case of a typical exchange reaction may be written as

$$a\,A_1 + b\,B_2 = a\,A_2 + b\,B_1 \qquad \ldots (1)$$

where A and B are molecules which have some one element as a common constituent and subscripts 1 and 2 indicate that the molecule contains only the light or the heavy isotope respectively.

The equilibrium constant for the reaction is given by

$$-RT \ln K = \triangle F^{\circ} \qquad \ldots(2)$$

where $\triangle F^{\circ}$ is the standard free energy change. For a reaction of the type shown in equation (1) this becomes

$$-RT \ln K = a F_{A_2} + b F_{B_1} - a F_{A_1} - b F_{B_2} \qquad \ldots (3)$$

The free energy is related to the partition function by the well-known equation

$$F = E_o + RT \ln N - RT \ln Q \qquad \ldots (4)$$

where E_o is the zero point energy of the molecule, N is Avogadro's number, and Q is the complete partition function for the molecule. If we consider the standard state to be for one molecule (instead of one mole) in unit volume the N drops out. Then, substituting equation (4) in equation (3) and simplifying, we obtain

$$K = \left[\frac{Q_{A_2}}{Q_{A_1}}\right]^a \Big/ \left[\frac{Q_{B_2}}{Q_{B_1}}\right]^b \; e^{-\left[\frac{aE_{oA_2} + bE_{oB_1} - aE_{oA_1} - bE_{oB_2}}{RT}\right]} \qquad \ldots (5)$$

Instead of taking E_o as the zero point energy of the molecule one can take it as the bottom of the potential energy curve for the molecule. Since the potential energy curves are practically identical for isotopic molecules $E_{oA_2} = E_{oA_1}$ and $E_{oB_2} = E_{oB_1}$. Thus the exponential term becomes unity and

$$K = \left[\frac{Q_{A_2}}{Q_{A_1}}\right]^a \Big/ \left[\frac{Q_{B_2}}{Q_{B_1}}\right]^b . \qquad \ldots (6)$$

Therefore, to calculate the equilibrium constant for an exchange reaction it is necessary to determine the ratio of partition functions for the isotopic molecules.

A fair approximation to the complete partition function for a polyatomic molecule is given as (78)

$$Q = g_e g_n \cdot \frac{(2\pi MkT)^{3/2}}{h^3} \cdot \frac{RT}{P} \cdot \frac{8\pi^2 (8\pi^3 ABC)^{1/2} (kT)^{3/2}}{\sigma h^3} \prod_i (1-e^{-u})^{-1} \quad \ldots (7)$$

where M is the molecular weight of the molecule considered, A, B, C are the three principal moments of inertia, σ is the symmetry number, g_e, g_n are the electronic and nuclear statistical weights respectively, and $u = \frac{hc\nu_i}{kT}$, where ν is the molecular vibrational frequency in cm^{-1}, and the product is to be taken over all i such frequencies.

In our case we are dealing with the ratios of partition functions for isotopic molecules.. Thus, from equation (7), after cancelling of terms, we have

$$\frac{Q_2}{Q_1} = \frac{\sigma_1}{\sigma_2} \left[\frac{A_2 B_2 C_2}{A_1 B_1 C_1}\right]^{1/2} \left[\frac{M_2}{M_1}\right]^{3/2} \prod_i \frac{e^{-\frac{u_{2i}}{2}}}{1-e^{-u_{2i}}} \cdot \frac{1-e^{-u_{1i}}}{e^{-\frac{u_{1i}}{2}}} \quad \ldots (8)$$

The ratios of the moments of inertia for polyatomic molecules are usually unknown and are difficult to calculate. Urey (13) has used the following reasoning to obtain further simplification. If the left and right sides of equation (8) are multiplied by $(m_1/m_2)^{3/2n}$, where m_1 and m_2 are the atomic weights of the isotopic atoms being exchanged, and n is the number of isotopic atoms being exchanged, and if the right side of equation (8) is multiplied and divided by the ratio $\prod_i \frac{u_{1i}}{u_{2i}}$, the equation becomes

$$\frac{Q_2}{Q_1}\left[\frac{m_1}{m_2}\right]^{3/2n} = \frac{\sigma_1}{\sigma_2}\prod_i \frac{u_{2i}}{u_{1i}} \cdot \frac{e^{-u_{2i}/2}}{1-e^{-u_{2i}}} \cdot \frac{1-e^{-u_{1i}}}{e^{-u_{1i}/2}} \qquad \ldots (9)$$

since by the Teller-Redlich Theorem

$$\left[\frac{M_2}{M_1}\right]^{3/2}\left[\frac{A_2\ B_2\ C_2}{A_1\ B_1\ C_1}\right]^{1/2}\left[\frac{m_1}{m_2}\right]^{3/2n}\prod_i \frac{u_{1i}}{u_{2i}} = 1 \qquad \ldots (10)$$

We then define new partition function ratios where

$$\frac{Q_2'}{Q_1'} = \frac{Q_2}{Q_1}\left[\frac{m_1}{m_2}\right]^{3/2n} = \frac{\sigma_1}{\sigma_2}\prod_i \frac{u_{2i}}{u_{1i}} \cdot \frac{e^{-u_{2i}/2}}{1-e^{-u_{2i}}} \cdot \frac{1-e^{-u_{1i}}}{e^{-u_{1i}/2}} \qquad \ldots (11)$$

It is obvious that the equilibrium constant K is given by

$$K = \left[\frac{Q'_{A_2}}{Q'_{A_1}}\right]^a \Big/ \left[\frac{Q'_{B_2}}{Q'_{B_1}}\right]^b \qquad \ldots (12)$$

Equation (11) has been simplified, for purposes of calculation, by Urey (13) and also by Bigeleisen and Mayer (79). We shall use the Bigeleisen and Mayer simplification here.

Let $u_{1_i} = u_{2_i} + \triangle u_i$, where u_{1_i} corresponds to the lighter molecule. Then $\triangle u_i$ is always positive and equation (11) becomes

$$\frac{Q_2'}{Q_1'} = \frac{\sigma_1}{\sigma_2}\prod_i \frac{u_{2_i}}{u_{2_i}+\triangle u_i} \cdot e^{\triangle u_i/2}\,\frac{1-e^{-u_{2i}+\triangle u_i}}{1-e^{-u_{2i}}} \qquad \ldots (13)$$

When $\triangle u_i$ is small, which is true for every case except hydrogen, Bigeleisen and Mayer (79) have shown

that equation (13) can be simplified to give

$$\frac{Q_2'}{Q_1} = \frac{\sigma_1}{\sigma_2}\left[1 + \sum_i \left[\frac{1}{2} - \frac{1}{u_{2i}} + \frac{1}{(e^{u_{2i}}-1)}\right] \Delta u_i\right] \qquad \ldots (14)$$

where the summation is over all i vibrational frequencies of the molecule, counting an n - degenerate frequency n times. The function $\left[\frac{1}{2} - \frac{1}{u_{2i}} + \frac{1}{e^{u_{2i}}-1}\right]$ has been termed the free energy function by Bigeleisen and Mayer (7) and given the symbol G (u). They have tabulated values of this function for values of u from 0 to 25.

Therefore, although the calculation of a single partition function would be extremely complicated, we can calculate, using equation (14), the partition function ratios for isotopic molecules from a knowledge of only the vibrational frequencies of the two molecules. One can determine the vibrational frequencies of the molecule containing the most abundant isotope from spectroscopic data; however the rare isotopic molecule usually is in such low concentration that its vibrational frequencies cannot be determined experimentally. The usual practice is to calculate the vibrational frequencies of the rare molecule from the frequencies of the abundant molecule by means of well-known "normal vibration equations" (30). Normal vibration equations relate the frequencies to force constants and atomic weights. By assuming the force

constants to hold for both isotopic species it is possible to calculate frequencies for both isotopic molecules, and then find the differences. While these calculated frequencies may be slightly in error the differences can be evaluated quite accurately. By using these differences and the experimentally observed frequencies for the abundant molecule, it is possible to calculate the fundamental frequencies for the molecule containing the rare isotope.

Theory of Kinetic Isotope Effects

Bigeleisen (81) has put the theory of isotope effects in unidirectional reactions into its most satisfactory form making only the assumptions inherent in the transition state hypothesis. His development is presented in the following.

Consider the reactions

$$A_1 + B + C \xrightarrow{k_1} P_1$$

$$A_2 + B + C \xrightarrow{k_2} P_2$$

where the subscripts 1 and 2 indicate the molecule in question contains only the light or the heavy isotope in question. The rate constants, k_1 and k_2, for these two reactions are given by the absolute rate theory as

$$k_1 = \frac{K_1 C_1^{\ddagger}}{C_{A_1} C_B} \cdot \left[\frac{k T}{2\pi m_1^*}\right]^{1/2} \frac{1}{\delta_1} \qquad \ldots (15)$$

$$k_2 = \frac{K_2 C_2^{\ddagger}}{C_{A_2} C_B} \cdot \left[\frac{k T}{2\pi m_2^*}\right]^{1/2} \frac{1}{\delta_2} \qquad \ldots (16)$$

where K is the transmission coefficient,

C_1, C_{A_2}, C_2, C_B, C_{A_1} are concentration terms,

$\neq$ refers to the activated complex,

δ is a hypothetical quantity considered as being the length of a potential box containing the activated complex,

$m^{\neq}$ is the effective mass of the activated complex along the coordinate of decomposition.

The concentration terms of equations (15) and (16) can be expressed in terms of partition functions. Choosing the minimum in the potential energy curve rather than the zero-point energy as our zero point for calculating partition functions we obtain the following expression for the ratio of rate constants

$$\frac{k_1}{k_2} = \frac{K_1}{K_2} \frac{Q'_{A_2}}{Q'_{A_1}} \frac{Q_1^{\neq\,\prime}}{Q_2^{\neq\,\prime}} \left[\frac{m_2^*}{m_1^*}\right]^{1/2} \qquad \ldots (17)$$

where

$$\frac{Q'_{A_2}}{Q'_{A_1}} = \frac{\sigma_1}{\sigma_2}\left[1+\sum_i^{3n-6} G(u_i)\cdot\triangle u_i\right]$$

$$\frac{Q_2^{\neq\,\prime}}{Q_1^{\neq\,\prime}} = \frac{\sigma_1^{\neq}}{\sigma_2^{\neq}}\left[1+\sum_i^{3n'-6} G(u_i^{\neq})\ \triangle u_i^{\neq}\right]$$

as defined by the equation (14).

Bigeleisen (81) has rearranged equation (17) to give

$$\frac{k_1}{k_2} = \frac{K_1}{K_2} \frac{\sigma_1}{\sigma_2} \frac{\sigma_2^{\neq}}{\sigma_1^{\neq}} \left[\frac{m_2^{\neq}}{m_1^{\neq}}\right]^{1/2} \left[1 + \sum_i^{3n-6} G(u_i) \Delta u_i - \sum_i^{3n'-6} G(u_i^{\neq}) \Delta u_i^{\neq} \right] \quad \ldots (18)$$

By making several approximations equation (18) may be used to calculate the ratio of rate constants for isotopic molecules from a knowledge of vibrational frequencies. For isotopic atoms heavier than hydrogen the ratio of the transmission coefficients is assumed to be unity. Usually the symmetry numbers are considered the same for the isotopic molecule and its activated complex.

By far the most serious restriction to the application of this theory to the calculation of kinetic isotope effects is the fact that we know very little about the nature of the activated complex. The ratio $m_2^{\neq}/m_1^{\neq}$ is described as the effective mass of the activated complex along the reaction coordinate. In practice this is usually taken as the reduced mass of the atoms involved in the bond being broken or formed.

Since we do not know the structure of the activated complex we cannot know its fundamental vibrational frequencies. Therefore, we cannot calculate the partition function ratio for the activated complexes of the two isotopic species. In general we can say that the activated complex will be intermediate in form between the reactants and

the products; thus we can calculate the two extreme limits for the ratio of rate constants. If we assume that the activated complex is identical with the starting material then $\sum G(u) \cdot \triangle u = \sum G(u^{\neq}) \cdot \triangle u^{\neq}$, and the ratio of the rate constants becomes the ratio of reduced masses. The other extreme arises if we assume that the activated complex is the same as the product molecule. In this case $\sum G(u^{\neq}) \cdot \triangle u^{\neq}$ is not necessarily the same as $\sum G(u) \triangle u$ and the ratio of rate constants will be either larger or smaller than the ratio of reduced masses depending on the values of the two free energy functions. In this thesis equation (18) will be used to calculate the ratio of rate constants for the unidirectional reduction of $S^{32}O_4^{=}$ and $S^{34}O_4^{=}$.

Calculation of Isotope Effects Involving the Sulphite Ion

Equilibrium Isotope Exchange Reactions: In 1951, Tudge and Thode (68) reported the calculation of partition function ratios for a number of ions and molecules containing sulphur, and calculated equilibrium constants for possible exchange reactions between these compounds. At that time no data was available on the vibrational frequencies of the sulphite ion so no calculations involving this ion were possible.

Millet (32) has presented evidence that sulphite is an intermediate in the reduction of sulphate by D desulphuricans. This has been confirmed in this laboratory (33) by the use of S^{34} enriched sulphate and sulphite as tracers.

Postgate (84) has shown that sulphite is reduced at a faster rate than sulphate by the bacteria. This evidence along with the results of Wallouch (77), previously presented, suggested that the rate-controlling step in the reduction of sulphate could be the step $SO_4^= \longrightarrow SO_3^=$. Wallouch's large temperature coefficient (Figure 2) suggested that another possibility was the exchange between sulphate and sulphite, catalyzed by the bacteria, followed by a step involving a kinetic isotope effect. In either case it appeared imperative to obtain vibrational data on the sulphite ion and calculate the theoretical values for these reactions.

Accordingly pure samples of sodium sulphite in aqueous solution were prepared in this laboratory and the infra-red and Raman spectra of the sulphite ion very kindly obtained by Dr. H. Bernstein, National Research Council, Ottawa. At about the same time Bernstein published the results (87), two other papers were published (85, 86) on the Raman spectra of the sulphite ion in solution. The values reported by the three groups agree very well with each other.

Bernstein (87) calculated the vibrational frequencies of $S^{34}\ O_3^{16=}$ using the mean values of the Raman and infra-red frequencies of the four modes of $S^{32}\ O_3^=$ in aqueous solution, dimensions of the ion from X-ray data,

and equations derived from a four constant potential function (80). For our calculations of the partition function ratio of the sulphite ion we have used as the vibrational frequencies of $S^{32}O_3^=$ the average of the values for the Raman lines in solution shown in Table II. The vibrational frequencies of $S^{34}O_3^=$ were calculated from these values using the isotope shift determined by Bernstein (87). The details of the calculation of the partition function ratio are given in Appendix 1.

TABLE II

Vibrational Frequencies of the Sulphite Ion

Ion	State	w_1 cm^{-1}	w_2 cm^{-1}	w_3^* cm^{-1}	w_4^* cm^{-1}	References
$S^{32}O_3^=$	solution	968	618	932	465	Raman (85)
$S^{32}O_3^=$	solution	966	612	925	471	Raman (86)
$S^{32}O_3^=$	solid	983	632	947	494	Raman (86)
$S^{32}O_3^=$	solution	967	620	933	469	Raman (87)
$S^{32}O_3^=$	solution	1002	632	954		I.R. (87)
$S^{32}O_3^=$	solid	1010	633	961	496	I.R. (87)
$S^{34}O_3^{16=}$	solution	972	623	937	457	calculated (87)

* doubly degenerate

The ratio of the partition functions for the sulphite ion, Q_2'/Q_1', was found to be 1.059 at 25°C and 1.068 at 0°C. The partition function ratios calculated by Tudge and Thode (68) were based on Urey's methods (13),

whereas the values calculated in this thesis follow Bigeleisen and Mayer (79). For purposes of comparison of the two methods a second calculation was made of the partition function ratio for the sulphate ion (see Appendix II). Values of 1.064 at 25°C and 1.096 at 0°C were obtained compared to the values of 1.038 and 1.101 calculated by Tudge and Thode (68). This was the only case where the Bigeleisen and Mayer method gave results differing from the values reported by Tudge and Thode.

In Table III we have assembled partition function ratios for several compounds and have calculated equilibrium constants for possible exchange reactions. In all cases the heavy isotope will concentrate in the compound listed at the left. The values enclosed in the heavy black lines were calculated from this work; all others are from Tudge and Thode (68).

From these partition function ratios the equilibrium constant for the exchange reaction

$$S^{34}O_3^{=} + S^{32}O_4^{=} = S^{32}O_3^{=} + S^{34}O_4^{=}$$

has been calculated to be 1.024 at 25°C increasing to 1.026 at 0°C (see heavy lines Table III). This is a typical temperature coefficient for an equilibrium exchange for isotopic reactions of sulphur. It is obvious that Wallouch's results, shown in Figure 2, cannot be explained on the basis of a temperature coefficient

TABLE III

Equilibrium Constants for Sulphur Exchanges

	$\frac{S^{34}O_4^=}{S^{32}O_4^=}$	$\frac{S^{34}O_3}{S^{32}O_3}$	$\frac{S^{34}O_3^=}{S^{32}O_3^=}$	$\frac{S^{34}O_2}{S^{32}O_2}$	$\left[\frac{CS_2^{34}}{CS_2^{32}}\right]^{1/2}$	$\left[\frac{S_2^{34}}{S_2^{32}}\right]^{1/2}$	$\frac{H_2S^{34}}{H_2S^{32}}$	$\frac{PbS^{34}}{PbS^{32}}$	$\frac{S^{=34}}{S^{=32}}$	°C
$\frac{O'_2}{O'_1}$	1.096	1.096	1.068	1.053	1.021	1.015	1.015	1.010	1.000	0°
	1.084	1.084	1.059	1.045	1.019	1.013	1.013	1.009	1.000	25°
$\frac{S^{34}O_4^=}{S^{32}O_4^=}$	1.000	1.000	1.026	1.041	1.073	1.080	1.080	1.085	1.096	0°
		1.000	1.024	1.037	1.064	1.070	1.070	1.074	1.084	25°
$\frac{S^{34}O_3}{S^{32}O_3}$		1.000	1.026	1.041	1.073	1.080	1.080	1.085	1.096	0°
			1.024	1.037	1.064	1.070	1.070	1.074	1.084	25°
$\frac{S^{34}O_3^=}{S^{32}O_3^=}$			1.000	1.014	1.046	1.052	1.052	1.057	1.068	0°
				1.013	1.039	1.045	1.045	1.050	1.059	25°
$\frac{S^{34}O_2}{S^{32}O_2}$				1.000	1.031	1.037	1.037	1.043	1.053	0°
					1.026	1.032	1.032	1.036	1.045	25°
$\left[\frac{CS_2^{34}}{CS_2^{32}}\right]^{1/2}$					1.000	1.006	1.006	1.011	1.021	0°
						1.006	1.006	1.010	1.019	25°
$\left[\frac{S_2^{34}}{S_2^{32}}\right]^{1/2}$						1.000	1.000	1.005	1.015	0°
							1.000	1.004	1.013	25°
$\frac{H_2S^{34}}{H_2S^{32}}$							1.000	1.005	1.015	0°
								1.004	1.013	25°
$\frac{PbS^{34}}{PbS^{32}}$								1.000	1.010	0°
									1.009	25°
$\frac{S^{34=}}{S^{32=}}$									1.000	0°
										25°

for an equilibrium exchange reaction.

It is worthwhile noting in particular one more equilibrium constant involving the sulphite ion. In 1945 Thode, Graham, and Ziegler (88) measured experimentally the equilibrium constant for the exchange reaction

$$HS^{32}O_3^- + S^{34}O_2 = HS^{34}O_3^- + S^{32}O_2 \quad \ldots 1$$

and found K 1.019 at 25°C. From Table III it is seen that K for the equilibrium

$$S^{32}O_3^{=} + S^{34}O_2 = S^{34}O_3^{=} + S^{32}O_2 \quad \ldots 2$$

is 1.013. Although we do not have the vibrational frequency data to calculate K for reaction (1), one would not expect K_1 and K_2 to differ appreciably. The agreement between theory and experiment is therefore quite good. It has been found (13) that the partition function ratios for the carbonate and the bicarbonate ion do not differ greatly. The fact that the equilibrium constants for reactions (1) and (2) are reasonably close to each other stands as indirect proof that the experimentally determined constant is reasonably accurate.

Kinetic Isotope Effect in Reaction $SO_4^{=} \longrightarrow SO_3^{=}$

It was suggested previously that the fractionation in the biological reduction of sulphate could occur in the reduction step from sulphate to sulphite. In a chemical reduction carried out and reported in this thesis the results strongly indicated that the fractionation was

occurring in the first S-O bond breakage. With the vibrational frequencies of the sulphite ion now known we are in a position to calculate the isotope effect for this reaction using various models.

Equation (18), from the theory, relating the ratio of rate constants to the free energy functions of the reactants and transition complex is (assuming $K_1 = K_2$ and $\sigma_1 \sigma_2^{\neq} = \sigma_2 \sigma_1^{\neq}$).

$$\frac{k_1}{k_2} = \left[\frac{m_2^*}{m_1^*}\right]^{1/2} \left[1 + \sum_i^{3n-6} G(u_i)\Delta u_i - \sum_i^{3n'-6} G(u_i^{\neq})\Delta u_i^{\neq}\right]$$

Before any calculations of the ratio of rate constants can be made we must make some assumptions regarding the structure of the activated complex.

Case I: The transition state is the same as the starting material. Essentially this means that there is no stretching of the sulphur-oxygen bond in the transition state. In this case the free energy functions of the reactant and activated complex are the same and the ratio of rate constants becomes

$$\frac{k_1}{k_2} = \left[\frac{m_2^*}{m_1^*}\right]^{1/2}$$

If we assume that the effective mass along the reaction coordinate is the reduced mass of the S-O bond being broken, we obtain

$$\frac{k_1}{k_2} = 1.010$$

Case II: The transition state is identical with the product of the reaction, in this case sulphite. The term $\sum_{i}^{3n-6} G(u_i) \triangle u_i$ is the free energy function for sulphate, calculated in Appendix II, and the term $\sum_{i}^{3n'6} G(u_i^{\ddagger}) \triangle u_i^{\ddagger}$ is the free energy term for sulphite, calculated in Appendix I. The ratio of the reduced masses remains the same. Substitution of the numerical values gives

$$\frac{k_1}{k_2} = 1.035 \text{ at } 25^{\circ}C.$$

$$\frac{k_1}{k_2} = 1.038 \text{ at } 0^{\circ}C.$$

Since the true configuration of the activated complex is undoubtedly intermediate in form between the two limiting cases just presented, the actual fractionation factor observed experimentally should fall between the lower limit of 1.010 and the upper limit of 1.035 at 25°C. The temperature coefficient calculated above is typical of most kinetic isotope effects and again cannot begin to explain the large temperature coefficient found by Wallcuch.

Sulphur Exchange between Solid Calcium Sulphate and Sulphate in Solution

One of the experimental difficulties in studying the bacterial reduction of sulphate was the maintenance of sufficient sulphate in the nutrient solution so that each sulphide sample produced was only a small fraction

of the total present; this is necessary in any study of isotope effects to obtain accurate values. One possibility was to add a large amount of a moderately soluble sulphate as an effectively infinite source of sulphate ion of constant concentration. This method is now in use in this laboratory using $CaSO_4 \cdot 2H_2O$ as the source of sulphate with excellent results. In this thesis barium sulphate and strontium sulphate were used for the same purpose. The question thus arose of the possibilities of an isotope effect in an exchange reaction of the type

$$CaS^{32}O_4 \cdot 2H_2O + S^{34}O_4^{=}\ (sol'n) = CaS^{34}O_4 \cdot 2H_2O + S^{32}O_4^{=}\ (sol'n)$$

This equilibrium has been calculated theoretically as well as measured experimentally.

The sulphate ion in solution is of the common tetrahedral XY_4 type of symmetry, classified in spectroscopy as T_d. It has four fundamental vibration frequencies $w_1 = 980$, $w_2 = 450$, $w_3 = 1114$, and $w_4 = 619$, all in cm^{-1}, of which w_2 is doubly degenerate and w_3 and w_4 are triply degenerate. The force constants for the sulphate ion are well known (80) and the isotopic shift has been calculated by Tudge and Thode (68). In Appendix II, as previously mentioned, we have calculated the ratio of partition functions for the sulphate ion in solution.

However, in a solid such as $CaSO_4 \cdot 2H_2O$ the effect

of the neighbouring calcium ions on the sulphate ion is to change the symmetry by causing a small displacement of the oxygen atoms so that we no longer have a tetrahedral XY_4 symmetry but an XY_2Z_2 type symmetry (89,90) classified by spectroscopists as C_2. This change of symmetry has the effect of splitting the degenerate vibrational levels of the sulphate ion into their components. This is shown by the frequencies assembled in Table IV for the sulphate ion in solution and for the sulphate ion of solid $CaSO_4 \cdot 2H_2O$.

Table IV

Vibrational Frequencies of $SO_4^=$ in Gypsum

Molecule	ν_1	$\nu_2(2)$	$\nu_3(3)$	$\nu_4(3)$	Reference
$SO_4^=$ solution	980	451	113.60	618.90	(91)
$CaSO_4 \cdot 2H_2O$	1006	415 492	1117 1138 1144	621 669	(89)
$CaSO_4 \cdot 2H_2O$	1008	415 494	1113 1135	618 672	(92)
$CaSO_4 \cdot 2H_2O$	1006	415 492	1115 1136	585 618 673	(93)

The force equations for ions of the C_2 type symmetry, as exhibited by the sulphate ion in the crystal of $CaSO_4 \cdot 2H_2O$, have not been worked out as yet. To calculate the vibrational frequencies for the sulphate ion of $CaS^{34}O_4 \cdot 2H_2O$ we have assumed the same isotopic shift for S^{34} substitution as Tudge and Thode (68) calculated for

the sulphate ion in solution. This is probably a good approximation since the perturbation of the sulphate in the gypsum crystal is quite small as indicated by the slight splitting of the degenerate levels.

Using the values for the vibrational frequencies as assembled in Table IV, and utilizing the assumptions regarding the isotope shift discussed above, the ratio of partition functions for the sulphate ion in $CaSO_4 \cdot 2H_2O$ was calculated from equation (14). The details of the calculation are given in Appendix III. For the solid we obtained a value of 1.085 at 25°C compared to 1.084 for the sulphate ion in solution. For the exchange reaction

$$Ca S^{32}O_4 \cdot 2H_2O + S^{34}O_4^{=}{}_{(sol'n)} = Ca S^{34}O_4 \cdot 2H_2O + S^{32}O_4^{=}{}_{(sol'n)}$$

K is 1.001 at 25°C.

Experimentally this was confirmed in the following manner. Ten grams of $CaSO_4 \cdot 2H_2O$ was mixed with 100 mls. of water and left at room temperature for approximately two weeks with occasional shaking. At the end of this time equilibrium was considered to have been reached. The solution and the solid were then separated from each other by filtration, and samples of sulphur dioxide prepared from the sulphate of each for mass spectrometric analysis. The details of preparation and mass spectrometry will be presented in the experimental section. The results of seven runs carried out in this manner are shown in Table V.

TABLE V

Equilibrium Constant for Exchange Reactions

$$CaS^{32}O_4 \cdot 2H_2O + S^{34}O_4^{=}(soln.) = CaS^{34}O_4 \cdot 2H_2O + S^{32}O_4^{=}(soln.)$$

Run #	$\frac{S^{32}O_4^{=}\ (soln.)}{S^{34}O_4^{=}}$	$\frac{CaS^{32}O_4\ (solid)}{CaS^{34}O_4}$	K
1	22.272	22.232	1.0018
2	22.311	22.252	1.0031
3	22.297	22.230	1.0030
4	22.262	22.252	1.0010
5	22.292	22.227	1.0029
6	22.247	22.217	1.0014
7	22.267	22.227	1.0018
		Average	1.0021

Experimentally we have found that at room temperature in the equilibrium between sulphate ion in solution and the sulphate ion in the gypsum crystal the S^{34} is favoured in the solid by 0.2%. Considering the assumptions made in the calculations this compares quite favourably with the theoretical value of 0.1%. As would be expected, the isotope effect is very small since no great change in the bonding of the sulphur occurs during the exchange.

Experimental

Mass Spectrometry

All samples were analysed as sulphur dioxide gas. The mass spectrometer used was essentially the one described by Wanless and Thode (94), with slight modifications as reported by Warren (64). It is a conventional 90° instrument modified to permit simultaneous collection and measurement, by means of a null method, of ion currents due to masses 64 ($S^{32}O_2^{16}$) and 66 ($S^{34}O_2^{16}$, $S^{32}O^{16}O^{18}$). The collection system is so constructed that only ion currents of these two masses strike the collector electrodes. A magnetic valve system permits rapid switching from standard gas to unknown thereby reducing the time required for analysis and making the rapid comparison of samples possible.

The analytical procedure for determining the isotope ratio of the unknown gas compared to the standard gas is as follows. The ion current from the more abundant peak (mass 64) is amplified by a vibrating reed electrometer and the output of this amplifier is applied across a 10,000 Ohm put-and-take potentiometer. The current from the peak of mass 66 is amplified by a similar amplifier, the output of which is balanced by a fraction of the voltage developed across the potentiometer. At the balance position there is zero output from the 66 amplifier. The output of this amplifier is fed into a recorder to which a test voltage is

applied to centre the recorder needle when there is zero output from the amplifier.

With the standard sulphur dioxide gas flowing into the mass spectrometer and the put-and-take potentiometer adjusted to indicate a balance on the recorder the instrument is calibrated by moving the potentiometer setting a known amount and measuring the displacement of the recorder pen. This calibration is done before and after each run of an unknown sample.

The magnetic valve is switched to allow the unknown sample to flow into the system. The balance point is shifted by the change in intensity of the 66 ion current, the 64 ion currents having been balanced previously on single collection. This change produces a displacement of the recorder pen which is directly proportional to the difference in the 64/66 ratio of the two samples. Since the standard has a known ratio the 64/66 ratio of the unknown can be calculated by comparing its displacement from the standard with the displacement caused by a known change in the potentiometer setting. Six measurements of the unknown's displacement and six for each calibration are taken.

The sensitivity of the instrument is controlled by the ion currents and the values of the resistors in the ion current amplification circuits. These are chosen

so that one centimetre displacement of the recorder pen corresponds to about 0.2‰ difference in 64/66 ratio of the samples.

The ion beam of mass 64 is made up of $S^{32}O^{16}O^{16}$ while mass 66 is composed of $S^{32}O^{16}O^{18}$, $S^{34}O^{16}O^{16}$, $S^{32}O^{17}O^{17}$, and $S^{33}O^{16}O^{17}$. These last two make a negligible contribution, thus

$$\frac{64}{66} = \frac{S^{32}O^{16}O^{16}}{S^{34}O^{16}O^{16} + S^{32}O^{16}O^{18}}$$

$$= \frac{1}{2\frac{O^{18}}{O^{16}} + \frac{S^{34}}{S^{32}}}$$

The value of O^{18}/O^{16} in the tank oxygen used for the preparation of samples has been determined as 0.00203. Hence

$$S^{32}/_{S34} = \frac{1}{\frac{66}{64} - 0.00416}$$

To determine the reproduceability of the instrument a large quantity of sulphur dioxide was prepared and separated into break-seal tubes by a Toepler pump; one of these samples was compared to the line standard at the start of each days work and periodically throughout the day. In Table VI we have collected values obtained for this secondary standard over a representative period of three months. These results show that during the course of one days operation the spectrometer gave results with a reproduceability of 0.02‰ but over a period of months a reproduceability of only 0.1‰.

TABLE VI

Mass Spectrometer Reproduceability

Date	‰ Difference*	S^{32}/S^{34}	Date	‰ Difference*	S^{32}/S^{34}
1 June	-0.110	22.148	13 July	-0.108	22.148
1 June	-0.100	22.143	27 July	-0.155	22.158
1 June	-0.102	22.148	3 Aug.	-0.146	22.159
7 June	-0.118	22.148	10 Aug.	-0.120	22.143
13 June	-0.096	22.143	24 Aug.	-0.125	22.153
13 June	-0.101	22.143	9 Sept.	-0.138	22.153
15 June	-0.141	22.153	9 Sept.	-0.133	22.153
28 June	-0.120	22.148	9 Sept.	-0.151	22.158
13 July	-0.116	22.148	Average	-0.122 ± 0.018≠	22.150 ± 0.005≠

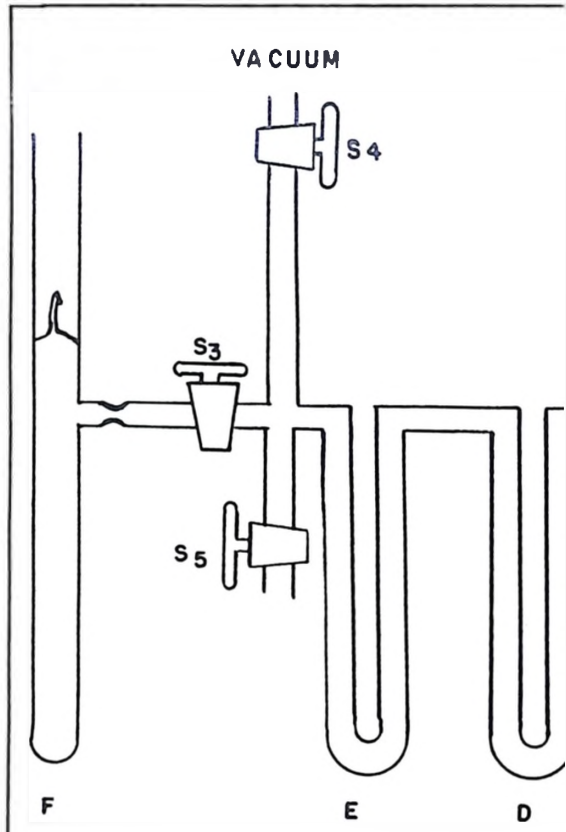
* compared to the same standard sample

≠ mean deviation

A gradual trend upwards in the S^{32}/S^{34} ratio of this secondary standard can be seen. This is probably due to slight changes in the standard gas and changes inherent in the mass spectrometer. Although this drift would be a serious factor in comparison of samples analyzed months apart, in the work reported in this thesis samples to be compared with each other were analyzed usually on the same day.

Preparation of Samples

All samples of sulphur dioxide were prepared by combustion of silver sulphide in an oxygen stream in a vacuum line. The vacuum line (Fig. 3) consisted of three


VACUUM
S4
S3
S5
F
E
D

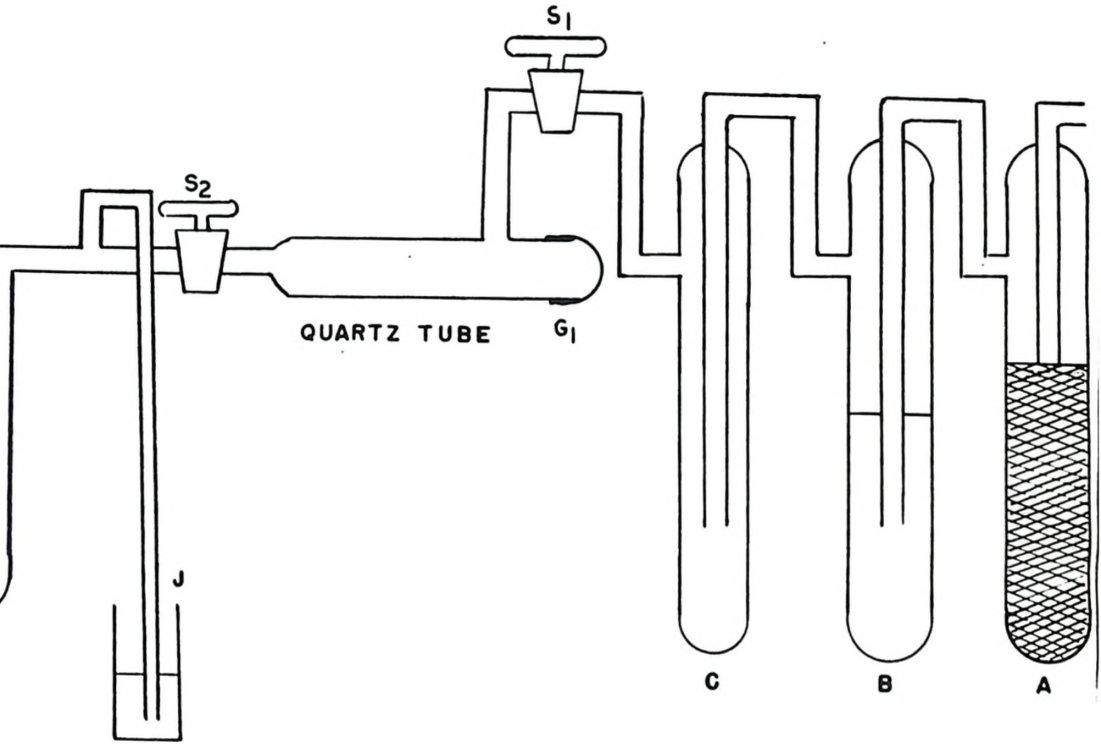


FIG. 3 COMBUSTION LINE

parts: an oxygen purifying train, a quartz combustion tube, and a collection line.

Tank oxygen was used for the combustion of the silver sulphide. It was purified before use by passing through an activated charcoal trap, A, cooled by dry-ice acetone mixture, a concentrated sulphuric acid bath, B, and a trap, C, cooled by liquid air. This procedure was considered to remove all carbon dioxide, moisture, and hydrocarbons from the gas.

The sample of silver sulphide (20-100 mg.) was placed in a nichrome boat and inserted into the quartz combustion tube through the ground glass joint G. The line was then evacuated through stopcock S_4 with S_1 closed. Following evacuation S_3 and S_4 were closed and the remainder of the line filled with oxygen through S_1. When the line was at atmospheric pressure, as indicated by the manometer J, stopcock S_5 was opened to allow the oxygen to flow through the line at the rate of 0.5 litres per minute. Liquid air was placed around the U-traps D and E to freeze the sulphur dioxide from the oxygen stream.

The sample of silver sulphide then was heated with a gas-oxygen flame for one to two minutes to insure complete combustion. Following the combustion the oxygen was allowed to stream through for about five minutes after which stopcocks S_5 and S_1 were closed and the line was evacuated. The sample of sulphur dioxide was allowed to

come to room temperature, refrozen, and pumped on to remove non-condensible gases trapped in the solid during the combustion. The sample was transferred to a break-seal tube F by distillation from a dry-ice acetone bath around D or E to the tube F cooled by liquid air. After the distillation was complete, as indicated by the pressure reading on a McLeod gauge, the sample was sealed from the line under vacuum.

To test the reproduceability of the burning procedure five samples of the same silver sulphide were burned and the sulphur dioxide samples were analyzed on the mass spectrometer with the results shown in table VII.

TABLE VII

Reproduceability of Combustion Procedure

Sample	Diff. ‰ from Standard	S^{32}/S^{34}
CT - 1	0.083	22.099
CT - 2	0.052	22.134
CT - 3	0.008	22.124
CT - 4	0.009	22.124
CT - 5	0.048	22.134
	average	$22.123 \pm 0.013^{\neq}$

$^{\neq}$mean deviation

It can be seen that the total variation is 0.1‰ and that the mean deviation is $\pm$0.013. Since most of the fractionation factors measured in this work are

considerably larger than those shown in the burning procedure these results were considered adequate.

In the burning procedure some sulphur trioxide is always produced. The equilibrium of the reaction $SO_2 + 1/2\ O_2 = SO_3$ is such that above 1200°C SO_2 is produced (95) in about 95% yield; as the temperature decreases the yield of SO_3 increases rapidly. The oxygen-gas flame used in the combustion of the sample certainly does not produce such high temperatures; however, the heat of combustion of pure silver sulphide is such that the resulting temperature is sufficiently high. However, it has been observed in this laboratory that diffuse samples of sulphide mixed with inactive ore must be burnt in an electric furnace at 1200 - 1400°C to obtain reproduceable results. It seems probable that the greatest cause of fractionation of the isotopes in the burning procedure is the failure to attain sufficiently high temperatures to ensure quantitative combustion to sulphur dioxide.

All samples of hydrogen sulphide whether produced by bacterial or chemical reduction were collected first as cadmium sulphide by trapping in a cadmium acetate-acetic acid buffer solution, and then converted to silver sulphide by the addition of silver nitrate to the precipitate. By this method a coarse, easily filtered precipitate was obtained. The silver sulphide was filtered on glass

wool, washed first with ammonium hydroxide to remove chlorides and carbonates, and then with water. It was dried overnight in an oven at 100°C before burning. Filtering on glass wool prevented the possibility of filter paper entering the combustion line and producing carbon dioxide; it had the added advantage that for small samples of sulphide, both sample and glass wool could be placed in the burning line to avoid mechanical losses.

For quantitative estimations of hydrogen sulphide a known excess of standard silver nitrate solution was added to the cadmium sulphide precipitate. After filtration the excess of silver ion was back-titrated with standard thiocyanate solution using ferric ion as indicator.

Sulphate samples were precipitated from solution by the standard barium chloride technique. For precipitation from bacteria media containing considerable sodium lactate it was found necessary to add a small amount of picric acid to aid in coagulation of the precipitate. Otherwise the barium sulphate remained colloidal and passed easily through the filter paper. Quantitative determinations of sulphate concentrations were done in the usual manner by ignition and weighing of the barium sulphate.

The barium sulphate samples were reduced to hydrogen sulphide by digestion with a reducing mixture of the following composition

HI (50%) 500 gms.

HCl (conc.) 480 mls.

H_3PO_2 (50%) 144 mls.

This method is a modification of that of Shirley and Pepkowitz (96). The mixture was boiled gently for about thirty minutes on preparation to remove traces of sulphur contaminants. About 100 mgs. of barium sulphate was added to a flask with 100 mls. of reducing mixture. The flask was fitted with a bubbler and a reflux condensor. A stream of nitrogen bubbling through the reaction mixture removed the hydrogen sulphide as it was produced. The gases were passed through a water trap to remove HCl vapours and then through a solution of cadmium acetate where the hydrogen sulphide was removed as cadmium sulphide.

To test the reproduceability of the reduction of barium sulphate eight portions of the same sample were reduced, burned, and analyzed on the mass spectrometer. The results of the mass spectrometric analysis with the per cent yield in the reduction are shown in Table VIII.

The results show that as long as the reaction proceeds essentially to completion (greater than 95%) the results are reproduceable to 0.1‰ but, as the analyses of samples 1 and 6 show, when the per cent reaction drops the discrepancies become larger. As reported in a later section of this thesis there is a 2‰ kinetic isotope effect in the reduction of a soluble sulphate by this method, the lighter

isotopic species, $S^{32}O_4^=$, reacting faster than the heavier species, $S^{34}O_4^=$. It was thus essential in the isotopic analysis of sulphate that the reduction of barium sulphate be carried out to completion.

TABLE VIII

Reproduceability of Barium Sulphate Reduction

Sample	% Yield of H_2S	S^{32}/S^{34}
1	88.0	22.104
2	97.5	22.085
3	98.5	22.095
4	100.0	22.090
5	97.5	22.090
6	92.3	(22.109) (22.114)
7	99.8	22.090
8	96.5	22.075
	average	22.093 $\pm$0.011$^{\neq}$

$^{\neq}$mean deviation

Where sulphite was used it was oxidized to sulphate by iodine and then precipitated as barium sulphate. Ford (97) has reported that this oxidation gives very reproduceable results as far as isotope ratios are concerned.

Cultivation of Bacteria

The original stock of D. desulphuricans used throughout this work was strain #7757 obtained from American Type Cultures, Washington, presumably as a

pure culture.

For fractionation experiments using continuous culture techniques during the first part of this work, and for much of the routine sub-culturing throughout Medium 1 was used to grow the bacteria. This medium originally employed by Starkey (98) had the following composition

NH_4Cl	1.00 g.
$MgSO_4 \cdot 7H_2O$	2.00 g.
Na_2SO_4	0.50 g.
K_2HPO_4	0.50 g.
$CaCl_2 \cdot 6H_2O$	0.10 g.
Sodium lactate	3.00 g.
$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	trace
Distilled water	1000 mls.

pH adjusted to 7.3

For solid tube cultures 2% agar-agar was added to this medium.

Medium 1 on autoclaving formed a precipitate which upon production of hydrogen sulphide turned black through formation of ferrous sulphide. This made the medium unsuitable for growth of bacteria when the cells were to be harvested by centrifugation and populations measured by turbidity. A medium was desired which permitted maximum growth of viable bacteria in the shortest possible time

with no formation of precipitate.

Butlin, Adams, and Thomas (99) have reported that 0.4% (w/v) yeast extract added to essentially medium 1 produces a greater growth of bacteria in a shorter time. D. desulphuricans require a reducing agent in the medium to maintain a low oxidation-reduction potential before growth will occur. Normally ferrous ion serves this purpose before growth and the product hydrogen sulphide after growth has begun. Grossman and Postgate (100) found that cells grown with only ferrous ion present as reducing agent were only 10% viable. They further found that cysteine or sodium sulphide could replace the ferrous ion and that with the use of these compounds greater growth was obtained with a much greater proportion of the cells viable. Making use of these observations we have employed the following medium with excellent results for growth of bacteria when the cells were to be harvested.

Medium 3a

NH_4Cl	1.00 g.
$MgSO_4 \cdot 7H_2O$	2.00 g.
Na_2SO_4	1.00 g.
K_2HPO_4	0.50 g.
$CaCl_2 \cdot 2H_2O$	0.10 g.
Sodium lactate	3.50 g.
Yeast extract	4.00 g.

Cysteine hydrochloride	0.60 g.
Distilled water	1000 mls.

pH adjusted to 7.3

With this medium no precipitate was produced upon autoclaving or upon production of hydrogen sulphide. The medium is essentially identical with that recently used by Grossman and Postgate (101) for harvesting cells.

Postgate (102) has reported the presence of an anaerobic contaminant nutritionally dependent on D. desulphuricans in growths of supposedly pure bacteria. When these growths were plated out for aerobic or anaerobic contaminants they appeared pure; however, when grown from a dilute inoculum in a stab culture in solid medium 4, given below, the contaminant appeared as a white colony in contrast to the black colonies of D. desulphuricans. During the course of the work reported in this thesis the working cultures were periodically subcultured in medium 4. No colonies other than those of D. desulphuricans were detectable. Periodic plating on the same medium under aerobic conditions showed no evidence of aerobic contaminants.

Medium 4

Bacto-tryptone	5.00 g.
Yeast extract	4.00 g.
Na_2SO_4	1.50 g.
$MgSO_4 \cdot 7H_2O$	1.50 g.
Glucose	10.0 g.

$Fe(NH_4)_2(SO_4)_2 \cdot 7H_2O$	trace
Agar-agar	2.00 g.
Distilled water	1000 mls.

Experimental Procedure for Fractionation Studies

Chemical Reduction of Sulphate

The fractionation in the chemical reduction of sulphate ion in solution by the reducing mixture of hydriodic, hydrochloric, and hypophosphorous acids was studied at temperatures of 50, 35, and 18°C. The concentration of reagents was

Na_2SO_4	0.81 moles/litre
HI	4.0 moles/litre
HCl	1.6 moles/litre
H_3PO_2	1.1 moles/litre

The effect of varying the concentration of the active reducing agent, hydriodic acid, was studied over a concentration range from 1.6 to 4.0 moles/litre using the same concentration of the other reagents as above.

The experimental procedure was to add the required amount of reagents to a three-neck flask of 200 mls. volume in a constant temperature bath. A total of seventy millilitres of reaction mixture was used. One neck of the flask was fitted with a gas bubbler, one neck was arranged to permit removal of samples, the third was fitted with a condensor and a gas outlet through a cadmium acetate trap. Nitrogen was bubbled through

the reaction mixture and the hydrogen sulphide produced swept out and collected as cadmium sulphide.

Sulphur dioxide samples were prepared from the sulphide and from a sulphate sample from the reaction mixture and analyzed on the mass spectrometer.
The fractionation factor was calculated as

$$f = \frac{S^{32}/S^{34}(\text{sulphide})}{S^{32}/S^{34}(\text{sulphate})}$$

Each sample of sulphide produced corresponded to less than 5% reduction of the sulphate. For low percentage reaction such as this the isotopic composition of the reactant does not change and the fractionation factor as calculated above is a true measure of the ratio of rate constants. For higher percentage reaction the observed fractionation must be corrected to zero reaction for a true measure of the ratio of rate constants.

Bacterial Reduction Experiments

Continuous Culture Six separate series studying the fractionation in the bacterial reduction of sulphate using continuous culture techniques were carried out, of which only the sixth D6, series, is reported here in detail. The experimental procedure was essentially as follows.

A nine-litre bottle of medium 1 (see Cultivation of Bacteria) was prepared and fitted with a bubbler, a funnel for aseptic addition of material,

a siphon for removal of material, and a gas outlet. The whole system was sterlized in the autoclave for one hour at 115°C. The unit was allowed to cool overnight in the sealed autoclave following which it was transferred to a constant temperature bath at 25°C and inoculated with about 10 mls. of an active culture grown in medium 1.

Nitrogen, purified of oxygen by passing through Fieser's solution, was bubbled through the bottle maintaining anaerobic conditions and sweeping out the hydrogen sulphide formed. This was collected as cadmium sulphide and prepared for mass spectrometry as previously described. Each time a sample of cadmium sulphide was removed the sulphate was precipitated from a sample of the medium to serve as a reference for comparison of the sulphide. Since each sulphide sample amounted to only about 1% of the total sulphate in the medium the experimentally measured fractionation factor was a true measure of the ratio of rate constants.

Following a lag period, usually lasting one to three days after inoculation, the culture began to produce hydrogen sulphide. Sterile solutions of sodium lactate and sodium or magnesium sulphate were added periodically to maintain the concentration of these metabolites. The production of hydrogen sulphide increased rapidly to a maximum and then began to decrease. The activity was increased by addition of the remaining

salts of the original nutrient medium.

Resting Cell Suspensions To permit a more rigid control over the variables of cell density and concentration of metabolites resting cell suspensions were used for most of the bacterial fractionation experiments reported in this thesis.

The method of resting cell suspensions consisted of growing the bacteria in a suitable medium (Medium 3a previously described), harvesting the cells by centrifugation, and resuspending them in a medium which did not contain any source of available nitrogen but did contain the essential metabolites - in our case sulphate and lactate. Under these conditions the bacteria could not multiply but they did for a period of twenty-four to forty-eight hours continue to carry on the usual metabolic processes of sulphate reduction and lactate oxidation. By controlling the number of bacteria used to prepare the cell suspension the population density could be maintained at a set level for reasonable lengths of time. The population decreased eventually, of course, due to death of the bacterial cells. This technique also permitted a more rigid control over concentration of metabolites.

The bacteria for use in resting cell suspensions were harvested from the growth obtained in 10 mls

to 2 litres of Medium 3a usually some sixty hours after inoculation. The greatest cell density and activity were found at this time. The cells were harvested by centrifugation for about fifteen minutes at 3200 r.p.m. (1600 x g), washed once with 2.5% NaCl solution, and then resuspended in sodium chloride solution of the same strength. Since we were interested chiefly in relative population densities from run to run rather than absolute value, the population density was controlled by turbidity measurements taken with a Model DU Beckman Spectrophotometer at 5200 A^{o}.

The procedure for fractionation studies was as follows.

The cells were harvested, a concentrated suspension made in sodium chloride solution and the turbidity measured. Cell suspensions for fractionation experiments were prepared by adding the required amount of this suspension to a solution of lactate and sulphate at the concentrations required by the experiment. The cell suspensions were set up in a constant temperature bath in Erlenmeyer flasks fitted through a rubber stopper with a bubbler and a gas outlet. Nitrogen, from which the oxygen had been removed by passing through Fieser's solution, was swept through the suspension to maintain anaerobic conditions and to

remove the hydrogen sulphide produced. This hydrogen sulphide was trapped as cadmium sulphide and handled in the usual manner. D. desulphuricans are not known to fix nitrogen under heterotrophic conditions although hydrogenase-producing strains have been reported (103) to fix nitrogen very slowly during autorophic growth. This report still requires direct experimental confirmation (104).

Preliminary experimentation showed that 300 mls. of bacteria suspension of an optical density of 0.10 to 0.15 when 0.05M in lactate and 0.05M in sulphate would produce at 30°C sufficient hydrogen sulphide for a mass spectrometric analysis in five to eight hours. During this time there was no decrease in turbidity indicating the bacterial suspension was stable and remained active for at least that long. Whenever conditions were changed so that the rate of production of hydrogen sulphide was decreased a greater volume of suspension was used to permit collection of the sample in a reasonable length of time.

Series of experiments with the cell suspensions were carried out to determine the effect of lactate concentration, sulphate concentration, temperature, age of cell suspension, and growth of cells upon the isotope effect in the reduction of sulphate to sulphide. The details of these various series are given below.

(a) Variation of lactate concentration. Several series were carried out at 30°C varying the lactate concentration from 0.001 moles/litre to 0.1 moles/litre, at a sulphate concentration of 0.01 to 0.03 moles/litre.

(b) Effect of bacterial growth. The effect of bacterial growth on the isotope effect in the sulphate reduction was determined by adding ammonium chloride and yeast extract, in the proportions used in Medium 3a, to two cell suspensions at 30°C after a sample of hydrogen sulphide had been produced under resting cell conditions. Two further samples of hydrogen sulphide were collected.

In a further run the bacteria were alternately subjected to growing conditions-by addition of ammonium chloride and yeast extract, and the resting cell conditions - by centrifugation and resuspension, to determine the effect of rapid changes between the extremes on the fractionation. This run was also done at 30°C.

(c) Variation of sulphate concentration. The sulphate concentration was varied in a series of cell suspensions from 1×10^{-5} moles/litre to 3×10^{-2} moles/litre, at a lactate concentration of 0.1 moles/litre and a temperature of 30°C. To maintain a supply of sulphate ions

at low concentrations an excess of a slightly soluble sulphate was added. Strontium sulphate was used to maintain a sulphate ion concentration of 6×10^{-4} moles/litre and barium sulphate was used for a sulphate ion concentration of 1×10^{-5} moles/litre. It has been found, as reported in a previous section of this thesis, that the equilibrium between sulphate in solution and a solid sulphate such a calcium sulphate involves only a very small isotope effect.

(d) Effect of temperature. The effect of temperature on the isotope fractionation in the bacterial reduction of sulphate was studied in two ways. Bacteria were acclimatized to temperatures of 24, 30, 36, and 40°C by repeated culture at one of these temperatures. Cell suspensions then were prepared at each of these temperatures from the acclimatized bacteria and sulphide samples collected. At each temperature a series of five runs was carried out varying the lactate concentration from 0.02 to 0.10 moles/litre at a sulphate concentration of 0.03 moles/litre.

The effect of temperature also was studied without previous acclimatization of the bacteria. One series acclimatized to 40°C was lowered to 30°C after production of a sample of hydrogen sulphide at the higher temperature and a further sample was collected. Another series, acclimatized to 36°C, produced a sample at that

temperature, and was then lowered successively to 30°C and to 24°C with samples collected at each of these temperatures.

(c) Effect of Age of cell suspension. Five cell suspensions with lactate concentration 0.10 moles/litre on sulphate concentration 0.06 moles/litre were set up at 36°C and samples of sulphide collected until the bacteria produced no more.

Reduction of Sulphite by Cell Suspensions: The isotope fractionation in the reduction of sulphite at 30°C by resting cell suspensions of D. desulphuricans was studied briefly over a range of lactate concentrations from 0.01 to 0.10 moles/litre at a sulphite concentration of 0.04 moles/litre. The effect of growth on the isotope effect was studied by the addition of ammonium chloride and yeast extract to two of the suspensions after production of one sample of hydrogen sulphide. Two further samples were collected.

Pure sodium sulphite was prepared immediately before use by adding dilute hydrochloric acid to commercial sodium sulphite and absorbing the evolved sulpher dioxide in sodium hydroxide solution in a nitrogen atmosphere. This sulphite gave no test for sulphate.

RESULTS

Chemical Reduction of Sulphate

The isotope fractionation observed in the chemical reduction of sulphate by the mixture of hydriodic acid, hydrochloric acid, and hypophosphorous acid, as well as the dependence of the fractionation factor on the temperature are shown in Table IX. For each run the hydrogen sulphide produced was only about 2% of the sulphate present; the experimentally determined fractionation factor is, therefore, a direct measure of the ratio of rate constants, k_1 and k_2, for the two reactions

$$S^{32}O_4^{=} \longrightarrow H_2S^{32} \qquad (1)$$

$$S^{34}O_4^{=} \longrightarrow H_2S^{34} \qquad (2)$$

This ratio of rate constants is seen to be 1.022, indicating a 2.2% faster rate of reaction for the lighter isotopic species. A calculation of this kinetic isotope effect was made (see theoretical section) on the assumption that the initial rate-controlling step involved the breaking of an S-O bond in the reduction of sulphate to sulphite. Upper and lower limits at 25°C of 1.035 and 1.011 for the fractionation factor were obtained, corresponding to the two extreme models

of the activated complex

(i) S-O bond completely broken, as in product,

(ii) S-O bond the same as in the initial state.

The intermediate isotope effect found experimentally suggests the rate-controlling step is the initial S-O bond breakage with an activated complex containing a stretched S-O bond intermediate between the initial and final states.

The isotope effect calculated on the basis of complete bond breakage in the activated complex showed an increase of 0.3% for a temperature decrease of 25°; the lower limit, on the other hand, showed no temperature coefficient. A 0.3% change in isotope effect is easily measurable with our procedures; one is, therefore, forced to the conclusion that the isotope effect is independent of temperature, contrary to theoretical expectations. This lack of temperature dependence could be explained by postulating that the extent of bond breakage decreases with a temperature decrease, resulting in a lower fractionation which compensates for the expected temperature effect. Such a hypothesis is not subject to experimental verification and must remain, therefore, a matter of conjecture.

The kinetics of the reaction were not studied extensively, but results indicated that the rate was dependent on the sulphate concentration, the hydrogen

TABLE IX

Chemical Reduction of Sulphate
Effect of Temperature on Isotope Fractionation

Sample #	Temp. °C	S^{32}/S^{34} ($SO_4^=$)	S^{32}/S^{34} ($S^=$)	Fractionation	Average Fractionation
C15-1	17.8	22.083	22.538	1.0206	
C15-2	17.8	22.065	22.564	1.0235	
C16-1	18.0	22.098	22.573	1.0215	1.022
C16-2	18.0	22.095	22.568	1.0214	
C14-1	35.0	22.098	22.543	1.0201	
C14-2	35.0	22.085	22.573	1.0221	
C 6-1	35.0	22.085	22.552	1.0212	1.022
C 6-2	35.0	22.075	22.594	1.0235	
C10-1	50.0	22.058	22.533	1.0215	
C13-1	50.0	22.083	22.533	1.0204	1.022
C13-2	50.0	22.055	22.558	1.0228	

TABLE X

Chemical Reduction of Sulphate
Effect of HI Concentration on Isotope Fractionation

Sample #	HI M./l.	S^{32}/S^{34} ($SO_4^=$)	S^{32}/S^{34} ($S^=$)	Fractionation	Average Fractionation
C13-1	4.0	22.083	22.533	1.0204	1.0215
C13-2	4.0	22.055	22.558	1.0228	
C11-1	3.2	22.053	22.533	1.0218	1.0211
C11-2	3.2	22.085	22.538	1.0205	
C12-1	1.6	22.075	22.543	1.0212	1.0212
C12-2	1.6	22.080	22.548	1.0212	

ion concentration, and the iodide ion concentration. The over-all reduction reaction can be written as

$$SO_4^{=} + 8I^- + 10H^+ \longrightarrow H_2S + 4H_2O + 4I_2$$

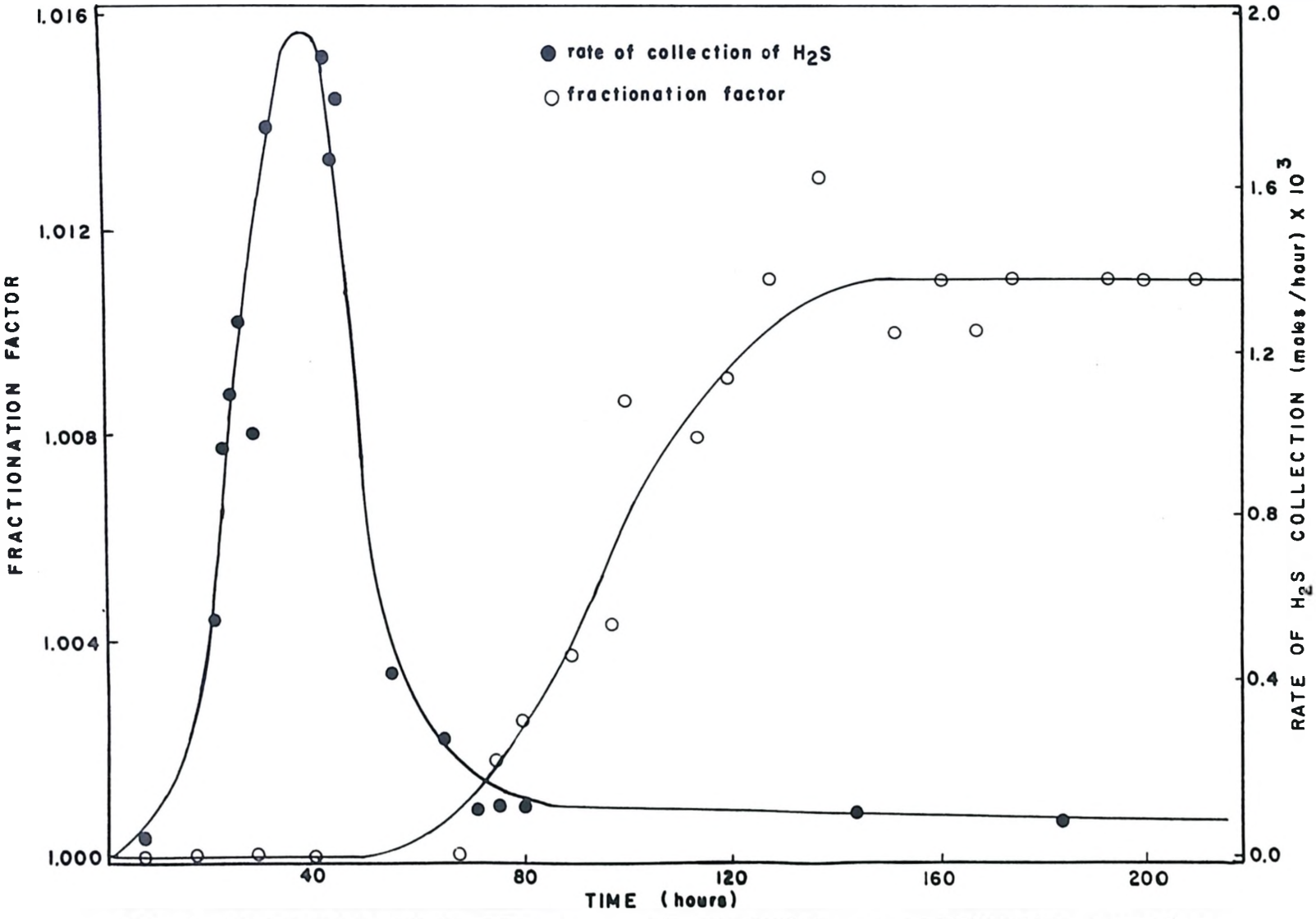
The iodine formed is then reduced to iodide ion by reaction with the hypophosphorous acid. The hydrochloric acid acts as a source of hydrogen ion and, in the case of reduction of barium sulphate, as a dissolving agent.

Attempts to study the fractionation in the reduction of sulphite by this reducing mixture led to difficulties on two accounts. The sulphite was decomposed to sulphur dioxide gas by the acidic medium. Further, the mixture of sulphur dioxide and sulphite ion reacted so rapidly with the hydriodic acid that large amounts of elemental sulphur were deposited rapidly, even in dilute solutions of the reactants. The formation of the elemental sulphur probably was due to a secondary reaction between hydrogen sulphide and iodine which occured before the iodine was reduced by the hypophosphorous acid. These observations on the rapidity of the reaction of sulphur dioxide with hydriodic acid indicate that the rate-controlling step in the reduction of sulphate is the first step, $SO_4^{=} \longrightarrow SO_3^{=}$ in support of the conclusions drawn from the agreement of the experimental fractionation factor to that calculated on this assumption.

The effect of the concentration of the reducing agent, hydriodic acid, on the fractionation factor is shown in Table X. It would not be expected that a change in the concentration of one of the reactants would affect the isotope effect unless such variations changed the rate-controlling step of the reaction. The results of Table X, showing no change of fractionation factor with varying hydriodic acid concentration, are to be expected; however, they do stand in direct contrast to the results to be presented concerning the bacterial reduction of sulphate.

Bacterial Reduction of Sulphate

Continuous Culture: The first run carried out by this author using the continuous culture techniques gave a fractionation factor between 1.000 and 1.003 at 25°C. Wallouch (77) at the same temperature had obtained a fractionation factor of 1.011 to 1.012. Several possibilities for the explanation of the result, such as contamination of bacterial culture, oxygen in the nitrogen stream, and variations of different strains of bacteria, where checked in subsequent runs. These experiments gave the lower fractionation factors repeatedly. In the series D6 the effect of the length of time for which the continuous culture was in operation was studied. The results of this run are shown, in part, in Figure 4


rate of collection of H_2S
fractionation factor
FRACTIONATION FACTOR
RATE OF H_2S COLLECTION (moles/hour) X 10^3
TIME (hours)
1.016
1.012
1.008
1.004
1.000
2.0
1.6
1.2
0.8
0.4
0.0
40
80
120
160
200

where we have plotted the fractionation factor as a function of time. Zero time was taken as the time of removal of the first sample of sulphide some three days after inoculation of the system with bacteria.

The results in Figure 4 show that for the first sixty hours after production of sulphide began there was no fractionation of the sulphur isotopes, the fractionation factor being 1.000; after sixty hours the fractionation factor began to rise gradually, eventually levelling off at 1.011 the same value previously obtained in continuous culture experiments (76,77). For comparison purposes we have also plotted in Figure 4 the rate of collection of hydrogen sulphide as a function of time. The rate of collection of hydrogen sulphide depends upon such things as the rate of flow of nitrogen through the system, the pH of the medium, and the hydrogen sulphide concentration in the medium. However, it should be a fairly good measure of the rate production of hydrogen sulphide by the bacteria.

The first part of the rate curve in Figure 4 is typical of the logarithmic phase of bacterial growth. The rapid decrease of the rate after the initial maximum cannot be explained on the basis of a decrease in bacterial population but must be caused by a decrease in the metabolic activity of the bacteria. The results

given in Figure 4 indicate a dependency of the fractionation factor upon the rate of reduction of sulphate. The lag period between the decrease in the rate and the increase in the fractionation factor can be explained by the hydrogen sulphide hold-up in the culture solution when the bacteria were very active. This hydrogen sulphide hold-up would tend to dilute the hydrogen sulphide produced later, causing a delay in the first observations of fractionation.

Resting Cell Studies:

The results shown in Figure 4 suggested that the fractionation of the sulphur isotopes was a function of the rate of production of hydrogen sulphide. It was decided, therefore, to change the technique of study to resting cell suspensions in order that the variables, cell density of bacteria, and concentration of metabolites, could be controlled more accurately. As has been shown in the results from the chemical reduction of sulphate no change in the isotope effect was to be expected with change in rate unless the mechanism of the reaction changed. The problem undertaken with the resting cell suspensions was to determine definitely whether the fractionation was a function of the rate, and if so to determine what change in the mechanism occurred to cause the change in the fractionation. The results presented show the effect of meta-

bolite concentrations, temperature, and growth conditions upon the rate and upon the fractionation of the isotopes. From these results we have been able to draw some definite conclusions concerning the mechanism of the reaction.

(a) Effect of Lactate Concentration: Postgate (105) has reported that the rate of sulphate reduction by D. desulphuricans using lactate as the source of hydrogen is dependent on the lactate concentration. Table XI summarizes the results obtained varying lactate concentration in the cell suspensions at 30°C at constant cell density. The rate in column four of the table is expressed as moles of hydrogen sulphide produced per hour per unit cell population. To permit a direct comparison of rates throughout the work with resting cells the unit of cell population arbitrarily was taken as 100 mls. of suspension of an optical density of 0.10.

Columns four and five of Table XI show that over the range of lactate concentration 0.006 to 0.010 moles/litre the rate and also the fractionation factor were essentially independent of the lactate concentration. This undoubtedly means that the enzyme systems involving the lactate were saturated with lactate; the rate was controlled by the amount of enzyme or coenzyme not the concentration of lactate.

TABLE XI

Bacterial Reduction of Sulphate

Effect of Lactate Concentration on Isotope Fractionation

Sample #	Optical Density	Lactate Moles/l.	Rate‡ x 10⁶	Fractionation Factor
R4-5	0.10	0.096	5.89	1.0127
R3-7	0.16	0.084	5.32	1.0086
R4-4	0.10	0.075	4.88	1.0122
R3-6	0.16	0.075	4.15	1.0102
R3-5	0.16	0.063	5.47	1.0109
R4-3	0.10	0.053	5.40	1.0108
R4-2	0.10	0.052	5.56	1.0117
R3-2	0.16	0.021	4.58	1.0104
R4-1	0.10	0.011	6.06	1.0120
R3-1	0.16	0.010	4.75	1.0106
R6-1 #1	0.12	0.0021	1.14	1.0103
R6-2 #2	0.12	0.0021	0.82	1.0147
R9-2 #1	0.09	0.0014	1.30	1.0126
R9-2 #2	0.09	0.0010	1.25	1.0145
R9-2 #3	0.09	0.0010	0.66	1.0148
R9-1 #1	0.09	0.0007	1.01	1.0131
R9-1 #2	0.09	0.0007	0.40	1.0145

‡ moles hydrogen sulphide per hour per unit cell population.

However, as the lactate concentration was decreased below 0.010 moles/litre the rate began to decrease. Although the variations in the fractionation factor were rather large there was a general trend to higher values at lower rates, reaching in the case of sample R19-1 a value of 1.0181. These results indicate that the isotope effect is dependent on the rate of sulphate reduction and increases as the rate is decreased by lowering the concentration of reducing agent. The fractionation factor of 1.011 obtained, on the average, in the region independent of lactate concentration is identical with that originally obtained by previous workers (76,77) at 25°C using continuous culture techniques.

(b) Effect of Bacterial Growth: It was apparent that the rate could not be increased sufficiently by increasing the lactate concentration to give the low fractionation found in the continuous culture at high metabolic activity. It has been reported (70, 106) that the rate of reduction of sulphate by D. desulphuricans is much greater during active growth than with resting cells. Therefore, ammonium chloride and yeast extract were added to two of the resting cell suspensions, R4-2 and R4-4, to permit growth of the bacteria. Subsequently two further samples of sulphide were collected under growing conditions and the fractionation compared to that obtained with resting cells. The results are shown in Table XII.

TABLE XII

Bacterial Reduction of Sulphate

Effect of Cell Multiplication on Isotope Fractionation

Sample	Optical Density	Time for Sample (hours)	Rate‡ X 10^5	Fractionation Factor
R4-2 #1*	0.099	7.0	5.56	1.0117
R4-2 #2		2.2	9.25	1.0099
R4-2 #3	0.20	1.7	14.4	1.0070
R4-4 #1*	0.099	6.7	4.88	1.0122
R4-4 #2		2.5	15.0	1.0099
R4-4 #3	0.19	1.5	16.8	1.0054

* Ammonium chloride and yeast extract added after these samples.

‡moles hydrogen sulphide/hour/unit cell population

The addition of the nitrogen source caused a rapid increase in the cell density shown by the increased optical density (column two); the cell population doubled within four hours after the addition of the source of nitrogen. This increase was accompanied by an increased rate of production of hydrogen sulphide per unit cell population; the actively growing cells carried out the sulphate reduction considerably faster than resting cells. As the rate of production of hydrogen sulphide increased the sulphur isotope fractionation decreased rather markedly.

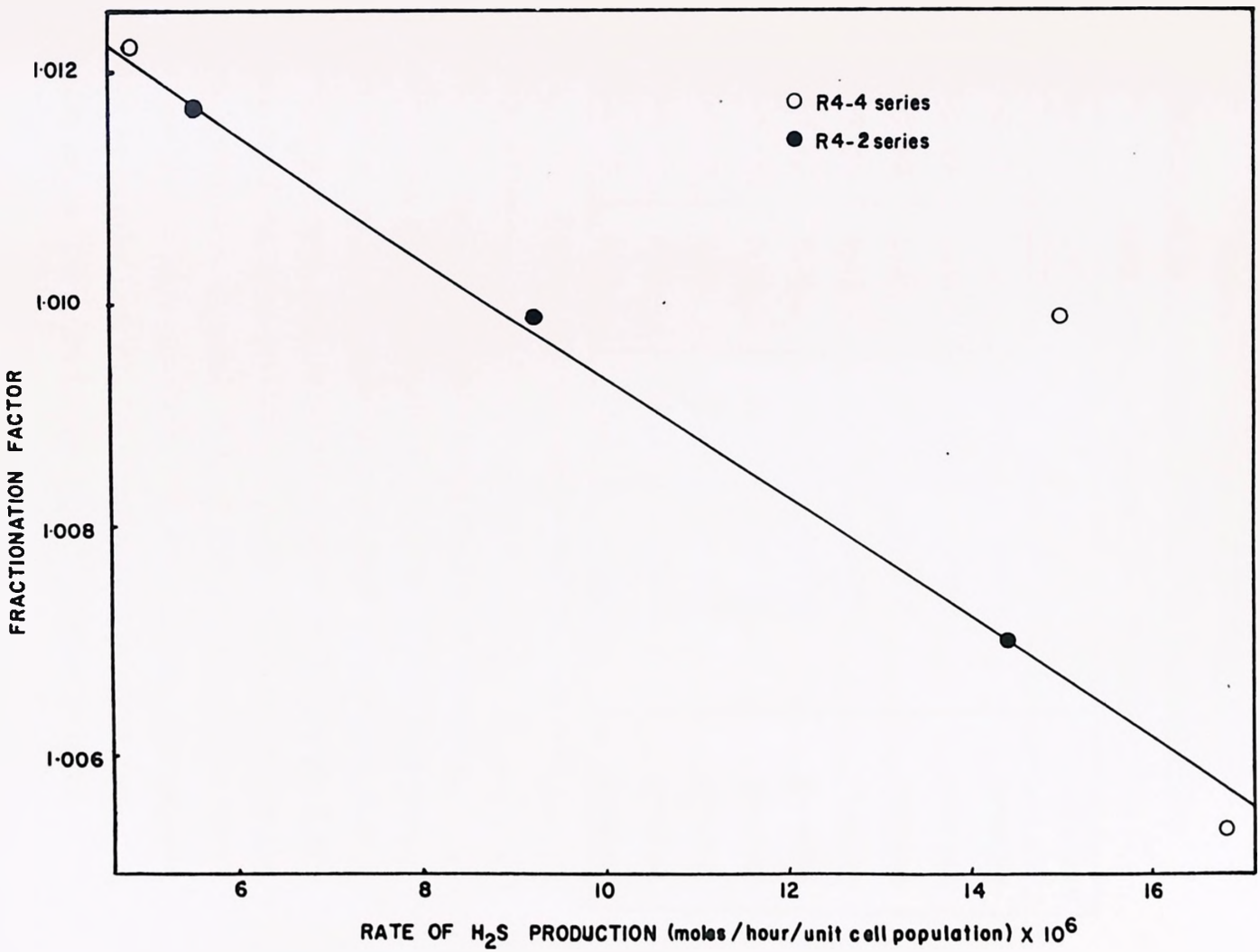


FIG. 5 DEPENDENCE OF ISOTOPE FRACTIONATION ON RATE DURING ACTIVE GROWTH

TABLE XIII

Bacterial Reduction of Sulphate

Effect of Rapid Changes From Growing to Resting

Conditions on Isotope Fractionation

Sample #	Optical Density	Rate ‡ x 10^6	Fractionation Factor	Conditions
R5-2 #1	0.22	18.8	1.0060	growing
R5-2 #2	0.11	10.9	1.0033	resting
R5-2 #3	0.11	32.6	1.0058	resting
R5-2 #4	0.11	16.8	1.0076	resting
R5-2 #5	0.11	69.2	1.0092	growing
R5-2 #6	0.20	37.7	1.0063	growing
R5-2 #7	0.30	25.4	1.0063	growing
R5-2 #8	0.11	19.3	1.0098	resting
R5-2 #9	0.11	13.1	1.0105	resting

‡moles hydrogen sulphide/hour/unit cell population.

That the fractionation factor is directly dependent upon the metabolic activity of the bacteria, as measured by the rate of production of hydrogen sulphide per unit cell population, is shown in Figure 5 by the straight line obtained on plotting the fractionation as a function of rate. Five of the six points lie very close to the straight line; the sixth point, corresponding to sample R4-4 #2, is considerably in error for unknown reasons. It is apparant from these results

that under conditions of active growth very little fractionation of the sulphur isotopes occurs.

There are two possible explanations of this result. First, two separate enzyme systems for the bacterial reduction process might be postulated. If one enzyme system were operative under resting cell conditions and involved a considerable isotope fractionation, and the other enzyme system were operative only during active growth of the bacteria and involved no isotope fractionation, then the above result could be realized. Second, two steps in a single mechanism might be competitive with regard to which is rate-controlling. If under conditions of active growth a step which did not involve the breaking of a S-O bond were rate-controlling, then no isotope fractionation would occur. On the other hand, if under conditions of resting cells the rate-controlling step did involve the breaking of a S-O bond, then considerable fractionation would result. Since one would not expect to find two separate enzyme systems in the same organism to carry out one reaction the second explanation appears by far the more likely. To distinguish between the two explanations further experiments were devised.

In these experiments the bacteria were studied in a manner which permitted rapid changes from conditions permitting rapid growth to conditions

of resting cells. The results assembled in Table XIII show that during these rapid changes in conditions the corresponding change in the isotope fractionation was quite gradual. If two separate mechanisms were operative, one under resting cell conditions involving considerable fractionation, and the other under growing conditions involving no fractionation, then one would expect rather abrupt changes in the isotope fractionation as the conditions were changed from the two extremes. On the other hand, if only one mechanism were operative and the change in fractionation depended on the change in the relative rates of two steps then one would expect a more gradual change in the observed isotope fractionation as the conditions were changed between the two extremes. The results shown in Table XIII support the second explanation and indicate that the effect of growth is a speeding up of the metabolic processes, especially the step in the reaction involving a large isotope effect.

(c) Effect of Sulphate Concentration: Since a decrease of the lactate concentration below the apparent saturation concentration has been shown to lower the rate of hydrogen sulphide production and cause an increase in the isotope fractionation, it was interesting to determine the effect of sulphate concentration on the rate and on the fractionation of the isotopes. The results

TABLE XIV

Bacterial Reduction of Sulphate

Effect of Sulphate Concentration on Isotope Fractionation

Run	$SO_4^=$ moles/litre	lactate moles/litre	Fractionation Factor
R3 (series)	0.029	varied	1.0100*
R4 (series)	0.010	varied	1.0115*
R9-3	0.004	0.10	1.0110
R16-1	0.0014	0.10	1.0130‡
R19-1	0.0006	0.11	1.0037
R19-2	0.0006	0.11	1.0031
R18-1	0.00001	0.10	0.9999

*each value average of five determinations

‡15.8% reaction, corrected to zero reactions by method of Stevens (107).

TABLE XV

Bacterial Reduction of Sulphate

Effect of Temperature on Isotope Fractionation*

Series #	Temperature °C	Average Rate ≠	Average Fractionation	Determinations
R17	24	5.30	1.0089	5
R3-R4	30	4.09	1.0105	10
R13	36	4.87	1.0123	5
R12	40	5.19	1.0107	5

≠moles/hour/unit cell population $\times 10^6$

*Bacteria previously acclimatized to temperature at which cell suspension maintained.

presented in Table XIV show that over the range from 0.03 moles/litre to 0.001 moles/litre the sulphate concentration had no effect on the fractionation factor. However, as the concentration was lowered still further the fractionation decreased to 1.003 at a sulphate concentration of about 6.10^{-4} moles/litre, as controlled by the solubility of strontium sulphate. At the concentration of sulphate given by a saturated solution of barium sulphate the fractionation factor apparently inverted with $S^{34}O_4^=$ reacting 0.3‰ faster than $S^{32}O_4^=$. This result will be discussed more fully in a later section of this thesis. When some of these runs were carried out no quantitative measure of the rates were obtained. Qualitatively it was observed that the rate was constant for a sulphate concentration above 0.01 moles/litre, but decreased considerably at lower concentrations.

(d) Effect of Temperature: Kallouch (77) using continuous culture techniques found a large temperature coefficient for the isotope effect in the bacterial reduction of sulphate (Figure 2). This could not be explained on the basis of a normal temperature coefficient of either an exchange or a unidirectional reaction as was shown in the theoretical section; it was important to see whether this was also a rate-controlled phenomenon. The effect of temperature on the rate and on the isotope

fractionation was studied in two manners. In one case the bacteria were acclimatized to the different temperatures by repeated sub-culturing at that temperature; in the other case the temperature of the cell suspensions was lowered abruptly without any previous acclimatization.

The fractionation of the isotopes in the hydrogen sulphide produced by cell suspensions reducing sulphate at temperatures from 24 to 40°C is shown in Table XV. In these four series the bacteria were first acclimatized to the temperature at which the cell suspensions were maintained by growth at that temperature. Column three shows that the rate of production of hydrogen sulphide was a minimum at 36°C, increasing slightly at both lower and higher temperatures. The fractionation factor was a maximum at 36°C, decreasing above and below this temperature. There would appear to be some degree of correlation between the size of the isotope effect and the rate of sulphate reduction although the variations in both, especially the rate, are too small to draw any definite conclusions.

In addition to the experiments described above a further study of the temperature effect was made in which the temperature of a cell suspension was lowered after each successive sample was produced; in this case

there was no previous acclimatization to the lower temperature. The suspensions of series R13 of the above table were lowered to 30°C, after production of samples at 36°C, and further samples were collected. The temperature then was lowered to 24°C and a third set of samples collected. Series R12 of Table XV originally produced samples at 40°C, after which the cell suspensions were lowered to 30°C and a second set of samples collected. The results from these two series are presented graphically in Figure 6 where the fractionation is plotted as a function of the rate of production of hydrogen sulphide per unit cell population. At the lower temperatures the rate of hydrogen sulphide production decreased considerably and the isotope effect increased. That the fractionation is again a function of the rate is shown by the reasonable agreement of the experimental points with the curve drawn through them.

The effect of temperature as shown in Figure 6 is to be contrasted with the effect as shown by the results of Table XV. When the bacteria were allowed to acclimatize themselves to a temperature by growth at that temperature they apparently were capable of adjusting their enzyme systems in such a manner as to enable them to carry on their metabolism at the normal rate regardless of the temperature change. However, when

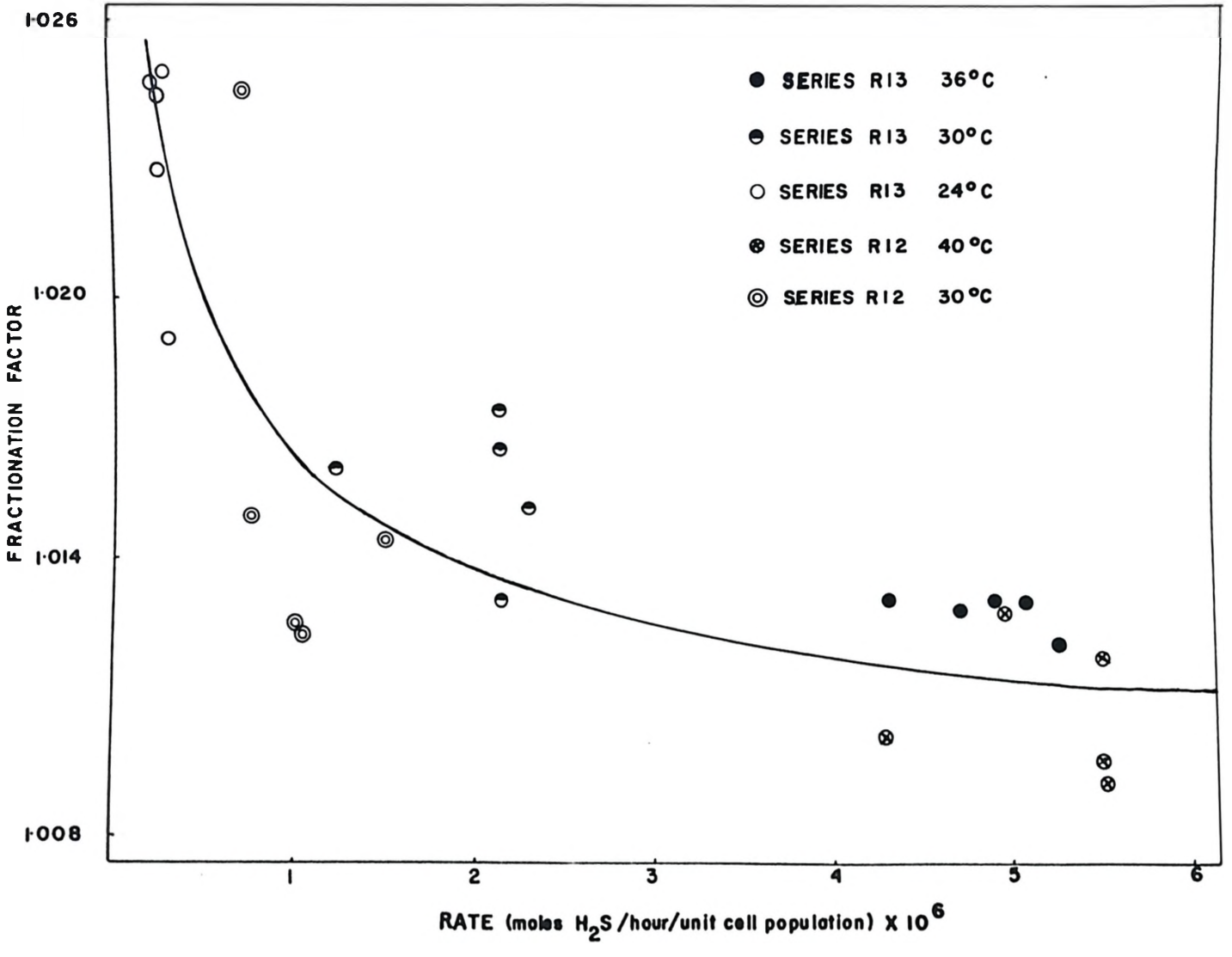


FIG. 6 EFFECT OF TEMPERATURE ON ISOTOPE FRACTIONATION

they were subjected in cell suspension to a sudden lowering of the temperature without previous acclimatization there was a large decrease in the metabolic rates which was reflected in an increase in the fractionation of the sulphur isotopes. This will be discussed more fully when we consider possible mechanisms of the reduction reaction.

(c) Effect of Age of Cell Suspension: The large decrease in rate at lower temperatures in the above runs necessitated maintaining the suspensions for long periods of time to produce sufficient hydrogen sulphide for analysis. Figure 6 indicated that the increase in the fractionation was caused by the decrease in the rate. However, there remained the possibility that the isotope fractionation was affected by the age of the cell suspension. To check this possibility five cell suspensions were set up at 36°C and samples of sulphide collected until no more hydrogen sulphide was produced. Six to nine samples of hydrogen sulphide were produced by each suspension. For each suspension the first sample produced and the last sample produced were analyzed. The results gave, on the average a fractionation factor of 1.014 for the first sample produced decreasing to 1.009 for the final sample. For the fifth suspension all the samples collected were analyzed with the results shown in Table XVI.

TABLE XVI

Bacterial Reduction of Sulphate

Effect of Age of Suspension on Isotope Fractionation

Sample #	Elapsed Time (Hours)	Fractionation Factor
R15-5 #1	4.7	1.0142
R15-5 #2	10.7	1.0137
R15-5 #3	20.7	1.0119
R15-5 #4	25.7	1.0110
R15-5 #5	34.4	1.0123
R15-5 #6	45.9	1.0109
R15-5 #7	51.9	1.0103
R15-5 #8	58.9	1.0105
R15-5 #9	79.6	1.0080

The fractionation factors in Table XVI fall into three groups. The isotope fractionation for the first two samples was 1.014, decreasing to 1.011 for the next six samples, and to 1.0080 for the last sample. This decrease in the isotope effect, especially the abrupt change, is difficult to explain. The cell population decreased with age making it impossible to correlate the observed fractionation with the rate of hydrogen sulphide production. The results do show, however, that the large increase in the isotope effect found previously as the temperature was lowered (Fig. 6) must be an effect of temperature not age, since aging of the cell suspensions has been shown to cause a decrease in the fractionation.

Reduction of Sulphite by Resting Cells

The sulphur isotope fractionation found in the reduction of sulphite by cell suspensions of D. desulphuricans at 30°C over a range of lactate concentrations is presented in Table XVII. The fractionation factor was found to average 1.011, identical with the value found for the sulphate reduction under similar conditions. In addition the rate of production of hydrogen sulphide per unit cell population was almost the same as the rate found in the reduction of sulphate. Postgate (84) has reported that cell suspensions of D. desulphuricans reduce sulphite at a faster rate than sulphate, with molecular hydrogen as the reducing agent. The rates were measured by the rate of hydrogen uptake, which was the same for both sulphate and sulphite. Since sulphite requires three moles of hydrogen for reduction compared to four moles for sulphate it was concluded that sulphite was reduced more rapidly. No measurements were made of the rate of hydrogen sulphide production by Postgate. Our results do not support Postgate's conclusions although the systems are not directly comparable because of the difference in the reducing agent.

TABLE XVII

Isotope Fractionation in Bacterial Reduction of Sulphite

Run #	Optical Density	Lactate (moles/l.)	Rate≠ x10⁶	Fractionation Factor
R10-2	0.18	0.012	6.46	1.0106
R10-3	0.18	0.024	7.81	1.0103
R10-4	0.18	0.048	5.56	1.0124
R10-5	0.18	0.083	3.09	1.0125
R10-6	0.18	0.119	2.65	1.0106

≠moles hydrogen sulphide/hour/unit cell population.

TABLE XVIII

Bacterial Reduction of Sulphite

Effect of Cell Multiplication on Isotope Fractionation

Sample #	Optical Density	Time for Sample(Hrs.)	Rate≠ x 10⁶	Fractionation Factor
R10-3 #1 *	0.18	4.5	7.81	1.0103
R10-3 #2		3.5	13.07	1.0061
R10-3 #3	0.32	2.6	20.19	1.0035
R10-5 #1*	0.18	4.5	3.09	1.0125
R10-5 #2		3.5	11.11	1.0072
R10-5 #3	0.27	2.0	18.52	1.0050

*ammonium chloride and yeast extract added to resting cell suspension after these samples

≠moles hydrogen sulphide, hour/unit cell population.

No evidence was obtained for sulphate formation during the reduction of sulphite contrary to the report of Spiegler (108) who claimed to have found an increase in the sulphate concentration during the reduction of a mixture of sulphate and sulphite in continuous culture.

In Table XVIII are tabulated the results obtained when ammonium chloride and yeast extract was added to two of the suspensions reducing sulphite. The optical density increased considerably indicating cell multiplication. At the same time these rapidly dividing cells showed an increased rate of sulphite reduction per unit population. As the rate increased the fractionation dropped accordingly. The result in the case of actively growing cells is seen to be identical for the reduction of both sulphite and sulphate.

Summary of Results

All the results presented show that the extent of the sulphur isotope fractionation in the bacterial reduction of sulphate is dependent on the rate of reduction, regardless of whether this rate is controlled by the temperature, concentration of metabolites, or conditions of growth. In Figure 7 the isotope fractionation obtained varying these conditions is plotted as a function of the rate of hydrogen sulphide production. It is seen that all the values fall on a smooth curve regardless of the condi-

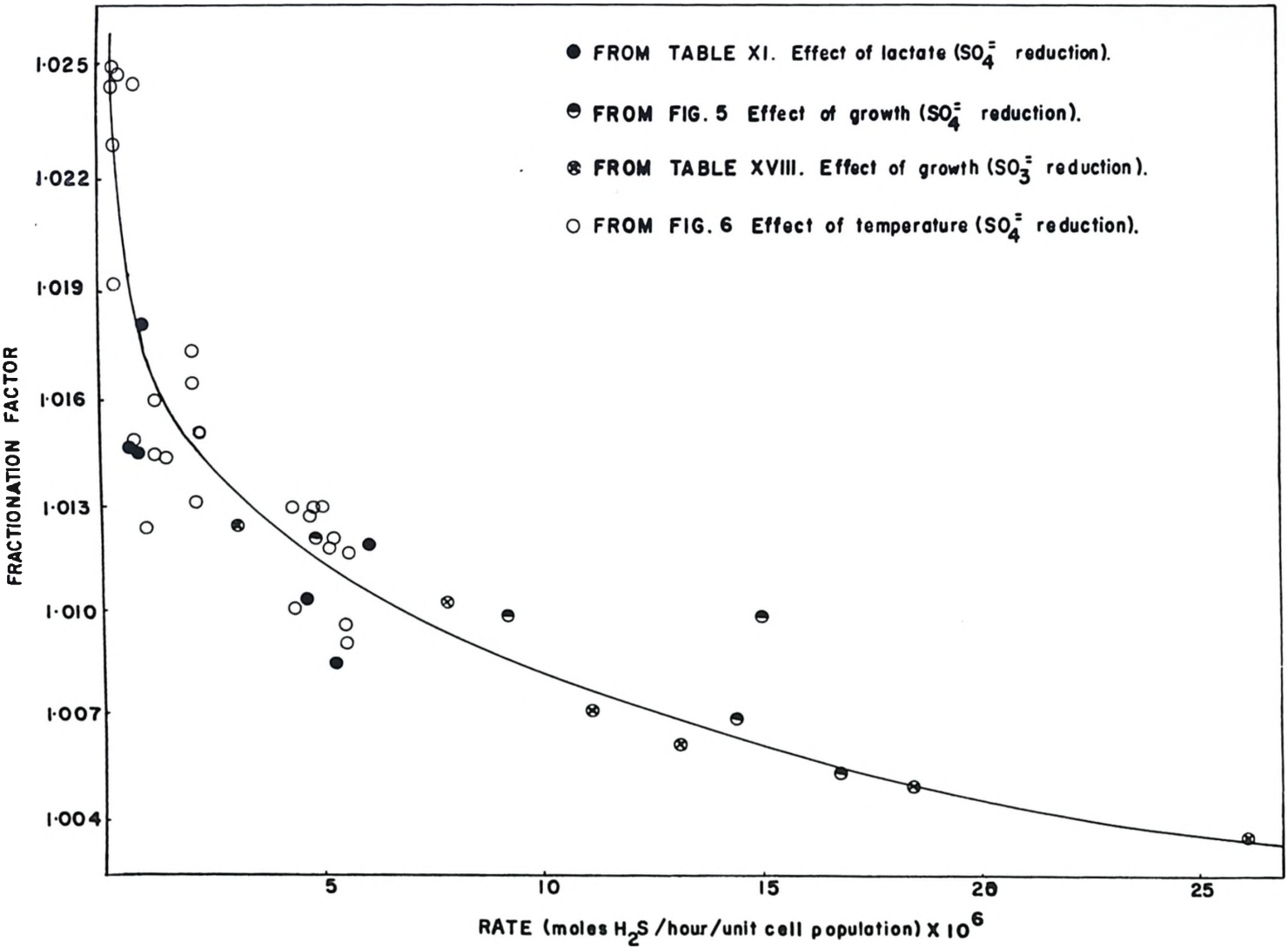


FIG. 7 ISOTOPE FRACTIONATION AS A FUNCTION OF RATE

tions of the experiment. On the same graph we have shown the isotope effect in the reduction of sulphite as a function of the rate. In this case also the fractionation of the sulphur isotopes appears to depend on the rate in much the same manner as for sulphate.

During the preparation of this manuscript, Kulp and co-workers (109) reported, in a brief note, that they had observed a large temperature coefficient for the isotope effect in the reduction of sulphate by D. desulphuricans, in essential agreement with Kallouch's earlier results from this laboratory. They suggested that the size of the isotope effect was affected by factors which influence the rate of growth, although they made no effort to explain the result nor did they suggest a mechanism. The experiments of this work show that the isotope effect varies considerably not only with temperature but also with concentration of metabolites and conditions of growth. The results show further that in every case the main effect of these variables is due to the rate of reduction of sulphate under the conditions of the experiment. Finally, the results taken together suggest a mechanism whereby the widely different isotope effects can be explained. This mechanism of the bacterial reduction of sulphate is discussed below.

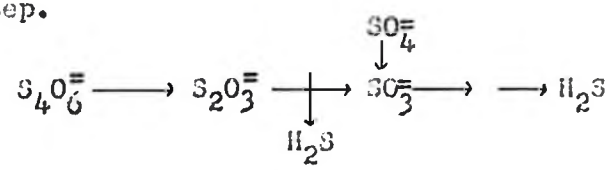
Discussion

It was mentioned previously that the large variation of isotope effect with rate could be explained by postulating a reaction sequence involving two consecutive steps either of which could be rate-controlling. If one of these steps involved a large fractionation of the isotopes and the other involved little or no isotope effect, one could expect a large variation in the observed fractionation depending on the relative rates of the two steps.

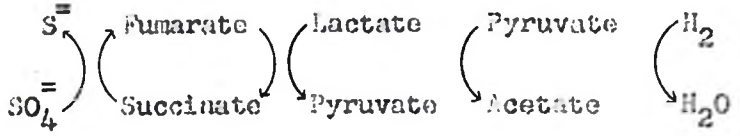
Our results show that at low rates of sulphate reduction the rate-controlling step in the bacterial sulphate reduction involves an isotope effect of at least 2.5‰. This compares with 2.2‰ obtained in the reduction of sulphate by chemical methods. For the chemical reaction it was shown that the rate-controlling step was the initial S-O bond breakage occurring in the reaction $SO_4^{=} \longrightarrow SO_3^{=}$. Comparison of the two isotope effects suggests, at once, that the same step is rate-controlling in the bacterial reduction at low rates as was rate-controlling in the chemical reduction. At high rates of sulphate reduction, during active growth, the rate-controlling step showed only a very small isotope effect. A small isotope effect

also was obtained at very low sulphate concentrations indicating that this rate-controlling step directly involved the sulphate ion in a reaction resulting in very little fractionation of the isotopes.

A great amount of work has been reported in the literature (82,84,110-117) showing that the reduction of sulphur compounds by *D. desulphuricans* occurs through the following sequence of reactions with a separate enzyme for each step.



It has been shown (101) that the reducing agent, such as lactate, acts to reduce sulphate and other compounds through a fumarate-succinate cycle outlined below. Electrons apparently are transferred between the two systems by a cytochrome system (117-120).



Lactate is thus directly involved in the sulphate reduction; a decrease in the lactate concentration decreases the rate of the reduction reaction.

The scheme for the sulphate reduction, outlined above, shows that sulphite is an intermediate in the reduction of sulphate although all attempts to isolate

sulphite in the bacterial cell have failed (82,113-116). These results along with other evidence from inhibition studies and relative rates of reduction (84,111,112) have shown that sulphite is reduced faster than sulphate in the bacterial cell. These reasons and especially the agreement of the isotope effect in the chemical reduction with the isotope effect at low rates of bacterial sulphate reduction lead us to the conclusion that at these low rates the rate-controlling step in the bacterial reduction is the first S-O bond breakage occurring in the reaction $SO_4^{=} \longrightarrow SO_3^{=}$ as in the case of the chemical reduction. Any step in the further reduction of sulphite cannot lead to an isotope effect since all the sulphite is reduced as rapidly as it is formed.

It has been observed in this laboratory (83) that sulphite and sulphate undergo some isotopic exchange in the presence of bacteria suspensions containing no lactate as reducing agent. This suggested that the isotope fractionation observed in the sulphate reduction might occur during this exchange. However, without a build-up of sulphite no exchange reaction could occur; in the experiments promoting the exchange sulphite was added to the suspensions. Further, this exchange occurs only very slowly with no reducing agent present and with only a small amount of lactate present does not occur appreciably in forty-eight hours,

considerably longer than our fractionation experiments.

The low isotope fractionation when the bacteria were rapidly growing could be explained by postulating a different mechanism for the reduction under these conditions. As discussed previously this second mechanism is considered to be highly unlikely both on a theoretical basis and from experimental results. The question then arises of the step in the reaction sequence which is rate-controlling during rapid sulphate reduction. Results show that this step involves very little fractionation of the isotopes. The same step is undoubtedly rate-controlling at very low sulphate concentration, where again a small isotope effect was observed. This decrease of isotope effect at very low sulphate concentration suggests that the rate-controlling step both in this case and during active growth is the pick-up of sulphate by the bacterial cells. The pick-up of sulphate by the cell would not involve a large isotope effect since there is no great change in the bonding of the sulphur during such a reaction.

The study of uptake of ions and molecules by cells (121-124) has suggested that negative ions such as sulphate cannot enter the bacterial cell passively due to the negative charge of the bacteria under physiological conditions (125, 126). The theory is that an active anion absorption must occur deriving its energy from the anaerobic fermentation

of carbohydrates.

Whether the rate of pick-up of sulphate is controlled by the diffusion of sulphate to the cells or by the active transfer through formation of a sulphate-enzyme complex in either case the isotope effect would be small. The isotope fractionation in diffusion is dependent on the square root of the ratio of the mass of the two isotopic species $S^{34}O_4^{=}$ and $S^{32}O_4^{=}$. Such an isotope effect would be very small for the highly hydrated ions present in solution; if any isotope effect were observed it would show the $S^{32}O_4^{=}$ reacting faster. On the other hand the formation of the enzyme complex in the active transfer would probably involve an equilibrium with the $S^{34}O_4^{=}$ favoured in the complex to a slight extent. The isotope effects in the two possible cases are in opposite directions but both would be quite small. The results at very low sulphate concentration (Table XV) showed the $S^{34}O_4^{=}$ reacting faster by 0.3‰ suggesting that the active transfer was rate-controlling. In view of the small effect this point perhaps should be studied further.

The postulate that the pick-up of sulphate is rate-controlling during active growth and at low sulphate concentration is in accord with Mitchell (127) who states that at low nutrient concentration the limiting factor is the rate of nutrient availability determined by the rate

of diffusion of nutrient to the cell surface or through the osmotic barrier.

The lowering of the temperature of the cell suspension would cause a greater decrease in the rate of the reduction step than in the rate of sulphate pick-up, since the reduction step would have a greater activation energy. That the fractionation increased as the temperature was lowered must mean that the reduction reaction became more rate controlling; this supports our postulates concerning the rate-controlling steps in the reduction reaction.

The few results obtained on the isotope fractionation in the bacterial reduction of sulphite suggest that in this reduction a reaction sequence occurs similar to that for sulphate. At high rates of reduction the isotope fractionation decreased probably because the pick-up of sulphite became rate-controlling.

It is interesting to see what variation of isotopic fractionation with rate could be expected theoretically from a reaction sequence of the type proposed for the bacterial reduction. The mechanism we have proposed involves the two steps pick-up of sulphate and reduction to sulphite competing for control of the rate and can be written as follows for the two isotopic species, where $SO_4^{=}$ (bound) is considered a sulphate inside the bacterial cell.

$$S^{32}O_4^{=}\ (\text{sol'n}) \underset{k_2}{\overset{k_1}{\rightleftharpoons}} S^{32}O_4^{=}\ (\text{bound}) \xrightarrow{k_3} S^{32}O_3^{=}$$

$$S^{34}O_4^{=}\ (\text{sol'n}) \underset{k_2'}{\overset{k_1'}{\rightleftharpoons}} S^{34}O_4^{=}\ (\text{bound}) \xrightarrow{k_3'} S^{34}O_3^{=}$$

McDaniel and Smoot (128) recently have dealt with the kinetics of a reaction sequence of this type. From their equations one can derive the expression given below for the fractionation factor, assuming a steady-state concentration of bound sulphate

$$\text{fractionation factor} = \frac{k_3}{k_3'}\left(\frac{1+\frac{k_2'}{k_1'}+\frac{k_3'}{k_1'}}{1+\frac{k_2}{k_1}+\frac{k_3}{k_1}}\right)$$

This equation shows that if the reduction step involves a large isotope fractionation while the first step, either equilibrium or unidirectional, involves essentially no isotope fractionation, the experimentally observed isotope effect will depend on the relative rates of the three reactions. Large variations in isotope fractionation would be expected if changes in experimental conditions caused changes in the relative rates of the various steps. From the above theoretical expression it was impossible to obtain a theoretical curve of fractionation factor versus rate of reduction which fitted the

experimental curve (Fig. 7) with any degree of accuracy, although the general shape of the two curves was similar.

The results presented have shown conclusively that the rate of metabolic processes in living cells can be controlled by the rate of nutrient up-take. This observation would be difficult to obtain by kinetic studies; this work is another example of the use of isotope effects in determining the mechanism of such complex reactions.

The maximum fractionation of the sulphur isotopes obtained in the reduction of sulphate by D. desulphuricans was 2.5‰. Analysis of sulphur and sulphate samples from the Cyrenaican Lakes of Africa where D. desulphuricans are known to be active gave isotope effects of 1.5 to 3.2‰ (Table I), in very good agreement with the results obtained in the laboratory controlled experiments. Our results have shown that the extent of the isotope fractionation is closely related to the environment of the organism; the spread is the isotope fractionation for the samples from Cyrenaica, collected at different times, is not unexpected. To explain more fully the mechanism of sulphur isotope fractionation in nature, experiments are in progress in this laboratory combining D. desulphuricans in a mixed culture with photosynthetic sulphide-oxidising bacteria such as are found in nature. It is hoped by this method to reproduce in the laboratory a simplified bacterial sulphur cycle such as is found in nature.

Appendix

I. Calculation of Q_2'/Q_1' for $SO_3^=$.

The ratio of partition functions is given by the following equation (equation (14), theoretical section)

$$\frac{Q_2'}{Q_1'} = \frac{\sigma_1}{\sigma_2}\left[1 + \sum_i^{3n-6} G(u_{2_i}) \cdot \Delta u_i\right]$$

where $u_{2_i} = \frac{hc\, w_{2_i}}{kT}$, $\Delta u_i = u_{1_i} - u_{2_i}$ and the summation is over all i frequencies, counting an n- degenerate frequency n times. The following values are used for hc/kT (129).

$$\frac{hc}{kT} = 4.8256 \times 10^{-3} \text{ at } 25^\circ C$$

$$\frac{hc}{kT} = 5.2663 \times 10^{-3} \text{ at } 0^\circ C.$$

The calculations of Q_2'/Q_1' at 25°C and 0°C are assembled in the two tables below. The G (u) values were taken from the table of Bigeleisen and Mayer (7)). The Δw_i values are those calculated by Bernstein and Evans (87) and the w_{1_i} values are the average of the Raman lines given in Table II. The w_{2_i} values were calculated from the w_{1_i} and Δw_i values.

Calculation of Q_2'/Q_1' for $SO_3^=$ (25°C)

i	w_{2_i}	w_{1_i}	Δw_i	u_{2_i}	Δu_i	$G(u_{2_i})$	$G(u_{2_i}) \cdot \Delta u_i$
1	954.5	967.0	12.5	4.60604	0.060320	0.29298	0.01767
2	613.6	616.6	3.0	2.96099	0.014478	0.21687	0.00314
3	923.5	930.0	6.5	4.45644	0.031366	0.28734	0.00901
3	923.5	930.0	6.5	4.45644	0.031366	0.28734	0.00901
4	456.3	468.3	12.0	2.20192	0.057910	0.17019	0.00986
4	456.3	468.3	12.0	2.20192	0.057910	0.17019	0.00986

$$\sum_{i}^{3n-6} G(u_{2_i}) \cdot \Delta u_i = 0.05855$$

$$\frac{Q_2'}{Q_1'} = 1.059 \text{ at } 25^\circ C.$$

Calculation of Q_2'/Q_1' for $SO_3^=$ (0°C)

i	w_{2_i}	w_{1_i}	Δw_i	u_{2_i}	Δu_i	$G(u_{2_i})$	$G(u_{2_i}) \cdot \Delta u_i$
1	954.5	967.0	12.5	5.02667	0.065829	0.30767	0.02025
2	613.6	616.6	3.0	3.23140	0.015800	0.23165	0.00366
3	923.5	930.0	6.5	4.86342	0.034231	0.30218	0.01034
3	923.5	930.0	6.5	4.86342	0.034231	0.30218	0.01034
4	456.3	468.3	12.0	2.40301	0.063200	0.18329	0.01158
4	456.3	468.3	12.0	2.40301	0.063200	0.18329	0.01158

$$\sum_{i}^{3n-6} G(u_{2_i}) \cdot \Delta u_i = 0.06775$$

$$\frac{Q_2'}{Q_1'} = 1.068 \text{ at } 0^\circ C.$$

II. Calculation of Q_2'/Q_1' for $SO_4^=$.

The Q_2'/Q_1' ratios for the sulphate ion were re-calculated using the G(u) values of Bigeleisen and Mayer (79) and the vibrational frequencies and isotope shift reported by Fudge and Thode (68). The calculations for 25°C and 0°C are summarized in the following tables

Calculation of Q_2'/Q_1' for $SO_4^=$ (25°C)

i	w_{2_i}	w_{1_i}	Δw_i	u_{2_i}	Δu_i	$G(u_{2_i})$	$G(u_{2_i})\cdot\Delta u_i$
1	980	980	0.000				
2	451	451	0.000				
3	1097.56	1113.60	16.04	5.29639	0.077403	0.31626	0.02448
3	1097.56	1113.60	16.04	5.29639	0.077403	0.31626	0.02448
3	1097.56	1113.60	16.04	5.29639	0.077403	0.31626	0.02448
4	615.55	618.90	3.35	2.97040	0.016166	0.21740	0.00351
4	615.55	618.90	3.35	2.97040	0.016166	0.21740	0.00351
4	615.55	618.90	3.35	2.97040	0.016166	0.21740	0.00351

$$\sum_{i}^{3n-6} G(u_{2_i})\cdot\Delta u_1 = 0.08397$$

$$\frac{Q_2'}{Q_1'} = 1.084 \text{ at } 25^\circ C.$$

Calculation of Q_2'/Q_1' for $SO_4^=$ (0°C)

i	W_{2i}	W_{1i}	ΔW_i	u_{2i}	Δu_i	$G(u_{2i})$	$G(u_{2i}) \cdot \Delta u_i$
1	980	980	0.0				
2	451	451	0.0				
3	1097.56	1113.60	16.04	5.78007	0.084471	0.33015	0.02789
3	1097.56	1113.60	16.04	5.78007	0.084471	0.33015	0.02789
3	1097.56	1113.60	16.04	5.78007	0.084471	0.33015	0.02789
4	615.55	618.90	3.35	3.24167	0.017642	0.23220	0.00410
4	615.55	618.90	3.35	3.24167	0.017642	0.23220	0.00410
4	615.55	618.90	3.35	3.24167	0.017642	0.23220	0.00410

$$\sum_{i}^{3n-6} G(u_{2i}) \cdot \Delta u_i = 0.09597$$

$$\frac{Q_2'}{Q_1'} = 1.096 \text{ at } 0^\circ C$$

III. Calculation of Q_2'/Q_1' for the Sulphate Ion of $CaSO_4 \cdot 2H_2O$.

The partition function ratio for the sulphate ion in $CaSO_4 \cdot 2H_2O$ was calculated using the values for $S^{32}O_4^=$ in gypsum as given in Table IV and the isotope shifts for sulphate ion in solution given by Tudge and Thode (63). The details of the calculationare given in the following table.

Calculation of Q_2'/Q_1' for Sulphate of $CaSO_4 \cdot 2H_2O$ (25°C)

i	w_{2i}	w_{1i}	Δw_i	u_{2i}	Δu_i	$G(u_{2i})$	$G(u_{2i}) \cdot \Delta u_i$
1	1006	1006	0.0				
2	415	415	0.0				
2	493	493	0.0				
3	1098.96	1115	16.04	5.30314	0.077403	0.31648	0.02450
3	1119.96	1136	16.04	5.40448	0.077403	0.31953	0.02473
3	1127.96	1144	16.04	5.44308	0.077403	0.32068	0.02482
4	581.65	585	3.35	2.80681	0.016166	0.20800	0.00336
4	617.65	621	3.35	2.98053	0.016166	0.21797	0.00352
4	668.65	672	3.35	3.22664	0.016166	0.23140	0.00374

$$\sum_{1}^{3n-6} G(u_{2_i}) \cdot \Delta u_1 = 0.08467$$

$$\frac{Q_2'}{Q_1'} = 1.085 \text{ at } 25^\circ C.$$

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