SULPHUR ISOTOPE EFFECTS
IN CHEMICAL AND BIOLOGICAL PROCESSES

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SCOPE AND CONTENT:

The sulphur isotope effect in the oxidation of aqueous sodium sulphite with molecular oxygen has been measured under a variety of conditions. The observed effect is compared with theoretical calculations based on the reaction mechanism. The uncatalyzed and enzyme-catalyzed sulphur isotope exchange between sulphate and sulphite has been examined. The oxidation of sulphide to internally-stored sulphur and the subsequent oxidation of the sulphur to sulphate by *Chromatium* have been studied. Sulphur isotope fractionation in mixed cultures of *Chromatium* and *Desulphovibrio desulphuricans* has also been examined. The results are discussed in relation to the natural variations in sulphur isotope abundances.

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INTRODUCTION

During the past twenty years, differences in the properties and variations in the natural abundances of stable isotopes have been studied extensively. In the field of reaction mechanisms, the determination of differences in the rates of reaction for different isotopic species has provided an insight into the nature of the transition state in the rate-controlling step.

Also, since variations in the natural abundances of stable isotopes were first observed, the measurement of such variations has proven useful in the elucidation of many geological problems. By studying the physical, chemical and biological processes which bring about such variations, workers in this field have learned a great deal concerning the history of our planet.

In particular, observed variations in the natural abundances of the sulphur isotopes have provided valuable clues concerning the origin of deposits of sulphur and its compounds. Laboratory experiments have shown that some biological processes lead to isotope fractionation while others do not. In the present work the metabolism of two species of sulphur bacteria, one a sulphate reducer, the other a sulphide oxidizer, has been studied for possible isotope effects. The results indicate a mechanism whereby large natural variations in sulphur isotope abundances may have been produced. Other experiments were carried out in order to determine the extent of isotope fractionation in the oxidation of aqueous sodium sulphite solution with molecular oxygen. The results of these experiments give some insight into the mechanism of this oxidation process.

Discovery of Isotopes

Soddy (1) first postulated the occurrence of isotopes in 1910 in order to explain certain chemically inseparable groups of elements in the disintegration chains of the radioactive elements. This postulate was presented in a more complete and generalized form by Soddy (2) and Fajans (3) independently in 1913. Soddy suggested the term "isotopes" (Gr., isos, equal; topos, place) since they occur in the same place in the periodic table.

The first indication of the occurrence of isotopes among the non-radioactive elements was provided by Sir J. J. Thomson (4) who was studying positive rays with the first mass spectrograph. In 1912 Thomson had observed a second parabola on his photographic plate near the one due to neon. Aston (5) in 1920 confirmed that this line was due to the isotope Ne^{22}. With the advent of better mass spectrographs, isotopes were soon discovered in all parts of the periodic table. Of particular interest is the discovery by Aston (6) of two of the three naturally-occurring heavy isotopes of sulphur, S^{33} and S^{34}, in 1927.

Several of the isotopes of the lighter elements, however, were first observed by spectroscopic techniques. Giauque and Johnston (7) observed lines in the atmospheric absorption spectrum which were attributed to O^{17} and O^{18}. Similarly, King and Birge (8) discovered lines due to

C^{13} in the spectrum of carbon at 2800°C. Naude (9) confirmed the existence of the oxygen and carbon isotopes, and also obtained evidence for the existence of the nitrogen isotope, N^{15}, in the ultraviolet absorption spectra of these elements. Urey, Brickwedde and Murphy (10) concentrated deuterium sufficiently by evaporation of hydrogen near the triple point so that they could observe it spectroscopically. Most of this early work has been reviewed by Aston (11).

Equilibrium Isotope Effects

Almost as soon as isotopes had been discovered, chemists and physicists began speculation concerning possible chemical and physical differences between them. Lindemann and Aston (12), in 1919, in a discussion of the possibility of separating isotopes, indicated that it could be done, at least theoretically. Lindemann (13) in the same year pointed out that isotopic differences would be larger if there were no zero-point energy. At this time the status of *Nullpunktsenergie* was uncertain. Since he could observe no differences in the vapour pressures of the lead isotopes, Lindemann concluded that zero-point energy did exist. Although his conclusion concerning the existence of zero-point energy was correct, his experimental observations were not. The vapour pressures of the lead isotopes do differ, but by an amount too small for Lindemann's techniques to detect.

Investigations into the differences in chemical properties of stable isotopes were of little significance until 1933, when Urey and Rittenberg (14) calculated the equilibrium constant for the reaction

$$H_{2(g)} + D_{2(g)} \rightleftharpoons 2\,HD_{(g)}$$

Rittenberg, Bleakney and Urey (15) during the following year determined experimental values of the equilibrium constant at 25, 397 and 468°C. The three values, 3.28, 3.62 and 3.75 at the respective temperatures are in good agreement with the theoretical values of 3.28, 3.73 and 3.82. Meanwhile, Crist and Dalin (16) were studying the exchange reaction

$$H_{2(g)} + HDO_{(g)} \rightleftharpoons HD_{(g)} + H_2O_{(g)}$$

Again the experimental value, 0.71 for the equilibrium constant, is in accord with the theoretical value of 0.62.

Thus encouraged, Urey and Greiff (17) by means of the methods of statistical mechanics were able to calculate equilibrium constants for many other isotopic exchange reactions from spectroscopic data. Their early calculations were for reactions involving the light elements, lithium, carbon, nitrogen and oxygen, with the halogens, chlorine and bromine. More convenient equations have recently been obtained by Bigeleisen and Mayer (18). By means of tables compiled by those authors and their simplified equation, the calculation of equilibrium constants becomes a simple matter, as long as the necessary vibrational frequencies of the molecule are known. Tudge and Thode (19) calculated equilibrium constants for exchange reactions involving several sulphur compounds. Recent publication of spectroscopic data for the sulphite ion (20, 21, 22) has made possible the calculation of exchange constants involving this species as well (23)

Since the theory of isotope exchange reactions was worked out on the basis of ideal gases, its success with reactions in solution has been interesting to theoreticians. The excellent agreement between calculated and measured values for isotope exchange constants has indicated that non-ideal effects in solution cancel out for the isotopic species. For instance, Weber, Wahl and Urey (24) measured the equilibrium constant for the reaction

$$2\,H_2O^{18}_{(l)} + CO^{16}_{2\,(g)} \rightleftharpoons 2\,H_2O^{16}_{(l)} + CO^{18}_{2\,(g)}$$

They found K to be 1.097 as compared with the theoretical value of 1.096. Thode, Graham and Zeigler (25) studied the reaction

$$HS^{32}O^{-}_{3(aq)} + S^{34}O_{2(g)} \rightleftharpoons HS^{34}O^{-}_{3(aq)} + S^{32}O_{2(g)}$$

and found K to be 1.019 at 25°C. For the same reaction involving sulphite rather than bisulphite ion, Harrison (23) calculated a value of 1.013 for the equilibrium constant. While in reasonable agreement, the discrepancy between the observed and calculated values indicates, perhaps, the influence of the proton in the bisulphite ion. More recently, Dunford, Harrison and Thode (26) determined experimentally the exchange constant for the reaction

$$S^{34}O_{2(g)} + H_2S^{32}O_{4(l)} \rightleftharpoons S^{32}O_{2(g)} + H_2S^{34}O_{4(l)}$$

over the temperature range from 200 to 400°C. Since suitable data for the sulphuric acid were unavailable, the value of the equilibrium constant could not be calculated. The theoretical value of the equilibrium constant for the reaction

$$S^{34}O_{2(g)} + S^{32}O_{4(aq)}^{=} \rightleftharpoons S^{32}O_{2(g)} + S^{34}O_{4(aq)}^{=}$$

however, was found to be very close to the experimental value for the reaction involving sulphuric acid. This agreement indicates that the two protons have only a slight influence on the sulphate ion.

As soon as equilibrium isotope effects had been confirmed, the separation of isotopes was seen to be possible, at least theoretically. Obviously, if one equilibrium step produces a small separation, many successive equilibrium steps will produce a large enrichment. Experimental confirmation was soon forthcoming.

In 1932 Washburn and Urey (27) reported that the residue from the electrolysis of water was enriched in deuterium. Lewis and Cornish (28) shortly afterwards succeeded in producing changes of 60 to 80 gamma (parts per million of density) in water by fractional distillation in a column with forty theoretical plates. The density change was attributed to enrichment of the heavy isotopes in the residue. At approximately the same time Keesom and his co-workers (29, 30, 31) separated the isotopes of neon by distillation near the triple point. Urey, Aten and Keston (32) in 1936 examined the possibility of separating the carbon isotopes by the bicarbonate-carbon dioxide exchange reaction, but found the equilibrium was attained too slowly for practical purposes. Meanwhile, Urey, Pegram and Huffmann (33) succeeded in producing a slight enrichment of O^{18} by fractional distillation of water. Deuterium was also concentrated in the process.

Urey's group at Columbia found, however, that separation of isotopes were best obtained using counter-current liquid-gas exchange columns. In 1939 Thode and Urey (34) reported the production of ammonia containing 72.8 per cent N^{15}. Sufficient material was produced so that many of the physical properties of N^{15} and its compounds could be investigated. Hutchison, Stewart and Urey (35), utilizing the exchange between cyanide ion and hydrogen cyanide, were able to produce 20 g. of C^{13} at a concentration of 25 per cent in the form of hydrogen cyanide gas. This is almost a 20-fold enrichment. Stewart and Cohen (36) in the same year reported the production of 3.2 g. of sulphur per day containing 25 per cent S^{34} per day. Recently, Spindell and Taylor (37), utilizing similar techniques, have produced nitric acid containing 99.8 per cent N^{15} from the exchange reaction

$$N^{15}O_{(g)} + HN^{14}O_{3(aq)} \rightleftharpoons N^{14}O_{(g)} + HN^{15}O_{3(aq)}$$

Other methods have also been employed. In 1938 Taylor and Urey (38) succeeded in producing partial separation of the isotopes of lithium, nitrogen (as ammonium ion) and potassium on long zeolite columns. By means of modern ion-exchange resins, Spedding, Powell and Svec (39) reported production of 99 per cent N^{15} from a series of columns with a total length of 600 feet. Isotope separation methods, including thermal diffusion, gaseous diffusion and high ion current mass spectrometry in addition to the foregoing, have now been developed to the point where the U. S. Atomic Energy Commission (40) is able to supply separated isotopes of virtually all the elements in the periodic table.

Kinetic Isotope Effects

If equilibrium isotope effects occur, then obviously kinetic effects should also occur. In 1939 Rollefson (41) found deuterium to be less reactive than hydrogen in the equation

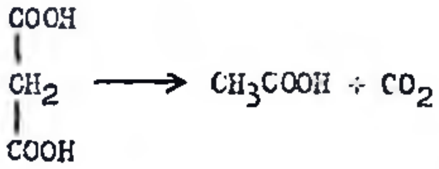
$$H_2 + 2Cl_2 + CO \longrightarrow COCl_2 + 2HCl$$

He attributed this difference to a higher activation energy for deuterium. Wynne-Jones (42) in the same year measured the relative ratio of ionization of the pseudo-acid, nitromethane. He found protons to ionize ten times faster than deuterons. Also in 1929 Farkas and Farkas (43) showed that the photochemical reaction of deuterium with chlorine was only one-third as fact as that of hydrogen. These workers postulated that the difference was due to a higher zero-point energy for hydrogen. This is the currently accepted view. Experimental verification has been provided by Gerö and Schmidt (44). These workers showed that hydrides are less stable than deuterides, in agreement with spectrometric data which indicate that the nuclear separation is greater for hydrides.

The calculations of Eyring and co-workers (45, 46, 47) and Evans and Polanyi (48), based on the theory of absolute reaction rates, provided theoretical support for the experimental results. Similar calculations were made by Farkas and Wigner (49) for reactions of the type

$$D + H2 \longrightarrow DH + H$$

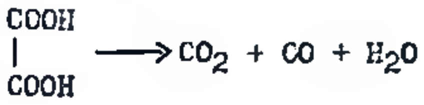
The first determination of a kinetic isotope effect involving an element heavier than hydrogen was reported in the literature in 1948. Beeck, Otvos, Stevenson and Wagner (50) that year reported a 29 per cent more frequent rupture of $C^{12}-C^{12}$ than $C^{12}-C^{13}$ bonds when propane was subjected to electron bombardment. Shortly thereafter, the same workers (51) found that $C^{12}-C^{12}$ bonds ruptured with 8 per cent higher frequency than $C^{12}-C^{13}$ bonds in the thermal cracking of propane. The following year Yankwich and Calvin (52) reported a similar effect in the decarboxylation of malonic and bromomalonic acids labelled with C^{14} in the carboxyl position. The reaction is



$$\begin{matrix} COOH \\ | \\ CH_2 \\ | \\ COOH \end{matrix} \longrightarrow CH_3COOH + CO_2$$

In malonic acid they found a 12 per cent preference for the C^{12} to appear in the carbon dioxide, rather than the acetic acid, indicating a preferential rupture of $C^{12}-C^{12}$ rather than $C^{12}-C^{14}$ bonds in the molecule. This is known as an intramolecular kinetic isotope effect. For bromomalonic acid, the same reaction showed a 40 per cent effect. This work was soon repeated by two other groups with materials of normal isotopic abundances and using mass spectrometric analysis. For the intramolecular case, Bigeleisen and Friedman (53) and Lindsay, Bourns and Thode (54) found a 2.0 and a 2.6 per cent isotope effect, respectively. These workers also reported a second effect called an intermolecular kinetic isotope effect, which is the preferential decarboxylation of an acid molecule containing C^{12} only, compared with one containing C^{13} in one of the carboxyl positions. This intermolecular effect was found to be about 3.5 per cent, favouring the molecule containing C^{12} only. Later, Yankwich and Promislow (56) re-investigated the C^{14} isotope effect in this reaction, using mass spectrometric techniques. Their results demonstrated that the C^{14} effect previously reported (52) was too high. The factor of 1.96 which they obtained between the C^{14} and C^{13} isotope effects is in good agreement with theoretical expectations.

Kinetic isotope effects have since been reported in many other reactions. In one of the earliest publications, Lindsay, McElcheran and Thode (57) noted two effects in the thermal decomposition of oxalic acid, which breaks down as follows:



$$\begin{matrix} COOH \\ | \\ COOH \end{matrix} \longrightarrow CO_2 + CO + H_2O$$

For oxalic acid with normal isotope abundances they found a 3.3 per cent enrichment of C^{13} in the CO_2 over the CO, and a 3.4 per cent preference for the rupture of C^{12}- C^{12} rather than C^{12}- C^{13} bonds. In another reaction, Stevens and Attree (58) found that ethyl benzoate labelled with C^{14} in the carboxyl position hydrolyzed 16 per cent more slowly than unlabelled material.

In 1949 Bigeleisen (55) published a simplified form of the Eyring equation (45) for kinetic isotope effects. His expression is more convenient to use and the results are easier to interpret, since there is a physical significance attached to each term. In general, the occurrence of a kinetic isotope effect is indicative of a change in bonding at the atom under consideration during the rate-controlling step. Considerable use is being made of this fact in reaction mechanism studies, particularly in organic chemistry.

Harrison (23), for example, has studied the kinetic sulphur isotope effect in the reduction of sodium sulphate by hydriodic acid. The fact that the S^{32} reacts 2.2 per cent faster than the S^{34} has been interpreted as indicating that one of the sulphur-oxygen bonds is being broken in the rate-controlling step.

Isotope Abundances in Nature

The postulates of Soddy (2) and Fajans (3) concerning isotopes indicated that leads of different radiogenic origins should have different atomic weights. Careful determinations by Richards and Lambert (59) in 1919 corroborated the theoretical expectations. The possibility, however, of finding similar differences among the stable elements was considered remote. As late as 1932 Aston (11) was able to state that variations in natural isotopic abundances could not be expected. Just two years elapsed before Emeleus et al. (60) detected density differences in water from various sources of as much as 8.6 gamma (parts per million of density). These differences they attributed to variations in deuterium content. Furthermore, they showed how various physical processes could produce the observed fractionation. Other workers found that ocean water had a higher deuterium content than either iceberg (61) or Lake Michigan (62) water.

Variations in the O^{18} content of oxygen from various sources have been studied extensively by Dole and his co-workers (63-70) since

1935. These workers also employed precise water density measurements ultimately capable of detecting differences as small as 0.1 gamma. With this precision the limiting factor becomes the ability to obtain sufficiently pure water without, at the same time, fractionating the isotopes. Variations in deuterium content were eliminated by electrolyzing the water to be analyzed, then recombining the oxygen with standard hydrogen gas. Dole investigated the O^{18} content of atmospheric oxygen (63) and water from various sources (64, 65), while Dole and Slobod (66) examined the oxygen from carbonate rocks and iron ores. This group also attempted to determine the cause of the observed variation between atmospheric oxygen and water. Dole and Jenks (67) studied the oxygen isotope effect in photosynthesis, while Dole, Hawkings and Barker (68) examined bacterial fractionation of the oxygen isotopes. Neither process could account for the differences actually observed in nature. Various workers in this laboratory (71, 72) have confirmed Dole's results, using mass spectrometric techniques. The work in this field up to 1952 has been admirably reviewed by Dole (69). More recently, Dole and Lane (70) have reported that the differences in O^{18} content between water and atmospheric oxygen are explicable on the basis of a preferential absorption of O^{16} by plants and animals during respiration.

The isotope abundances of oxygen in silicate rocks have been studied by Baertschi (73) and Baertschi and Silverman (74). These workers found that sedimentary silicates are enriched in O^{18} by up to 2.4 per cent with respect to igneous rocks. The isotope geology of oxygen with special reference to silicates has been discussed by Silverman (75).

Another element of great geological significance which has been the object of intensive study is carbon. The earliest work was reported by Nier and Gulbransen (76) in 1939. Mass spectrometric analyses indicated that limestones were enriched in C^{13}, while plants were depleted. The maximum variation reported at this time was 5 per cent. In 1950 Trofimov (77) examined the C^{12}/C^{13} ratio in meteorites. Variations of 2.5 per cent were reported, with the average value well within the range reported for terrestrial materials.

Mars (78) repeated and extended the earlier investigations of carbon isotope abundances. His results confirmed those of previous workers. Wickman, Blix and von Ubisch (79), in a systematic examination of carbonate minerals and limestones, found definite generic relationships between the C^{13} content and the geological history of the sample. The results bore out theoretical considerations. Later publications by Wickman (80, 81) reported C^{13} abundance data for plants from different localities and biological families. The former variable appeared to be directly related to the carbon isotope abundances. This, Wickman (81) attributed to variations in the intensity of the carbon dioxide cycle with climate.

A very complete C^{13} abundance survey was published in 1953 by Craig (82), who examined carbonates, terrestrial and marine plants, and fossil carbon. His data showed no age correlation for limestone, coal or fossil wood. He did, however, observe variations in C^{13} content of limestone which he attributed to a temperature effect. This geological thermometer has been of considerable interest to geologists.

Also, in 1953, Buchanan, Nakao and Edwards (83) conducted experiments with balanced aquaria which were sealed for as long as three years in an attempt to demonstrate carbon isotope abundance variations in a controlled manner. Their results were in excellent agreement with data from natural sources: organic materials were depleted in C^{13}, while carbonates and shells were enriched. Recently Taylor (84) has studied the carbon isotope effect in the bacteriological decarboxylation of pyruvic acid. On the basis of the 1.5 per cent effect observed, he explained the occurrence of C^{13} depleted carbonates in the cap rocks of the salt domes of Louisiana and Texas, which had been studied by previous workers (85).

Sulphur Isotope Abundances in Nature

Sulphur is another element of widespread occurrence and geological significance which has been extensively studied from the viewpoint of variations in isotopic abundances. The first data were published in 1949 by Thode, Macnamara and Collins (86). They reported variations in S^{34} content up to 5 per cent in samples of pyrite, native sulphur, sulphate, and hydrogen sulphide from various locations. Contrasted with the variations in terrestrial sulphur compounds, Macnamara and Thode (87) reported that meteoritic sulphur had a constant isotope ratio which was, moreover, virtually identical with the value for igneous sulphides. Thode and his co-workers therefore postulated that meteoritic sulphur represented the primordial abundance of the isotopes from which base level all subsequent terrestrial fractionations have occurred. More recent work, in this laboratory and elsewhere, using higher precision mass spectrometers, has confirmed the constancy of the meteoritic sulphur isotope ratio (88, 89, 90).

Most of the isotope abundance data obtained to date for sulphur from various sources are displayed in Figure 1. The δ value given at the left of Figure 1 is the per mil enrichment of S^{34} compared to meteorites as the standard. It is defined as follows:

$$\delta = \left(1 - \frac{S^{32}/S^{34}\ \text{sample}}{S^{32}/S^{34}\ \text{standard}}\right) 1000$$

An examination of Figure 1 reveals several broad relationships. Sulphates are, in general, enriched in S^{34}, while sulphides are depleted. Sea water sulphate is quite constant at a value of +19.5 per mil (89, 90). Sedimentary sulphates occur both above and below this value. The ratios for igneous sulphides are grouped close to meteorites, but slightly lower, sedimentary sulphides, on the other hand, are usually depleted in S^{34}, but by widely varying amounts. Some sulphides, however, are very highly enriched. The total spread in the isotope ratio is about 8 per cent. The causes of these large variations have been the subject of intensive research, including the present work.

Tudge and Thode (19) pointed out in 1950 that the maximum spread in sulphur isotope ratios, 8 per cent, is close to the equilibrium value for the exchange reaction

$$S^{32}O_4^{=} + H_2S^{34} \rightleftharpoons S^{34}O_4^{=} + H_2S^{32}$$

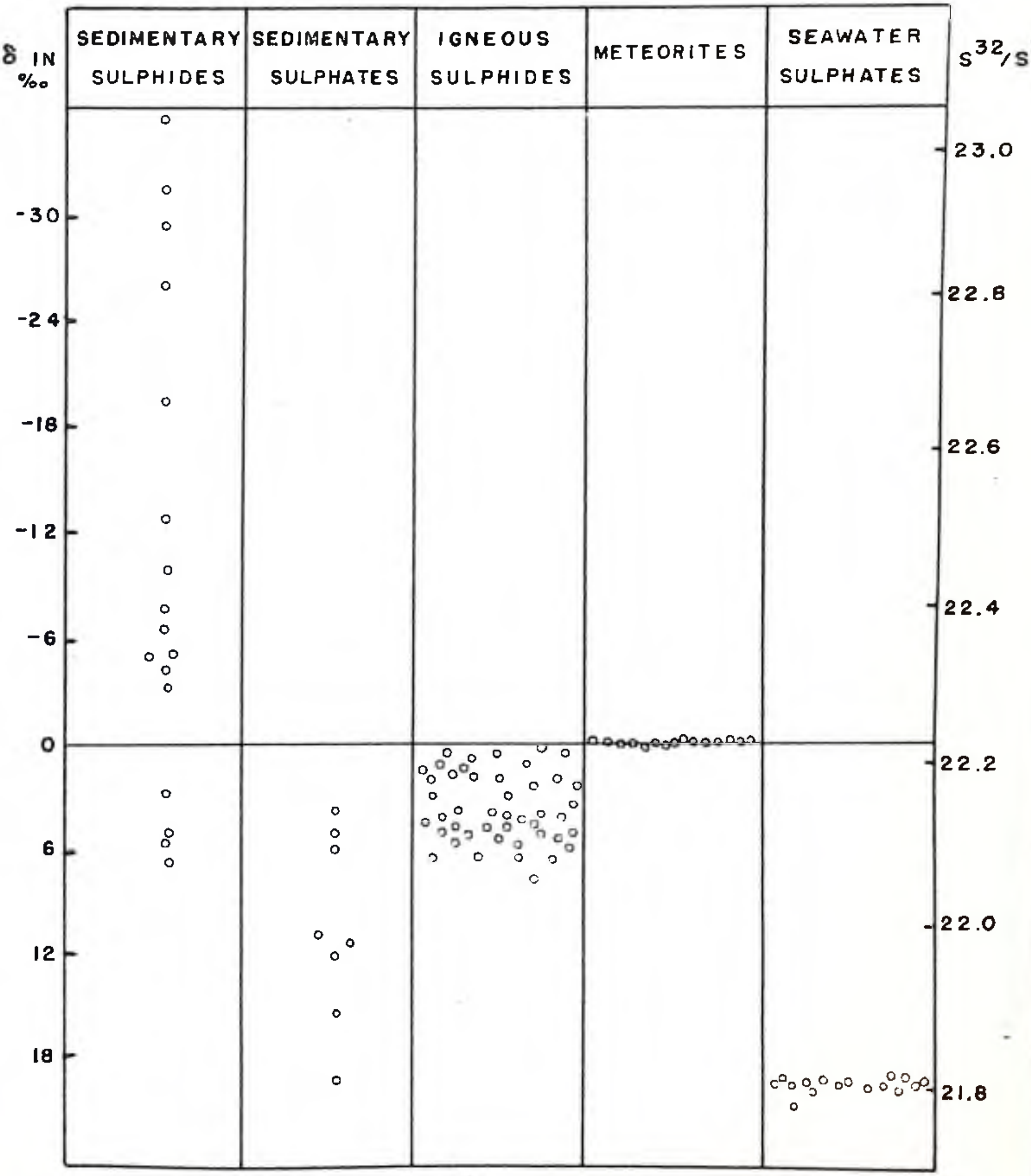


FIGURE I SULPHUR ISOTOPE DISTRIBUTION IN NATURE

The calculated equilibrium constant is 1.075 at 25°C. (recalculated by Harrison (23) as 1.070). This remarkably close agreement led Tudge and Thode (19) to postulate that the biological sulphur cycle was acting as an exchange mechanism, tending to produce the most favoured distribution of the isotopes. The sulphur cycle is represented schematically below.

BIOLOGICAL SULPHUR CYCLE

Indirect evidence for the action of the sulphur cycle was given by Thode, Macnamara and Fleming (91), who reported that the ratios for sedimentary pyrites showed a correlation with the age of the sample. The most recent specimens had the highest negative δ values, while older samples approached nearer and nearer to the meteoritic base level. Because isotope fractionation between sulphides and sulphates seemed to begin about 800 million years ago, the authors suggested that the biological sulphur cycle became significant at approximately that time. Since then it has been functioning at an ever increasing rate, with the result that sediments about 100 million years old show a difference of about 8 per cent in the isotope ratios of sulphide and sulphate, which is nearly the equilibrium value for this isotope exchange reaction.

Direct evidence for the fractionation of isotopes in the operation of the sulphur cycle was first obtained from an examination of the Cyrenaican lakes in Africa. Butlin (92) and Butlin and Postgate (93) have described these shallow sloughs where the action of anaerobic reducing and photosynthetic oxidizing bacteria produce annually a layer of elemental sulphur on the beds of the lakes. Results of isotope abundance analyses obtained by Macnamara and Thode (94) and Harrison (23) are shown in Table I.

TABLE I

Isotope Fractionation in Cyrenaican Lakes

S^{32}/S^{34}		Fractionation $S^o/SO_4^=$	Reference
$SO_4^=$	S^o		
21.88	22.57	1.032	94
22.190	22.518	1.015	23

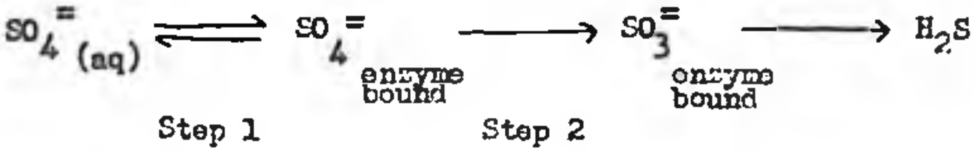
Here, in a location where the sulphur cycle is definitely operative, the difference in source sulphate and product sulphur is as high as 3.2 per cent.

Another example of an isolated system where the sulphur cycle has been active is given by the salt domes of Louisiana and Texas. The isotope ratios of the various forms of sulphur in these domes were reported by Thode, Wanless and Wallouch (85) in 1954. Here the average difference between elemental sulphur and associated gypsum was 3.9 per cent, with sulphide another 1 per cent depleted in S^{34}.

In order to explain these observed fractionations, detailed studies of various parts of the biological sulphur cycle have been undertaken. Miller (95) had reported in 1949 that anaerobic sulphate-reducing bacteria could be isolated from salt dome core samples. Subsequently Thode, Kleerekoper and McElcheran (96) found that these bacteria produced hydrogen sulphide which was about 1 per cent depleted in S^{34} with respect to the source sulphate. Wallouch (97) extended this work to include a study of the effect of temperature. He found a temperature coefficient which was too large to be explained on the basis of either a simple unidirectional or an equilibrium process. The highest observed fractionation, however, was still too small to account for the large spread in natural samples.

Meanwhile, other aspects of the sulphur cycle were being investigated. McElcheran (98), working with species of Thiobacillus, demonstrated that these bacteria did not fractionate the isotopes in the oxidation of sulphur to sulphate. The author has repeated this work, using T. thio-oxidans, with precisely the same results. The plant metabolism of sulphate was studied by Ishii (99). She demonstrated that sulphur metabolism in algae and mustard plants produced no isotope fractionation. Both these results were interpreted as indicating that the pick-up of sulphur and sulphate, respectively, by the organisms was the rate-controlling step.

Since only bacterial reduction of sulphate appeared to produce any separation of the isotopes, Harrison (23) examined the metabolism of these organisms more thoroughly. He found that the bacteria, *Desulphovibrio desulphuricans*, gave isotope fraction factors from 1.000 to 1.025, depending on the conditions of growth. When the metabolic rate was high, or the sulphate concentration extremely low, the fractionation factor approached unity. On the other hand, when the metabolic rate was very low owing to a low organic metabolitic concentration or a decrease in temperature, the isotope effect approached the maximum value of 2.5 per cent. This was comparable to the 2.2 per cent effect reported by Harrison (23) for the chemical reduction of sodium sulphate with hydriodic acid. On the basis of these findings, Harrison postulated a mechanism in which either of two steps could be rate-controlling.



$$SO_{4\,(aq)}^{=} \underset{\text{Step 1}}{\rightleftharpoons} \underset{\text{enzyme bound}}{SO_4^{=}} \underset{\text{Step 2}}{\xrightarrow{\qquad}} \underset{\text{enzyme bound}}{SO_3^{=}} \longrightarrow H_2S$$

The enzyme pick-up of sulphate ion in Step 1 should involve little or no isotope effect since bonding of the sulphur atom is not intimately involved. The breaking of the first sulphur to oxygen bond in Step 2 would have a large isotope effect similar to that found for the chemical reduction of sulphate, that is, 2.2 per cent. If Step 1 is rate-controlling, as it would be when the subsequent rate of reduction was very rapid, or the sulphate concentration very low, isotope fractionation would be observed in the overall process. On the other hand, if Step 2 is rate-controlling, the maximum effect would be observed. This condition would hold when the metabolism of sulphate was proceeding at a very slow rate. Intermediate isotope effects would occur when both Step 1 and Step 2 were partially rate-determining. This postulate fits the experimental data very well. Even the largest fractionation factor observed falls short however, of the values reported for the Cyrenaican lakes and the salt domes of Louisiana and Texas. Corroboration of Harrison's results has since been supplied by the work of Jones, Starkey, Feely and Kulp (100) and Jones and Starkey (101).

Other factors were now felt to be worth studying in an attempt to elucidate the mechanism whereby the sulphur isotopes have been fractionated to such a great extent in nature. Butlin and Postgate (93) had observed vigorous colonies of photosynthetic oxidizing bacteria in the Cyrenaican lakes which were apparently intimately associated with the production of elemental sulphur. Since these bacteria also represented another part of the biological sulphur cycle, they were obviously worthy of attention. Furthermore, since both reducers and oxidizers grow together in nature, they should grow together in laboratory experiments which would thus simulate a natural sulphur cycle. Studies of this nature form the basis of the present thesis.

Sulphur Bacteria

Two species of sulphur bacteria were used in this investigation. One species was a reducing type, while the other was an oxidizer. Brief descriptions follow.

Beijerinck (102) in 1895 attributed the hydrogen sulphide production in mud to bacterial reduction of sulphate. Nine years later van Delden (103) isolated such bacteria in pure culture. Since then they have been studied extensively by a great number of workers, owing to their great economic importance, both as agents of corrosion and water pollution, and as producers of elemental sulphur in nature. These bacteria have been variously called *Vibrio*, *Spirillum* and *Sporovibrio desulphuricans*. At present they are generally referred to as *Desulphovibrio desulphuricans*, henceforth abbreviated to *D. desulphuricans*. Morphologically, they are comma-shaped, motile rods, about 2 μ in length. Strains have been isolated which can survive high salt concentrations, high pressures and temperatures up to 80°C. (104). All strains are obligate anaerobes.

Only some aspects of their metabolism have been elucidated. Postgage (105) found the following stoichiometric relationships for the reduction of various sulphur compounds by autotrophic strains of *D. desulphuricans*:

$$
\begin{aligned}
SO_4^{=} + 4H_2 &\longrightarrow S^{=} + 4H_2O \\
SO_3^{=} + 3H_3 &\longrightarrow S^{=} + 3H_2O \\
S_2O_3^{=} + 4H_2 &\longrightarrow 2SH^{-} + 3H_2O
\end{aligned}
$$

Investigations of the inhibitive action of selenate (105,106), and the relative rates of reduction of various sulphur compounds led Postgate (107) to postulate the following sequence for the reduction of sulphate.

$$SO_4^{=} \xrightarrow{H_2} SO_3^{=} \longrightarrow \left[\begin{array}{l}\text{Unknown intermediates}\\ \text{containing 1 atom of S}\\ \text{per divalent ion}\end{array}\right] \longrightarrow S^{=}$$

Recently Millett (108) obtained direct evidence for the occurrence of sulphite as an intermediate in the metabolism of D. desulphuricans. Other workers (109) have reported the preparation of cell-free extracts which will reduce thiosulphate and sulphite to hydrogen sulphide, but so far no one has isolated a sulphate reductase.

The other genus of bacteria used in this study was Chromatium. These bacteria were first observed in crude culture by Winogradsky (110) in 1887. Much confusion surrounded these organisms until van Niel (111) conducted his definitive study of them in 1931. He demonstrated conclusively that these organisms are autotrophic, photosynthetic obligate anaerobes; that is, they are capable of synthesizing all their organic requirements from carbon dioxide and water, using light as the sole energy source. In the process, the oxygen, which in green plant photosynthesis is evolved as a gas, is disposed of by the oxidation of sulphur compounds. Van Niel (111) postulated the following stoichiometric relationship for these bacteria:

$$2CO_2 + H_2S + 2H_2O \longrightarrow 2(CH_2O) + H_2SO_4$$

where (CH_2O) represents the average reduction state of the carbon in the cell material. Morphologically, Chromatium are motile, ovoid to kidney-shaped, single cells about 5μ long.

Several aspects of the biological sulphur cycle involving these two genera of bacteria are the major subject of this dissertation. By means of various techniques, the author has investigated the sulphur isotope fractionation in the oxidation of hydrogen sulphide by Chromatium. Mixed cultures of Chromatium and D. desulphuricans have been studied as an example of a limited sulphur cycle, such as occurs naturally in the Cyrenaican lakes. Certain purely chemical studies are reported which involve the oxidation of aqueous sodium sulphite solution with molecular oxygen. This reaction, initially thought to be a simple bond formation process, has proven to be more complicated. It has, however, led to some interesting theoretical speculations. Finally, the isotopic exchange of sulphur atoms between aqueous sulphate and sulphite ions has been investigated both in neutral solution and in the presence of resting cell suspensions of D. desulphuricans. This unique use of an intact organism as an isotope exchange catalyst has given interesting results which may be of value in other metabolic studies.

THEORY

Equilibrium Isotope Effects

In 1935 the theory of isotope effects in equilibrium reactions was discussed by Urey and Greiff (17). Since then both Urey (112) and Bigeleisen and Mayer (18) have simplified the treatment until it is now possible to calculate equilibrium exchange constants from spectroscopic data alone.

The general equation for an isotopic exchange reaction may be written as

$$aA_1 + bB_2 \rightleftharpoons aA_2 + bB_1 \qquad (1)$$

where A and B are molecules with one element in common, which has two isotopes labelled 1 and 2. Throughout the discussion subscript 1 refers to the lighter isotopes.

From elementary thermodynamics it is known that

$$-RT\ln K = \Delta F^{o} = aF_{A_2} + bF_{B_1} - aF_{A_1} - bF_{B_2} \qquad (2)$$

From statistical mechanics it is also known that

$$F = E_o - RT\ln Q/N \qquad (3)$$

where Q is the partition function, N is Avogadro's number and E_o is the zero point energy of the molecule. If the standard state is considered to be one molecule rather than one mole in unit volume, N drops out.

Substituting equation (3) in equation (2), the following relationship is obtained:

$$K = \left[Q_{A_2}/Q_{A_1} \right]^a \cdot \left[\frac{Q_{B_2}}{Q_{B_1}} \right]^b \cdot e^{-\left[\frac{aE_{o_{A_2}} + bE_{o_{B_1}} - aE_{o_{A_1}} - bE_{o_{B_2}}}{RT} \right]} \qquad (4)$$

Since the potential energy curves are identical for both isotopic molecules, equation (4) may be simplified by assuming that the zero point energy is the bottom of the potential energy curve. Hence $E_{o_{A_2}} = E_{o_{A_1}}$ and $E_{o_{A_1}} = E_{o_{A_2}}$ and equation (4) reduces to

$$K = \left[\frac{Q_{A_2}}{Q_{A_1}} \right]^a \Bigg/ \left[\frac{Q_{B_2}}{Q_{B_1}} \right]^b \qquad (5)$$

The problem now is to find the ratios of the partition functions for the two isotopically substituted molecules. For all elements except hydrogen both the translational and rotational partition functions assume their classical values. Simple considerations (18) indicate that the classical partition functions cannot lead to an isotope effect. Thus the vibrational partition functions, which are quantized, are the only ones which need to be considered. The form of the vibrational partition function is:

$$Q_{vib} = \prod_i \frac{e^{-u_i/2}}{1 - e^{-u_i}} \qquad (6)$$

where $u_i = \frac{hc\omega}{kT}$ and the product is taken over all the vibrational frequencies of the molecule, taking an n-fold degenerate level n times.

The ratio of the partition functions for the isotopically substituted molecules, considering only the necessary vibrational contributions, is given by:

$$\frac{Q_2'}{Q_1'} = \frac{\sigma_1}{\sigma_2} \prod_i \frac{u_{2_i}}{u_{1_i}} \cdot \frac{e^{-u_{2_i}/2}}{1 - e^{-u_{2_i}}} \cdot \frac{1 - e^{-u_{1_i}}}{e^{-u_{1_i}/2}} \qquad (7)$$

where Q' is the partial partition function and is the symmetry number.

K can now be obtained by substituting equation (7) in equation (5). This unwieldy expression, fortunately, may be simplified (18).

Let $u_{1_i} = u_{2_i} + \Delta u_i$ where, as before, subscript 1 refers to the lighter isotope. In this way Δu_i is always positive. If this simplification is employed, equation (7) becomes:

$$\frac{Q_2'}{Q_1'} = \frac{\sigma_1}{\sigma_2} \prod_i \frac{u_{2_i}}{u_{2_i} + \Delta_i} \cdot e^{\Delta u_i/2} \cdot \frac{1 - e^{-u_{2_i} + \Delta u_i}}{1 - e^{-u_{2_i}}} \qquad (8)$$

For every case but hydrogen Δu_i is small enough so that equation (8) may be taken as

$$\frac{Q_2'}{Q_1'} = \frac{\sigma_1}{\sigma_2} \left[1 + \sum_i \left(\frac{1}{2} - \frac{1}{u_{2_i}} + \frac{1}{e^{u_{2_i}} - 1} \right) \Delta u_i \right] \qquad (9)$$

Again the summation is taken over all i frequencies, counting an n-fold degenerate level, n times. The function $\left(\frac{1}{2} - \frac{1}{u} + \frac{1}{e^{u} - 1} \right)$ has been termed the free energy function by Bigeleisen and Mayer, and given the symbol G(u). These authors have calculated and tabulated values of G(u) for values of n from 0 to 25 (18).

Equation (9) allows the calculation of partition function ratios for isotopically substituted molecules from a knowledge of vibrational frequencies only. Spectroscopic data will supply the frequencies for the more abundant isotope, but recourse is often made to the normal vibration equations in order to calculate the frequencies for the rarer isotope which usually cannot be observed directly. From the partition function ratios thus calculated for the molecules in question, the equilibrium constant is readily obtained through the use of equation (5).

Kinetic Isotope Effects

A theoretical treatment of kinetic isotope effects has been given by Bigeleisen (55) based on the theory of absolute reaction rates developed by Eyring (45) and Evans and Polanyi (48). Only assumptions inherent in this theory are made.

Consider the generalized reactions

$$A_1 + B + C \;....\qquad P_1$$

$$A_2 + B + C \;....\qquad P_2$$

where A differs only in the isotopic substitution of one atom, subscript 1 referring to the lighter species. From the absolute rate theory

$$k_1 = \frac{K_1 C_1^{\ddagger}}{C_{A_1} C_B \;....} \left(\frac{kT}{2\pi m_1^*}\right) \frac{1}{\delta} \qquad (10)$$

$$\text{and } k_2 = \frac{K_2 C_2^{\ddagger}}{C_{A_2} C_B} \cdots \left(\frac{kT}{2\pi m_2^*}\right)^{\frac{1}{2}} \frac{1}{\delta_2} \tag{11}$$

where

k is the specific rate constant;

K is the transmission coefficient;

m^* is the effective mass of the reacting molecules along the reaction co-ordinates;

δ is the length of the path across the top of the potential energy barrier;

and $\ddagger$ refers to the transition state.

Since $\delta_1 \approx \delta_2$, the ratio of the rate constants may be written as

$$\frac{k_1}{k_2} = \frac{K_1}{K_2} \quad \frac{C_1^{\ddagger}}{C_2^{\ddagger}} \frac{C_{A_2}}{C_{A_1}} \left(\frac{m_2^*}{m_1^*}\right)^{\frac{1}{2}} \tag{12}$$

From statistical mechanics, it is known that the concentration terms may be replaced by partition functions, so that equation (12) becomes:

$$\frac{k_1}{k_2} = \frac{K_1}{K_2} \; \frac{Q_1^{\ddagger}}{Q_2^{\ddagger}} \; \frac{Q_{A_2}}{Q_{A_1}} \left(\frac{m_2^*}{m_1^*}\right)^{\frac{1}{2}} \tag{13}$$

Previous considerations have shown that the total partition function ratios may be replaced by partial partition function ratios of the form

$$\frac{Q_2'}{Q_1'} = \frac{\sigma_1}{\sigma_2} \left[1 + \sum_i G(u_{2_i}) \Delta u_i\right] \tag{14}$$

The two appropriate equations of this form are substituted into equation (13) and rearranged to give the Bigeleisen kinetic isotope effect equation

$$\frac{k_1}{k_2} = \frac{K_1}{K_2} \; \frac{\sigma_{A_1} \sigma_2^{\ddagger}}{\sigma_{A_2} \sigma_1^{\ddagger}} \left(\frac{m_2^*}{m_1^*}\right)^{\frac{1}{2}} \left[1 + \sum_i G(u_{2_i}) \Delta u_i - \sum_i G(u_i^{\ddagger}) \Delta u_i^{\ddagger}\right] \tag{15}$$

This equation permits calculation of the ratio of rate constants for reactions involving isotopically substituted molecules, provided sufficient data are available. In practice, the transmission co-efficients and symmetry numbers are considered to be sufficiently similar for each isotopic species so that their ratios are unity and hence may be ignored. Generally, the reduced mass of the transition state along the reaction co-ordinate is taken as the reduced mass of the single bond directly involved in the reaction. Since little is known concerning the vibrational frequencies of the transition state, two limiting cases are usually considered.

Case I: In this case the activated complex is considered to be the same as reactant; that is, the bond involved is not affected in the rate-controlling step. The two G(u) terms are then equal and cancel out, leaving only the reduced mass term.

Case II: In this case the activated complex is considered to be the same as products. Now $G(u^{\ddagger})\Delta u^{\ddagger}$ may be calculated and, for this case, will assume its smallest value since the bond in question has been completely broken. Hence the ratio of the rate constants will be a maximum.

Normally, the actual value for $G(u^{\ddagger})\Delta u^{\ddagger}$ is intermediate between these two extremes. In some processes, however, such as elimination reactions, Case I has no physical significance, since the atom under consideration may not be at all affected in the rate-controlling step. If this is the case, a zero isotope effect will be observed.

The usefulness of the Bigeleisen equation has been well established for reactions involving a bond which is being broken. Uncertainty, however, surrounds its applicability to bond-formation reactions. Some workers feel that the equation cannot be used since in the initial state, that is, separated atoms, there is no minimum in the potential energy curve on the reactants side. Since isotope effects depend on differences in zero-point energies, this would seem to rule out the possibility of an effect in bond formation. For simple diatomic molecules this is probably true. For polyatomic molecules, however, substituents around the reaction centre would quite probably exert repulsive forces on an incoming group. These forces would produce a maximum in the potential energy curve corresponding to the transition state. Different isotopic species would then require different energies to reach this maximum, and hence would react at different rates. On this basis, isotope effects should be expected in bond-formation reactions.

Moreover, if the bond in the activated complex is sufficiently strong, the free energy term for the transition state might be large enough to counter-balance both the initial free energy term and the mass term, resulting in a reverse isotope effect; that is, one in which the heavy isotope reacts faster than the light one. Such an effect, however, has never been convincingly demonstrated. Two examples have been studied in this laboratory. Sheppard, Bader and Bourns (113) observed a zero sulphur isotope effect in the addition reaction of bisulphite with various aldehydes and ketones. More recently, Hayes (114) detected a small nitrogen isotope effect in the formation of quaternary ammonium salts. Moreover, the temperature co-efficient appeared to be in the right direction for a true bond formation process. The magnitude of this nitrogen isotope effect was, however, very near the limit of the precision of the analysis techniques. Hence the data, while indicative of an isotope effect in bond formation, are not conclusive.

EXPERIMENTAL

Chemical Procedures

Preparation of Pure Sodium Sulphite

Sodium sulphite solutions with very low concentrations of sulphate were required for various parts of this work. They were prepared immediately prior to use by the following method. The apparatus shown schematically in Figure 2 was employed. Sodium sulphite (reagent grade) in aqueous solution was added slowly from the header flask, B, to boiling, dilute hydrochloric acid in flask A. A stream of pure nitrogen swept the sulphur dioxide produced into a dilute solution of sodium hydroxide in flask C. Sufficient caustic was placed in C so that the desired amount of sodium sulphite would result when the sulphur dioxide had neutralized the absorber solution. When pH 7.0 - 7.5, determined with narrow range pH paper, had been attained, the pure sodium sulphite solution was poured into a glass-stoppered volumetric flask and diluted to the mark. An aliquot was usually taken for analysis, while the rest was used for the experiment at hand. This method produced sodium sulphite which gave a negative test for sulphate ion with barium chloride.

Preparation of Sodium Sulphate

For most of the experiments reported here, reagent grade sodium sulphate was completely satisfactory. In a few cases, however, sulphate and sulphite of nearly identical S^{32}/S^{34} ratios were required.

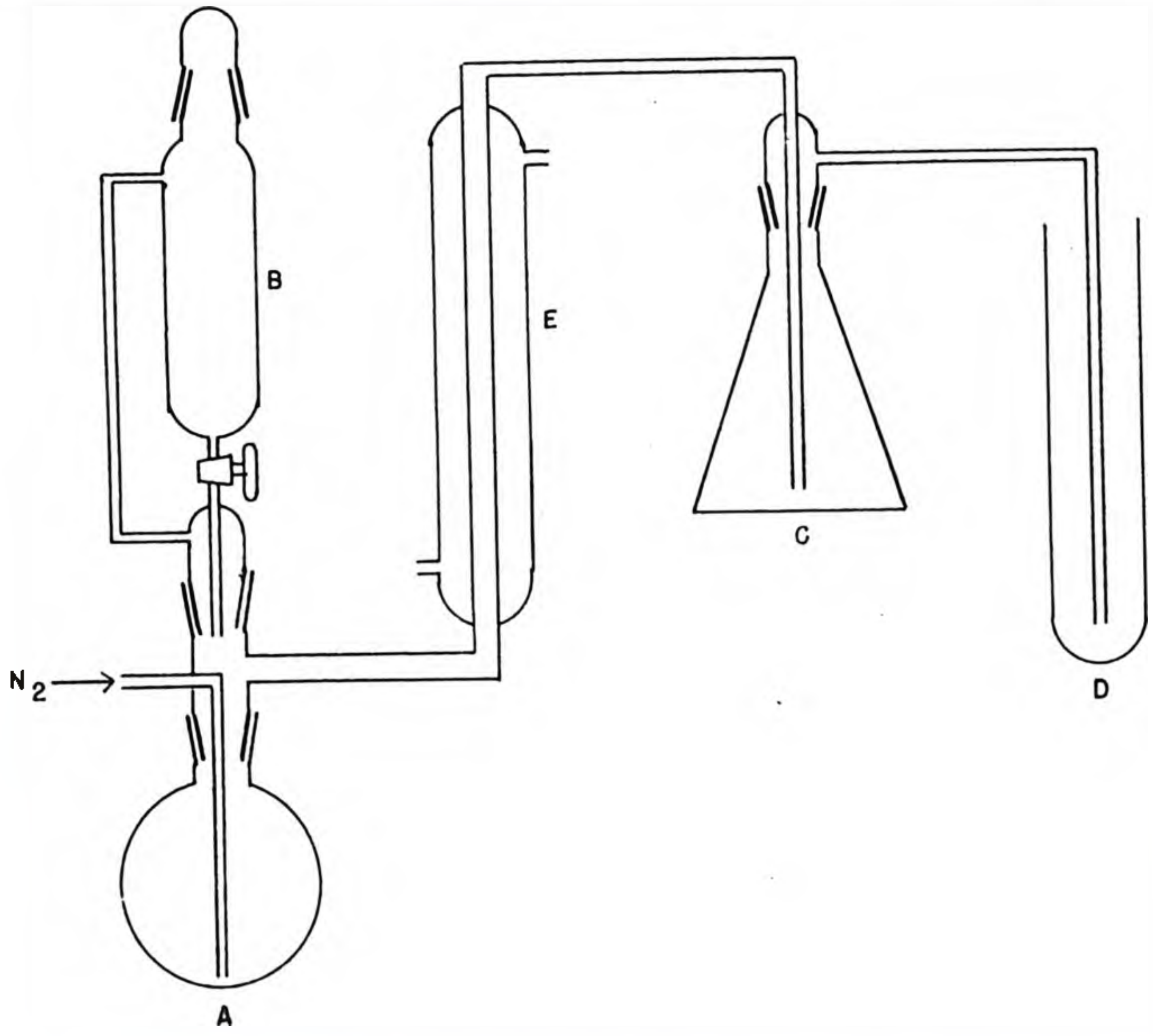


FIGURE 2 SULPHITE PREPARATION APPARATUS

Unfortunately, the reagent grade materials at hand differed in this respect by more than 0.5 per cent. Hence it was necessary to oxidize the sodium sulphite to sulphate with hydrogen peroxide in order to obtain material differing by less than 0.1 per cent in S^{34} abundance from the sodium sulphite. The excess oxidant was removed by heating the solution on a steam bath.

Separation of Sulphide, Sulphur, Sulphite and Sulphate

Solutions containing from two to four of these sulphur species were encountered in the course of this work. Separation procedures were simple and quantitative.

In the general case, sulphide was first precipitated with an unbuffered solution of cadmium acetate. Filtration removed the precipitate which was retained for further treatment.

The filtrate from the first step was then placed in flask A of the apparatus shown in Figure 2. Dilute hydrochloric acid was added from the header flask B. With a slow stream of nitrogen passing through the system, the solution in A was brought to a boil. The sulphur dioxide released from the sulphite was swept with the nitrogen into iodine solution contained in flask C and the large test tube, D. Sufficient iodine solution was used to oxidize all the sulphur dioxide to sulphate. Meanwhile, any elemental sulphur present in flask A distilled out and collected in the condenser, E. The solution was boiled for at least one hour in order to ensure total separation.

The sulphates in flask A and the two iodine absorber solutions were precipitated from boiling solution in the normal manner. Barium chloride was used as the precipitant. More manageable precipitates could be obtained if a few drops of saturated picric acid solution were added prior to precipitation. This was especially true in the case of the bacteria experiments using resting-cell suspensions with a fairly high concentration of sodium lactate. The precipitates, after digestion, were removed on filter paper, ignited and weighed. This gave a quantitative measure of both the sulphite and sulphate originally present.

The elemental sulphur was removed from the condenser by heating acetone under reflux in the apparatus for a half-hour. After the acetone was removed by evaporation on a steam bath, the sulphur was oxidized to sulphate with nitric acid and bromine, using the method of Hontoir (115). The sulphate was then treated in the usual manner to give a quantitative determination of the sulphur.

The cadmium sulphide obtained in the first step was reconverted to hydrogen sulphide by boiling in dilute hydrochloric acid. The apparatus shown in Figure 2 was also used for this process. The hydrogen sulphide was swept with a stream of pure nitrogen into cadmium acetate-acetic acid buffer solution. In order to obtain a more manageable precipitate, the cadmium sulphide was converted to the silver salt by the addition of silver nitrate solution. Filtration was carried out on glass wool. The precipitate was washed twice with concentrated ammonium hydroxide in order to remove silver chloride and carbonate, then rinsed with water several times before being dried for at least 24 hours in an oven at 100°C. The use of glass wool instead of filter paper prevented the contamination of the sample with combustible material which would yield carbon dioxide in the burning procedure. Also, mechanical losses were reduced since, for small samples, the glass wool and silver sulphide could be placed in the combustion chamber together.

A quantitative estimation of the sulphide was obtained by adding a known excess of standard silver nitrate solution to the cadmium sulphide. After filtration the filtrate was back-titrated with a standard solution of ammonium thiocyanate with ferric sulphate as the indicator.

Modifications of this general separation procedure were also employed. In cases where only sulphate and either sulphide or sulphite were present, the separation could be carried out in one step. By adding acid and boiling, the gaseous component was swept with a stream of nitrogen into the appropriate absorber solution. The separated compounds were then treated in the usual manner.

Tests indicated that these procedures were quantitative and did not introduce spurious isotope effects.

Reduction of Barium Sulphate

Barium sulphate samples were reduced to hydrogen sulphide by a modification of a method due to Popkowitz and Shirley (116). The reducing mixture had the following composition:

HI (47%)	500 ml.
$HCl_{(conc.)}$	820 ml.
H_3PO_2(50%)	240 ml.

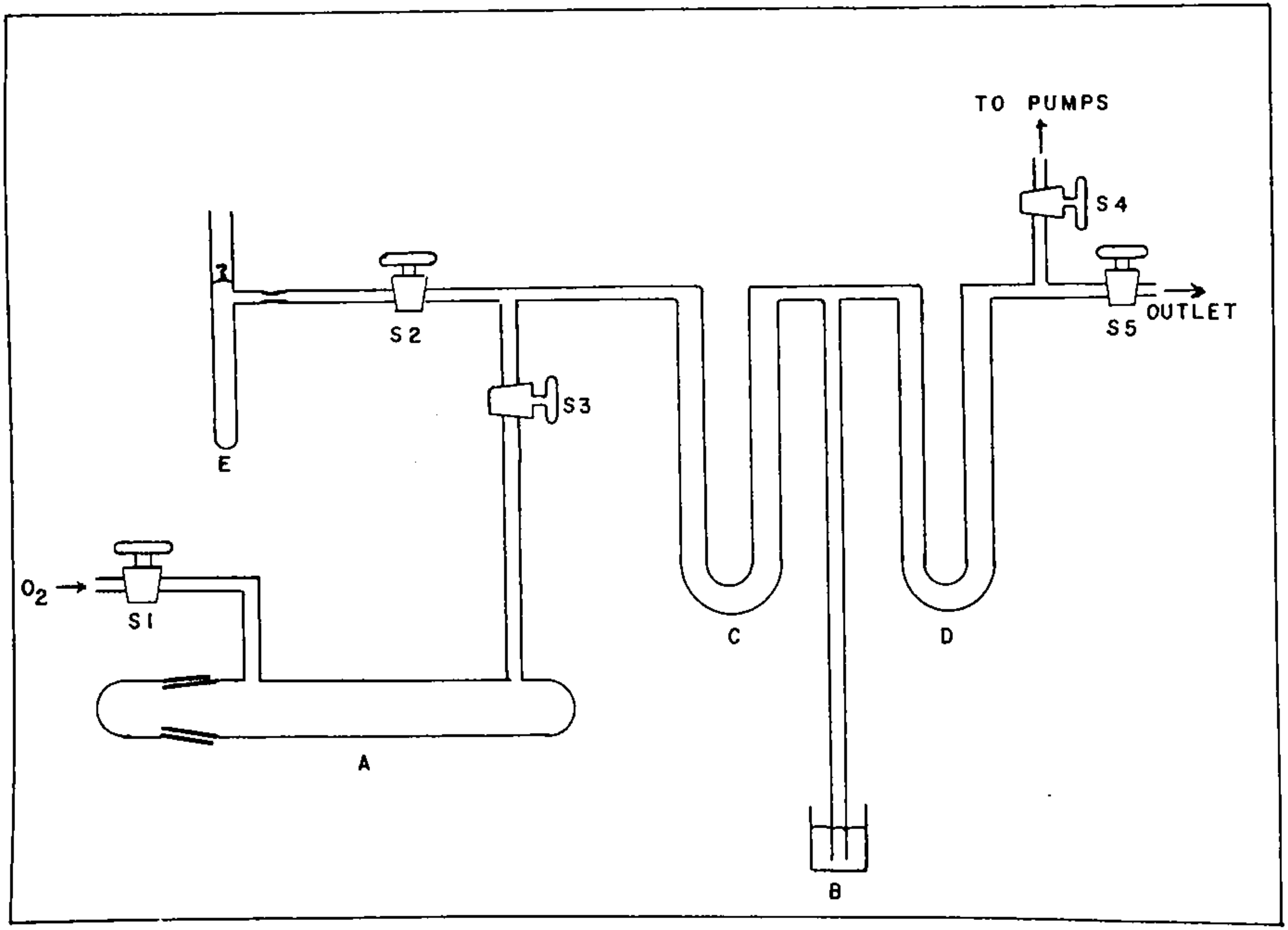


FIGURE 3 COMBUSTION APPARATUS

An outline of the burning procedure follows. At least 15 mg. of silver sulphide contained in a small nickrome boat was placed in the quartz tube. The system was evacuated, then filled with pure oxygen, which continued to flow during the burning. The sample was heated for about two minutes with a gas-oxygen flame. The sulphur dioxide produced was frozen from the oxygen stream in the two traps C and D, which were cooled with liquid oxygen. After combustion was complete, the oxygen was pumped off. Thawing and subsequent refreezing in one trap, followed by further pumping, removed non-condensable gases. When a pressure of 10^{-3} mm. of Hg had been attained as determined by a McLeod gauge, the sulphur dioxide was distilled from the trap, now at dry ice temperature, into one of the sample tubes at liquid air temperature. This step removed water vapour from the sample. When the transfer was complete, the sample tube was sealed off at the constriction designed for that purpose. Harrison (23) reported that this procedure gave a mean deviation of 0.01 per cent in the isotope ratio.

In order to transfer the sample to the gas handling system of the mass spectrometer, a short iron rod was placed on top of the break-seal and a stopcock and ground glass joint sealed in place. After this section of the tube was evacuated, the break-seal was crashed by lifting the iron rod with a pair of magnets and dropping it.

Mass Spectrometry

Sulphur isotope ratios were determined with a Nier-type, 90°, simultaneous collection mass spectrometer. The design and operation of this machine have been reported by Wanless and Thode (117), with modifications by Warren (89).

All samples were analyzed as sulphur dioxide. A magnetically operated valve system permitted rapid switching from a standard gas to the unknown. These gases were contained in separate gas-handling systems. The collector assembly was so designed that only masses 64($S^{32}O^{16}O^{16}$) and 66 ($S^{34}O^{16}O^{16}$; $S^{32}O^{16}O^{18}$) could reach the detector plates. Two vibrating reed electrometers provided amplification of the ion currents.

At the beginning of an analysis, the pressures of the standard and unknown gases were adjusted to give identical mass 64 ion currents. Then, with standard gas flowing into the tube, the electronics were switched to simultaneous collection. A suitable fraction of the mass 64 ion current was selected by a put-and-take potentiometer in order to balance the mass 66 current. At the balance point, no current was produced by the mass 66 amplifier, whose output was fed to a Brown recorder. The pen of the recorder was held in a central position by a test voltage. When the valves were switched, allowing the unknown gas to enter the analyzer tube, an imbalance was produced in the circuit, resulting in a voltage output from the mass 66 amplifier. This caused the pen to shift position on the recorder chart. The amount of this shift was directly proportional to the difference in the 64/66 ratios for the two gases. The sensitivity of the instrument was determined before and after each analysis by shifting the potentiometer setting by a known amount. Six measurements of the displacement were made for each calibration and sample run. Values of resistances in the amplification circuits and magnitudes of the ion currents were so adjusted that a 1 cm. displacement of the pen corresponded to a difference in 64/66 ratio of about 0.2 per cent.

From the known 64/66 ratio of the standard sulphur dioxide and the values of the displacements of the recorder pen, the 64/66 ratio of the unknown gas was calculated. In order to obtain the S^{32}/S^{34} ratio, a correction had to be applied for the contribution of the $S^{32}O^{16}O^{18}$ molecule to the mass 66 ion current. The contributions of $S^{33}O^{16}O^{17}$ and $S^{32}O^{17}O^{17}$ were neglected because of their extremely low probability of occurrence. The following relationships obtain:

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

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

The value of the O^{18}/O^{16} ratio has been determined as 0.00208. Therefore

$$\frac{S^{32}}{S^{34}} = \frac{1}{\frac{66}{64} - 0.00416}$$

and the desired S^{32}/S^{34} ratios may be calculated. Previous work (22) had shown that the mass spectrometer was capable of a precision of ± 0.02 per cent during the course of a single day. This precision decreased to about ± 0.1 per cent over a period of months. Most of the present work, however, required only the comparison of samples analyzed the same day.

Cultivation of Bacteria

Desulphovibrio desulphuricans

The initial culture of these bacteria was obtained from American Type Culture Collection in Washington, D. C. The strain, designated No.7757, was the same as had been utilized in the previous studies by Harrison (23). Sub-cultures were grown on a medium essentially the same as used by Grossman and Postgage (118) and Harrison (23).

Medium 1.

NH_4Cl	1.0 g.
$MgSO_4 \cdot 7H_2O$	2.0 g.
Na_2SO_4	1.0 g.
K_2HPO_4	0.5 g.
$CaCl_2 \cdot 6H_2O$	0.1 g.
NaCl	10.0 g.
Sodium lactate	3.5 g.
Difco yeast extract	9.0 g.
Cysteine hydrochloride	0.6 g.
Distilled water	1000 ml.

The pH was adjusted to 6.8 - 7.9 with sodium hydroxide.

About 150 ml. of medium in 250 ml. Erlenmeyer flasks was sterilized in an autoclave for one hour at 15 lbs./sq.in. pressure. After inoculation with a sterile wire loop, the cultures were incubated in a cabinet maintained at 30°C. This medium supported vigorous growth which reached a maximum in about 60 hours. The lack of any precipitate in the medium rendered it especially valuable for the production of bacteria to be harvested for resting-cell suspensions. Healthy cultures were maintained by transferring growth to fresh medium every four to seven days. Periodic examination of the cultures by microscopic and culturing techniques revealed no contaminants.

Chromatium

The initial culture of these bacteria was designated *Chromatium* National Collection of Industrial Bacteria No.8348. The medium for sub-culturing was basically that of Larson (119) with slight modifications.

Medium 2.

KH_2PO_4	1.0 g.
NH_4Cl	1.0 g.
$MgCl_2 \cdot 6H_2O$	0.5 g.
$CaCl_2 \cdot 2H_2O$	0.1 g.
NaCl	10.0 g.
Distilled water	1000 ml.

To this, before autoclaving, was added 10 ml. of the following solution of trace elements:

Fe	as	$FeCl_3$	50.0 mg.
B	as	H_3BO_3	20.0 mg.
Zn	as	$ZnSO_4 \cdot 7H_2O$	10.0 mg.
Co	as	$Co(NO_3)_2 \cdot 6H_2O$	5.0 mg.
Cu	as	$CuSO_4 \cdot 5H2O$	0.5 mg.
Mn	as	$MnCl2 \cdot 4H2O$	0.5 mg.

The foregoing was autoclaved for one hour at 15 lb./sq.in pressure; then the following, which had been sterilized by filtration through a porcelain bacteriological filter, was added.

$NaHCO_3$	2.0 g.
$NaS \cdot 9H_2O$	2.0 g.
Sodium lactate	2.0 g.
Distilled water	sufficient to dissolve

The pH was adjusted to 8.5 with phosphoric acid.

Growth occurred best in completely filled 8 oz. screw-capped bottles in an illuminated cabinet at 30°C. Maximum growth was attained in 6 to 8 days. Transfer cultures were prepared every 8 days in order to maintain healthy growth. Microscopic examination revealed no contaminants.

Experimental Procedures for Fractionation Studies

Chemical Oxidation of Sulphite

The sulphur isotope effect in the oxidation of sulphite to sulphate with molecular oxygen in aqueous solution has been studied. The effects of three variables, temperature, pH, and oxygen concentration, have also been examined.

The oxidations were carried out using pure sulphite prepared as previously described. The apparatus consisted of a 300 ml. three-necked flask fitted with a reflux condenser, gas bubbler and outlet tube. The latter, so arranged that it reached the bottom of the reaction flask, led directly into flask A of the separation apparatus shown in Figure 2. The gas bubbler had a sintered glass disc on the end for more effective dispersion of the incoming gases. Oxygen and nitrogen flow rates were measured with separate calibrated differential-pressure capillary flowmeters. Regulation was achieved by the combined action of bleeder valves and two-stage air reduction gauges. The reaction vessel was maintained at a constant temperature with a thermostated bath containing water for runs below 50°C. and oil at higher values.

An experimental run was carried out as follows. Approximately 10 g. of sodium sulphite in 100 ml. of water was prepared. A 5 ml. aliquot was taken for analysis while the rest was placed in the thermostated bath. When it had reached the desired temperature, the solution was poured into the reaction vessel through which oxygen and nitrogen were already flowing at the desired rates. The reaction was allowed to proceed to about 5 per cent completion. For this small an extent of reaction, the isotope ratio of the remaining reactant does not change appreciably. Hence the ratio $\frac{S^{32}/S^{34}\ \text{product}}{S^{32}/S^{34}\ \text{reactant}}$ could be taken as a direct measure of the ratio of the rate constants for the reaction. When the desired extent of reaction had been attained, the solution was forced by air pressure into the separation apparatus which contained boiling dilute hydrochloric acid. The separation of the two ions was fast, with virtually no further oxidation occurring. Quantitative and isotopic analyses were carried out as previously described.

Oxidation of Sulphur Compounds by Chromatium

Chromatium are capable of oxidizing sulphide to sulphur which is stored internally until the sulphide supply is exhausted. In the second step, the internally-stored sulphur is oxidized to sulphate.

The isotope fractionation in both these steps was studied. The first process was examined both in growing cultures and resting-cell suspensions, while the second stage was studied only in growing cultures.

For the experiments with growing cultures, several bottles of Medium 2 were prepared and inoculated at the same time. A sample of initial culture medium was taken for quantitative and isotopic analyses of the sulphide content. The cultures were incubated in the usual manner. Since the rate of oxidation was dependent on the illumination, care was taken to keep the bottles equidistant from the light source. After differing lengths of time, the bottles were removed and analyzed for sulphide or sulphate. Both quantitative and isotopic analyses were made. Since sulphate appeared in the medium only after the sulphide supply was exhausted, somewhat longer incubation times were required to obtain this product.

This method gave reasonably good results. The spread in values, however, was somewhat larger than desired. Also, previous work with the reducing bacteria (23) had shown that resting-cell techniques gave better control over several variables and hence more reproducible results. Therefore, further experiments on the bacterial oxidation of sulphide to sulphur were carried out using resting-cell suspensions.

Chromatium were harvested by centrifugation at 3200 r.p.m. (1000 x gravity) for 15 minutes from a suitable number of active, six-to-eight-day old cultures. The bacteria were rinsed once with 2.5 per cent solution, then suspended in a phosphate buffer solution of the following composition:

Medium 3.

KH_2PO_4	1.0 g.
$NaHCO_3$	2.0 g.
$Na_2S \cdot 9H_2O$	2.0 g.
$NaCl$	10.0 g.
Water	1000 ml.

The pH was adjusted to 8.0 - 8.5 with H_3PO_4. The relative population density was determined by measuring the optical density of a solution of the bacteria at 575 mu. with a Beckman model DU spectrophotometer. Eight-ounce, screw-capped bottles were completely filled with the cell suspensions. Incubation was carried out in an illuminated cabinet at 30°C. Quantitative sulphide determinations were made on the original medium and on each culture bottle after a suitable incubation period. The rate of oxidation was calculated for each suspension in terms of moles of substrate oxidized per hour per unit cell population, where unit cell population was 100 ml. of suspension with an optical density of 0.1. Isotope ratio analyses were made on each sulphide sample.

Mixed Culture Studies

In order to simulate more closely natural conditions, mixed cultures containing both reducing and oxidizing bacteria were grown and studied. Since the effects to be observed might have required long growth periods, only continuous culture methods appeared applicable.

The culture vessel used was a nine-litre glass jar with facilities for the aseptic addition of fresh medium and the removal of samples. The bottle was also fitted with a motor-driven stirrer and a bubbler through which pure nitrogen could be passed in order to sweep hydrogen sulphide from the medium. A thermostated water bath maintained the culture vessel at 30°C. Both the water bath and culture vessel had clear glass sides so that the medium could be illuminated by means of two 18 in. 15 watt fluorescent lamps.

The culture medium employed had been originally used by Butlin and Postgate (93) for similar mixed culture studies.

Medium 4.

Na_2SO_4	3.0 g.
Sodium lactate	2.0 g.
NH_4Cl	1.0 g.
KH_2PO_4	1.0 g.
$MgCl_2 \cdot 6H_2O$	0.5 g.
$CaCl_2 \cdot 6H_2O$	0.1 g.
$NaHCO_3$	2.0 g.
$NaCl$	10.0 g.
Difco yeast extract	1.0 g.
Trace elements (as in Medium 2)	10.0 ml.
Distilled water	1000 ml.

The pH was adjusted to 6.8 - 7.4 with sodium hydroxide. In one run to be reported, the Na_2SO_4 was replaced with 10 g. of $CaSO_4$. This gave a more constant supply of sulphate ion but introduced other complications which will be discussed later.

At the start of a run, eight litres of Medium 4 were autoclaved for 1 1/2 hours at 15 lb./sq.in. pressure. The culture bottle, after cooling overnight, was placed in the thermostated bath and inoculated with several millilitres of medium from healthy cultures of both D. desulphuricans and Chromatium. The fluorescent lamps were turned on and pure nitrogen was started bubbling through the medium. Buffered cadmium acetate absorber solution removed any hydrogen sulphide from the nitrogen stream. An increase in turbidity, indicative of growth, was evident after about 24 hours, but the first hydrogen sulphide appeared only after three to four days. By the end of another week, the culture became a deep reddish-purple, owing to the grosth of Chromatium.

A few days after active growth had become established, all connections to the culture vessel were clamped, thus isolating it completely. Periodically samples of the medium were removed for quantitative and isotopic analyses of the sulphide and sulphate present. After five to ten samples had been taken, fresh nutrient solution was added in order to maintain a sufficient volume. The fresh nutrient had the same composition as the original medium, except that extra sodium sulphate and lactate were sometimes added in order to maintain suitable concentrations, or to vary conditions. These continuous cultures were maintained in a healthy and active state for as long as three months.

One pure culture of D. desulphuricans was prepared and grown in exactly the same manner as the mixed cultures. Conditions were identical including the medium used, illumination, and methods of sampling and analyses.

The possible occurrence of growth factors in mixed cultures has also been examined. The possibility existed that one species of bacteria produced a substance which would affect the metabolism of the other species in such a way as to increase the isotope fractionation. Since they are more amenable to control, resting-cell suspensions were used in this work. These were prepared as outlined previously. Two slight differences in the preparation of D. desulphuricans should be noted. First, the optical density was determined at 525 mu., rather than at 575 mu. as for Chromatium; secondly, a different medium was used.

Medium 5.

K_2HPO_4	1.0 g.
Na_2SO_4	5.0 g.
Sodium lactate	1.0 g.
NaCl	10.0 g.
Distilled water	1000 ml.

As before, Medium 3 was used for Chromatium.

Cell-free medium from active cultures, henceforth called "used medium", was prepared by centrifuging the bacteria out of the cultures. The centrifugate was then passed through a bacteriological filter, resulting in an optically clean solution. In the case of "used" Chromatium medium, the hydrogen sulphide was removed at room temperature by placing the solution under reduced pressure produced by a water aspirator.

Suitable quantities of "used medium" were added to resting-cell suspensions of the appropriate bacteria. Suspensions of *Chromatium* were incubated and analyzed in the manner previously described for the fractionation studies. Erlenmeyer flasks, fitted with nitrogen bubblers and buffered cadmium acetate traps, contained the suspensions of *D. desulphuricans*. The temperature of 30°C. was maintained by placing the flasks in a thermostated water bath. Subsequent analyses were carried out in the usual manner.

Some further tests were conducted only with resting-cell suspensions of *D. desulphuricans*. To a series of suspensions, various combinations of nutrients were added. Some received "used" *Chromatium* medium; others were given equal quantities of fresh *Chromatium* medium; still others were kept as a control. After the first hydrogen sulphide samples had been produced, further additions were made. To flasks of each group were added ammonium chloride and yeast extract. These materials induced active growth. Further samples of sulphide and sulphate were taken from each suspension. Thus the combined effects of active growth and a possible growth factor could be observed. Also, the effect of the other nutrients in the "used" *Chromatium* medium could be eliminated.

Isotope Exchange between Sulphate and Sulphite

The isotope exchange reaction

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

has been studied at three temperatures, 25, 30 and 100°C. Both S^{34} enriched sulphate and materials with normal isotope abundances were employed.

S^{34} enriched sodium sulphate was prepared from enriched barium sulphate produced in this laboratory several years ago. The insoluble barium salt was converted to sulphur dioxide by burning it in a stream of oxygen in an electric furnace heated to 1400°C. The sulphur dioxide was removed from the gas stream with a dilute solution of sodium hydroxide. Subsequently, the sodium sulphite was oxidized to sulphate with hydrogen peroxide. The excess oxidant was destroyed by heating. The isotopic analysis for this material was carried out on a 180° single collection mass spectrometer.

Most of the exchange experiments were made in the same apparatus used for the sulphite oxidations. Known quantities of sulphite and sulphate in 100 ml. of water were placed in the reaction flask maintained at the desired temperature with a thermostated bath. Pure nitrogen was bubbled through the solution at a moderate rate. In the runs at 100°C. the amounts of sulphate and sulphite were about the same as encountered in the oxidation studies; that is, about 10 g. of sodium sulphite and 200 mg. of sodium sulphate. In the rest, about 200 mg. of each salt was used. Enriched sulphate and normal sulphite were used in the run at 25°C. only. After suitable reaction times, separations and analyses were carried out, as previously described.

Several experiments were conducted in which a resting-cell suspension of D. desulphuricans acted as a catalyst for the sulphate-sulphite exchange reaction. Medium 5 was used with slight modifications. The sodium lactate was eliminated, and about 3 g. of sulphite added. The sulphate concentration was reduced to about 1 g./l. Most of the runs were made in 8 oz. sealed bottles in a cabinet maintained at 30°C. In a few cases, 500 ml. Erlenmeyer flasks were used. These were fitted with nitrogen bubblers and buffered cadmium acetate traps. Temperature regulation was achieved with a constant temperature bath. The resting-cell suspensions of D. desulphuricans were prepared in the usual manner. After a suitable reaction period, of the order of days, the sulphur compounds were separated and analyzed in the prescribed manner.

RESULTS AND DISCUSSION

Chemical Oxidation of Sodium Sulphite

The sulphur isotope effect in the oxidation of aqueous sodium sulphite has been studied under a variety of conditions. Data showing the effect of temperature are given in Table II. The observed values of the isotope effect for several runs at each temperature are reported. Since the extent of reaction was less than 5 per cent in each case, the ratio $\frac{S^{32}/S^{34}{}_{SO_4^=}}{S^{32}\ S^{34}{}_{SO_3^=}}$ is a direct measure of the ratio of the overall rate constants k_1/k_2 for the reactions

$$S^{32}O_3^= + [O] \xrightarrow{k_1} S^{32}O_4^=$$

$$S^{34}O_3^= + [O] \xrightarrow{k_2} S^{34}O_4^=$$

Hence a number less than unity for this ratio indicates that the heavy isotope is reacting faster than the light. This is the case for the present reaction under all conditions studied. The fractionation factor in Table II increases from 0.9947 to 0.9975 in a regular manner as the temperature increases from 12°C. to 100°C. Since the precision of the experimental techniques leads to a reproducibility of better than ± 0.1 per cent in the fractionation factor for the average of several runs, the observed temperature co-efficient is definitely significant. Clearly, therefore, in the oxidation of sulphite to sulphate with molecular oxygen there is a sulphur isotope effect with S^{34} concentrating

TABLE II

Chemical Oxidation of Sulphite to Sulphate
Effect of Temperature on Isotope Fractionation

Run No.	Temperature °C.	S^{32}/S^{34}		Fractionation Factor	
		$SO_3^=$	$SO_4^=$	Observed	Average
17	12.1	22.190	22.056	0.9940	
18	12.2	22.195	22.056	0.9937	
47	12.5	22.232	22.144	0.9960	
48	12.5	22.230	22.119	0.9950	0.9947
14	25.0	22.022	21.911	0.9950	
15	25.0	22.190	22.095	0.9957	0.9954
19	38.0	22.175	22.075	0.9955	
20	37.8	22.190	22.090	0.9955	0.9955
21	49.7	22.175	22.095	0.9964	
22	49.7	22.208	22.119	0.9960	0.9962
23	62.3	22.208	22.080	0.9942	
24	61.8	22.210	22.173	0.9983	
41	62.5	22.212	22.134	0.9965	
42	62.5	22.212	22.168	0.9980	0.9968
25	74.5	22.202	22.109	0.9958	
26	75.0	22.195	22.139	0.9975	
39	75.0	22.207	22.153	0.9976	
40	75.0	22.210	22.153	0.9974	0.9971
28	87.5	22.200	22.139	0.9973	
38	87.5	22.212	22.153	0.9973	0.9973
30	100.0	22.200	22.141	0.9973	
35	99.5	22.219	22.163	0.9975	
36	99.5	22.207	22.153	0.9976	0.9975

in the sulphate. Furthermore, the effect is temperature dependent since it varies from 0.53 per cent to 0.25 per cent as the temperature increases from 12°C. to 100°C. This is an average temperature co-efficient of 0.003 per cent/° C.

The effects of oxygen partial pressure and pH of the solution were also examined. The results are tabulated in Tables III and IV. These data indicate that neither variable has an appreciable effect on the fractionation factor.

The observed isotope fractionation may, of course, be due to a kinetic effect, an equilibrium effect, or a combination of both. In this connection, Voge (120) has reported that no exchange occurs between sulphate and sulphite ions in an aqueous solution. These experiments have been repeated under the conditions of the sulphite oxidation experiments. The results, which will be presented in the next section of this thesis, confirm those of Voge.

There is, however, the further possibility that an equilibrium exchange reaction occurs between the reactant and an intermediate product of the oxidation which would wholly or in part mask a true kinetic isotope effect. In order to determine if such is the case, certain theoretical calculations of isotope effects have been made. Abel (121) has postulated the following reaction sequence for the oxidation:

$$SO_3^{=} + O_2^{-} \rightleftharpoons SO_3^{-} + 2O^{-} \qquad (1)$$

$$SO_3^{-} + H^{+} \rightleftharpoons HSO_3 \qquad (2)$$

$$HSO_3 + OH \longrightarrow SO_4^{=} + 2H^{+} \qquad (3)$$

TABLE III

Chemical Oxidation of Sulphite to Sulphate
Effect of Oxygen Partial Pressure on Isotope Fractionation

Run No.	O_2 Partial Pressure (atmos.)	S^{32}/S^{34}		Fractionation Factor	
		$SO_3^=$	$SO_4^=$	Observed	Average
43	0.1	22.183	22.085	0.9956	
44	0.1	22.178	22.099	0.9964	0.9960
17	0.5	22.190	22.056	0.9940	
18	0.5	22.195	22.056	0.9937	
47	0.5	22.232	22.144	0.9960	
48	0.5	22.230	22.119	0.9950	0.9947
49	1.0	22.225	22.119	0.9952	
50	1.0	22.217	22.134	0.9963	0.9956

N.B.: Temp. = 10.1°C.

TABLE IV

Chemical Oxidation of Sulphite to Sulphate
Effect of pH on Isotope Fractionation

Run No.	pH	S^{32}/S^{34}		Fractionation Factor	
		$SO_3^=$	$SO_4^=$	Observed	Average
17	7.0	22.190	22.056	0.9940	
18	7.0	22.195	22.056	0.9937	
47	7.0	22.232	22.144	0.9960	
48	7.0	22.230	22.119	0.9950	0.9947
45	10.0	22.180	22.090	0.9960	
46	10.0	22.163	22.075	0.9960	0.9960

N.B.: 10.1°C.

Steps (1) and (2) involving simple electron and proton transfers are probably very rapid while Step (3) is rate-controlling. Since proton addition has very little effect on the bonding at the sulphur atom, Step (2) should have a negligible isotope effect. Both steps (1) and (3), however, may lead to appreciable isotope effects. The former would be an equilibrium isotope effect involving exchange between sulphite ion and sulphite radical, whereas the latter would be a kinetic isotope effect in a bond-formation reaction. The overall isotope fractionation would then depend on the extent to which Step (1) and Step (3) are rate-controlling.

There are three different assumptions which may be made in this regard. The first assumption is that the equilibrium between sulphite ion and sulphite radical is rapidly established and that this exchange is the sole cause of the isotope effect. In this case the partition function ratio for sulphite radical must be calculated. It may be obtained from the approximate relationship

$$Q'_{SO_3^-} = (Q'_{SO_3^=} \cdot Q'_{SO_3})^{\frac{1}{2}}$$

The second assumption is that the equilibrium in Step (1) is slow and that a kinetic isotope effect occurs in Step (3). Calculation of a kinetic isotope effect necessitates a knowledge of the free energy term for the transition state in the Bigeleisen equation (equation 15, theoretical section). This is not generally known. If, however, the assumption is made that the transition states for reduction and oxidation are identical, then calculations can be made since Harrison (23) has studied the kinetic isotope effect in the reduction of sulphate in considerable detail. From his data the free energy term for the transition state can easily be obtained. This term may then be used in the Bigeleisen equation in order to calculate the kinetic isotope effect in the oxidation processes. Of course, the transition states for the oxidation and reduction reactions will only be identical if the reaction mechanisms are the same for both processes. In view of the difference in reaction conditions, this assumption may not be very accurate. It is, however, the best that can be made.

The third assumption is that the equilibrium is rapid, resulting in an equilibrium isotope effect which is then superimposed on a kinetic isotope effect in Step (3). The overall effect in this case would be the product of the equilibrium isotope effect in Step (1) and the kinetic isotope effect in Step (3).

Calculations of isotope effect have been made for all three cases. Values of Q' for sulphite, sulphur trioxide and sulphate at 0°C. have been given previously (19, 23). Calculations for these quantities at 50°C. and 100°C. are given in the Appendix. Table V gives the observed isotope effect at 0°C., 50°C. and 100°C., together with the calculated values based on the three assumptions.

TABLE V

Chemical Oxidation of Sulphite to Sulphate
Calculated and Experimental Values for the Isotope Effect

Temp. °C.	Fractionation Factor			
	Observed	Calculated		
		Assumption I	Assumption II	Assumption III
0	0.9947	0.987	1.007	0.994
50	0.9962	0.991	1.008	0.999
100	0.9975	0.992	1.011	1.003

The data of Table V indicate clearly that the third assumption leads to a theoretical value of the isotope effect which is closest to the observed value, especially at 0°C. The calculated temperature co-efficient is larger than that actually observed, but this is not surprising in view of the assumptions made. In fact, Harrison (23) observed no temperature co-efficient at all in the reduction process, although one was predicted by the theory. From these considerations, therefore, the isotope effect in the oxidation of aqueous sodium sulphite with molecular oxygen appears to be due to a combination of a fast equilibrium exchange reaction between sulphite ion and sulphite radical in the first step of the oxidation, combined with a kinetic isotope effect favouring the S^{32} in the subsequent oxidation of sulphite radical to sulphate.

Isotope Exchange between Sulphate and Sulphite

Chemical Exchange

The possible isotope exchange reaction between sulphate and sulphite ions in neutral, aqueous solution had been previously investigated by Voge (120). No exchange was observed. These data were, however, not entirely conclusive. Consequently, the following investigation was conducted.

Two experimental methods were employed. In one, S^{34} enriched sulphate was used. The tracer was added as sulphate rather than sulphite since the latter could easily be oxidized by oxygen impurities, thus masking a possible exchange reaction. After equilibrium, the sulphate and sulphite were separated and analyzed. Isotope exchange would result in an enrichment of the sulphite in S^{34}. In the second method sulphate and sulphite with identical, normal sulphur isotope abundances were employed. As before, the sulphate and sulphite were mixed for a suitable period of time, after which they were separated and analyzed. In the case of exchange the $S^{32}/S^{34}{}_{SO_3^=}/S^{32}/S^{34}{}_{SO_4^=}$ ratio would approach the calculated equilibrium constant for the reaction

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

This equilibrium exchange constant was calculated to be 1.024 at 25°C. (23). On the other hand, the isotope ratios for sulphate and sulphite would not change in the absence of exchange.

Data for experiments at three temperatures, 24.9°C., 30.0°C. and 100°C., are given in Table VI. S^{34} enriched sulphate was used only in the run of 24.9°C. In this case the sulphate was analyzed only at the end of the experiment while both initial and final samples of sulphite were analyzed. In runs of the other temperatures both components were analyzed before and after mixing.

TABLE VI

Chemical Exchange between Sulphate and Sulphite

Temp. °C.	Time hours	Sulphur Compound	S^{32}/S^{34}		Fractionation Factor	
			Initial	Final	Initial	Final
24.9	2.0	$SO_4^=$	6	6.515	3.7	3.34
		$SO_3^=$	22.163	21.768		
30.0	191.6	$SO_4^=$	22.193	22.188	1.0012	1.0020
		$SO_3^=$	22.217	22.232		
100.0	1.5	$SO_4^=$	22.227	22.222	1.0002	1.0002
		$SO_3^=$	22.228	22.222		

The data for 100°C. are an average of three runs. Hence the precision is somewhat better than ± 0.1 per cent in the fractionation factor.

In the run with S^{34} enriched sulphate at 24.9°C., a decrease of 1.8 per cent in the isotope ratio of the sulphite was observed. Complete exchange, however, would have resulted in a 40 per cent decrease. Thus at most there has been only 5 per cent exchange in the period of the experiment and this small amount could be due to slight contamination of the sulphite with S^{34}-enriched sulphate during the preparation of the samples.

The runs at 30°C. and 100°C. also show no isotope exchange. The fractionation factors before and after equilibration show no appreciable change. These results then are in complete accord with those of Voge (120); that is, no significant isotope exchange occurs between sulphate and sulphite ions in neutral, aqueous solution.

Bacteria-catalyzed Exchange

The possibility of catalyzing the isotope exchange reaction between sulphate and sulphite with the sulphate reductase in resting-cell suspensions of D. desulphuricans was investigated. Unfortunately, side-reactions tended to obscure the exchange. Even though no carbon source was present, the bacteria slowly produced hydrogen sulphide from the sulphate and sulphite. Also, sulphite became oxidized to sulphate. Whether this occurred by strictly chemical action of oxidizing impurities, or by bacterial action, or by a combination of the two could not be ascertained. Owing to these side-reactions, the concentrations, as well as the isotope ratios of the sulphite and sulphate, were measured in an effort to establish whether exchange in fact occurred. A further complication arose when, in the early runs, some sulphur could not be recovered. Eventually, this loss was attributed to the formation of thiosulphate from sulphide and sulphite under the influence of the bacterial enzymes. Fortunately, calculations indicated that the unrecovered sulphur in every case had the isotope ratio to be expected for sulphide produced by bacterial reduction of the sulphate or sulphite.

Data for three groups of experiments are given in Table VII. The three series, A, B and C, differed mainly in the population densities of the resting-cell suspensions of the bacteria. In all cases the initial sulphate and sulphite isotope ratios differed by less than 0.1 per cent. Columns three and four in Table VII give the per cent change in the S^{32}/S^{34} ratio for the sulphate and sulphite, respectively, at the end of each run. The fifth column contains the data for the per cent difference between the isotope ratio of the initial sulphite and the by-product sulphide or sulphur. In the sixth column are listed the calculated per cent depletions in S^{34} of the unrecovered sulphur. The last two columns contain the values of the fractionation factors which resulted between sulphite and sulphate, and sulphide and sulphate.

The data in Table VII clearly indicate that some isotope exchange occurred between sulphate and sulphite in the presence of bacterial enzymes. That this is the case may be seen from the following considerations.

First, in each case the sulphate became enriched in the heavy isotope while the sulphite was depleted.

Secondly, the sulphate became much more enriched in S^{34} than can be accounted for by assuming that the oxidation of the sulphite to sulphate in one of the side-reactions involved an isotope effect similar to that observed in the chemical oxidation of sulphite. At 30°C. this

TABLE VII

Bacteria-catalyzed Exchange between Sulphate and Sulphite

Run No.	Time hours	Per cent change in S^{32}/S^{34} ratio				Fractionation Factor	
		$SO_4^=$	$SO_3^=$	$S^=$	Unrecovered	$SO_3^=/SO_4^=$	$S^=/SO_4^=$
A - 1	65.0	+ 1.15	- 0.36	- 2.88	- 4.30	1.0151	1.0403
B - 1	17.6	+ 0.35	- 0.07	---	- 0.66	1.0042	---
B - 2	27.7	+ 0.31	- 0.13	+ 0.22	- 0.43	1.0044	1.0009
B - 3	40.8	+ 0.49	- 0.05	- 0.31	- 1.89	1.0054	1.0080
B - 4	64.8	+ 0.64	- 0.22	- 1.39	- 0.93	1.0086	1.0203
B - 5	93.8	+ 0.71	- 0.18	- 1.80	- 1.34	1.0089	1.0251
C - 1	68.5	+ 0.73	- 0.22	---	- 1.38	1.0096	---
C - 2	92.3	+ 0.64	- 0.56	---	- 0.44	1.0120	---
C - 3	115.5	+ 0.71	- 0.36	- 3.14[a]	- 0.40	1.0107	1.0387

[a] This sample was elemental sulphur.

Note: Optical Densities

Series A	1.82
Series B	0.33
Series C	0.66

isotope effect has the value of about 0.5 per cent. Assuming that, from an infinite reservoir, a quantity of sulphite equal to the sulphate originally present were oxidized in a reaction with this 0.5 per cent isotope effect, the total sulphate after reaction would be enriched by only 0.25 per cent. Obviously in the actual experiments the enrichment from this source would be considerably less.

A third consideration is the possible effect of the reduction process on the isotope ratios of the sulphite and sulphate. Since the reduction under these conditions involves an isotope effect of about 2 per cent, an appreciable change in the isotope ratio of the reactant would be expected. Most workers agree, however, that sulphite is reduced more rapidly than sulphate by these bacteria (23, 107), whereas the data in Table VII indicate that it was the sulphate which was becoming depleted in S^{32}.

The last consideration concerns the effect of the other side reaction, that is, the formation of thiosulphate, on the equilibrium process. Ames and Willard (122) have shown that thiosulphate and sulphite undergo an exchange reaction involving the central sulphur atom of the former, but that the two sulphur atoms in thiosulphate do not exchange with each other. Hence the formation of thiosulphate should have little or no influence on the sulphate-sulphite exchange.

In view of these four considerations, the only explanation for the observed fractionation which occurs between sulphate and sulphite in the presence of resting-cell suspensions of D. desulphuricans is that some isotopic exchange takes place, catalyzed by the bacterial enzymes.

The maximum fractionation factor obtained between sulphate and sulphite was 1.0151, comparied with the theoretical value of 1.025. This discrepancy is probably due to the reduction of the sulphite which would tend to enrich this ion in S^{34} while the exchange process would act in the opposite direction. Probably better agreement between the experimental and calculated exchange constants would be observable if this side-reaction could be completely blocked.

Oxidation of Sulphur Compounds by *Chromatium*

Chromatium oxidize sulphide to sulphate in two steps. In pure cultures, as long as sulphide is present in the culture medium, oxidation proceeds only as far as sulphur which is stored internally in the form of granules. When the supply of sulphide is exhausted, the internally-stored sulphur is oxidized through a second reaction sequence to sulphate. The first step has been examined for the possible occurrence of sulphur isotope effects in both growing cultures and resting-cell suspensions. Only growing cultures, however, have proven convenient for studying sulphur isotope fractionation in the second part of the process.

The isotope fractionation data for the oxidation of sulphide to sulphur in growing cultures are shown in Table VIII. Analyses from two series of cultures are included. They are arranged in order of increasing per cent oxidation as shown in the third column. The fractionation factor in the fourth column is the ratio of the rate constants for the two reactions:

$$S^{32=} \xrightarrow{k_1} S^{32^\circ} + 2e^-$$
$$S^{34=} \xrightarrow{k_2} S^{34^\circ} + 2e^-$$

TABLE VIII

Bacterial Oxidation of Sulphide to Sulphur Fractionation in Growing Cultures

Sulphide Sample No.	Time hours	Oxidation per cent	Fractionation k_1/k_2
A - 1	61.0	9.2	1.0000
A - 2	132.2	17.6	0.9964
A - 3	113.7	19.2	1.0011
A - 4	85.2	20.0	0.9990
A - 5	111.5	46.9	1.0007
B - 1	117.5	63.1	1.0037
A - 6	179.8	64.9	0.9991
B - 2	144.5	84.6	1.0001
B - 3	244.7	87.3	0.9964
B - 4	167.5	91.1	0.9999
A - 7	204.5	93.3	1.0011
B - 5	259.7	97.0	1.0005

The ratio k_1/k_2 was calculated from a knowledge of the extent of reaction and the isotope ratios for initial and unreacted sulphide using the equation:

$$\left(\frac{k_2}{k_1} - 1\right) = \frac{\log p}{\log(1 - f)}$$

where f is extent of reaction and p is isotope ratio of initial material divided by the isotope ratio of unreacted material.
From the data for k_1/k_2 in Table VIII there appears to be no sulphur isotope fractionation in the oxidation of sulphide to sulphur by Chromatium.

With growing cultures, however, reproducible conditions are difficult to obtain since each culture must be inoculated separately. Therefore, further studies of this initial oxidation step were made using resting-cell suspensions of Chromatium. With this technique conditions within a given series of suspensions were more nearly identical while, in addition, the results obtained with different series of samples could be compared more directly.

The four series of experiments were carried out with resting-cell suspensions of Chromatium in order to determine the isotope fractionation, if any, in the oxidation of sulphide to internally-stored sulphur. The results are reported in Table IX.

As before the analyses are arranged in order of increasing extent of reaction. Also included in the table are the optical densities and rate of oxidation. The latter are expressed as moles substrate oxidized per hour per unit cell population where unit cell population is taken as 100 ml. of suspension with an optical density of 0.10

TABLE IX

Bacterial Oxidation of Sulphide to Sulphur Fractionation in Resting Cell Suspensions

Sulphide Sample No.	Time hours	Oxidation per cent	Optical Density	Rate[a] x 10^6	Fractionation k_1/k_2
B - 1	51.5	23.8	0.59	0.90	1.0051
A - 1	21.5	29.9	0.72	0.83	1.0007
B - 2	92.8	31.5	0.54	0.29	1.0067
A - 2	44.0	36.9	0.72	0.51	1.0030
A - 3	73.3	38.3	0.72	0.32	1.0005
D - 1	24.7	44.7	0.56	1.89	1.0000
B - 3	73.5	57.0	0.59	0.67	1.0000
B - 4	194.2	58.7	0.59	0.26	0.9964
B - 5	122.8	67.9	0.59	0.48	0.9988
B - 6	147.0	68.7	0.59	0.40	1.0014
C - 1	50.3	75.8	1.11	0.58	1.0005
D - 2	95.2	78.4	0.56	0.69	1.0011
C - 2	42.0	79.3	1.11	0.72	1.0000
A - 4	93.7	87.7	0.72	0.59	1.0003
C - 3	69.3	91.6	1.11	0.50	0.9978

[a] Moles hydrogen sulphide oxidized per hour per unit cell population

These are the same units previously employed by Harrison (23) in his studies of *D. desulphuricans* so that direct intercomparisons are possible. The fractionation factor given in the last column is again the ratio of rate constants for the oxidation of the two sulphur isotopes S^{32} and S^{34}. The data indicate little or no isotope effect. As expected, the results show less variation than those in Table VIII for growing cultures. From these data it appears that the overall mechanism of the oxidation of sulphide to internally-stored sulphur by *Chromatium* is such that isotope fractionation does not occur.

The oxidation of internally-stored sulphur to sulphate has been studied only in growing cultures. The data are given in Table X. Here the fractionation factor is the ratio of the rate constants for the reactions:

$$S^{32} + 4\,[O] \xrightarrow{k_1} S^{32}O_4^{=}$$

$$S^{34} + 4\,[O] \xrightarrow{k_2} S^{34}O_4^{=}$$

The values of k_1/k_2 were obtained from the isotope ratios of initial sulphur and product sulphate and extent of reaction using the well known relationship

$$\frac{k_1}{k_2} = \frac{\log(1 - f)}{\log(1 - rf)}$$

where f is extent of reaction and r is isotope ratio of initial material divided by isotope ratio of product.

In this step also there appears to be no appreciable isotope effect. Hence in the overall oxidation of sulphide to sulphate by

TABLE X

Bacterial Oxidation of Sulphur to Sulphate
Fractionation in Growing Cultures

Sulphate Sample No.	Time hours	Oxidation per cent	Fractionation k_1/k_2
A - 1	118.5	8.3	0.9998
A - 2	117.0	15.7	0.9978
A - 3	143.6	24.6	0.9985

Chromatium no sulphur isotope fractionation occurs.

Similar results have been previously reported (98, 101) for the oxidation of elemental sulphur to sulphate by species of *Thiobacillus*. Since no biological oxidation process studied to date does lead to sulphur isotope fractionation, the reduction processes are apparently responsible for most of the fractionation which occurs in nature.

Mixed Culture Studies

Experiments with mixed cultures of the oxidizing bacteria, *Chromatium*, and reducing bacteria, *D. desulphuricans*, were conducted in an attempt to simulate the natural sulphur cycle in a controlled manner. Data for two such runs, differing slightly in detail, are reported and discussed here. Also given are the results for a continuous pure culture of *D. desulphuricans* which was studied for purposes of comparison. The pertinent data for the preliminary experiment designated Mixed Culture A, including the apparent fractionation between sulphate and sulphide and the concentration of these two compounds, are displayed in Figure 4. In this experiment, calcium sulphate was used as the sulphate source. Because of the flocculent nature of the solid calcium sulphate, the culture vessel could not be agitated since the increase in turbidity reduced the amount of light available for the *Chromatium* too severely. Since the medium could not be agitated, the concentration of sulphate ion in solution, which is reported in Figure 4, was usually somewhat less than the saturation value. During periods of active growth, the sulphate concentration dropped markedly. For the first 32 days nitrogen was bubbled slowly through the culture vessel, but the flow was discontinued thereafter. The apparent fractionation factor is given by the ratio $\dfrac{S^{32}/S^{34}{}_{S^{=}}}{S^{32}/S^{34}{}_{SO_4^{=}}}$ for sulphide and sulphate removed from the same sample of culture medium.

Figure 4 shows the apparent fractionation factor rose rapidly during the first ten days of the experiment. A maximum value of 1.021 was reached. Growth became less and less vigorous from the 18th to the 22nd day. On the 22nd day fresh medium including 0.9 g. of ferrous sulphate was added. Biological activity increased markedly. This was reflected in a rise of the sulphide concentration and a drop in the sulphate concentration. The apparent fractionation factor also rose between the 25th and 35th day from a value of 1.014 to 1.018, finally becoming almost constant at a value of 1.017. This is somewhat higher than the values reported by previous workers for the unidirectional reduction of sulphate to sulphide by pure cultures of reducing bacteria

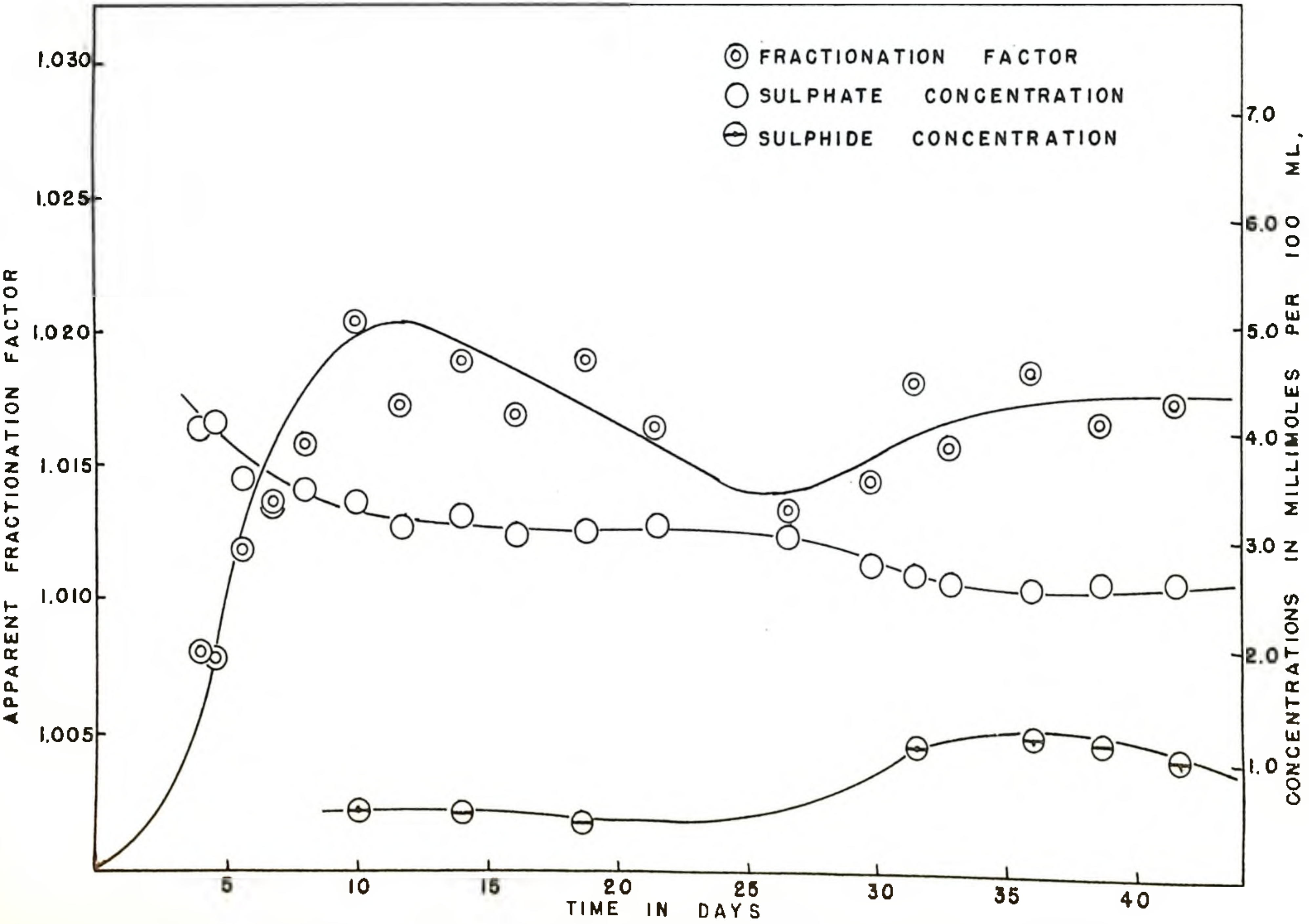


FIGURE 4 FRACTIONATION OF SULPHUR COMPOUNDS IN MIXED CULTURE A

under similar conditions but is very much smaller than the calculated equilibrium constant for the exchange reaction

$$H_2S^{34} + S^{32}O_4^{=} \rightleftharpoons H_2S^{32} + S^{34}O_4^{=}$$

which is 1.070 at 25°C. (23).

The technical difficulties inherent in using a solid substrate led to a further experiment in which a soluble salt was used as the sulphate source. In this run, designated as Mixed Culture C, the medium could be stirred prior to sampling, without the deleterious effects previously incurred. Stirring resulted in more uniform conditions throughout the culture vessel, as well as ensuring more representative samples. Nitrogen flow was halted after the first sample of medium had been taken. Otherwise, the methods were identical with those for Mixed Culture A.

For the first twenty days the results, displayed in Figure 5, were very similar to those of Mixed Culture A. The apparent fractionation factor rose rapidly to a maximum value of 1.019, then started to fall slightly. At the 19th day fresh medium was added which contained 50 ml. of 60 per cent sodium lactate solution and extra quantities of trace elements and ammonium chloride. This resulted in a rapid increase in the metabolism of the bacteria which rapidly depleted the supply of sulphate and increased the sulphide concentration in the culture. During the same period the apparent fractionation factor rose rapidly to a maximum value of 1.031. On the 30th day further fresh medium was added. The addition at this time of fresh sulphate with a higher isotope ratio than that remaining in the medium decreased the apparent

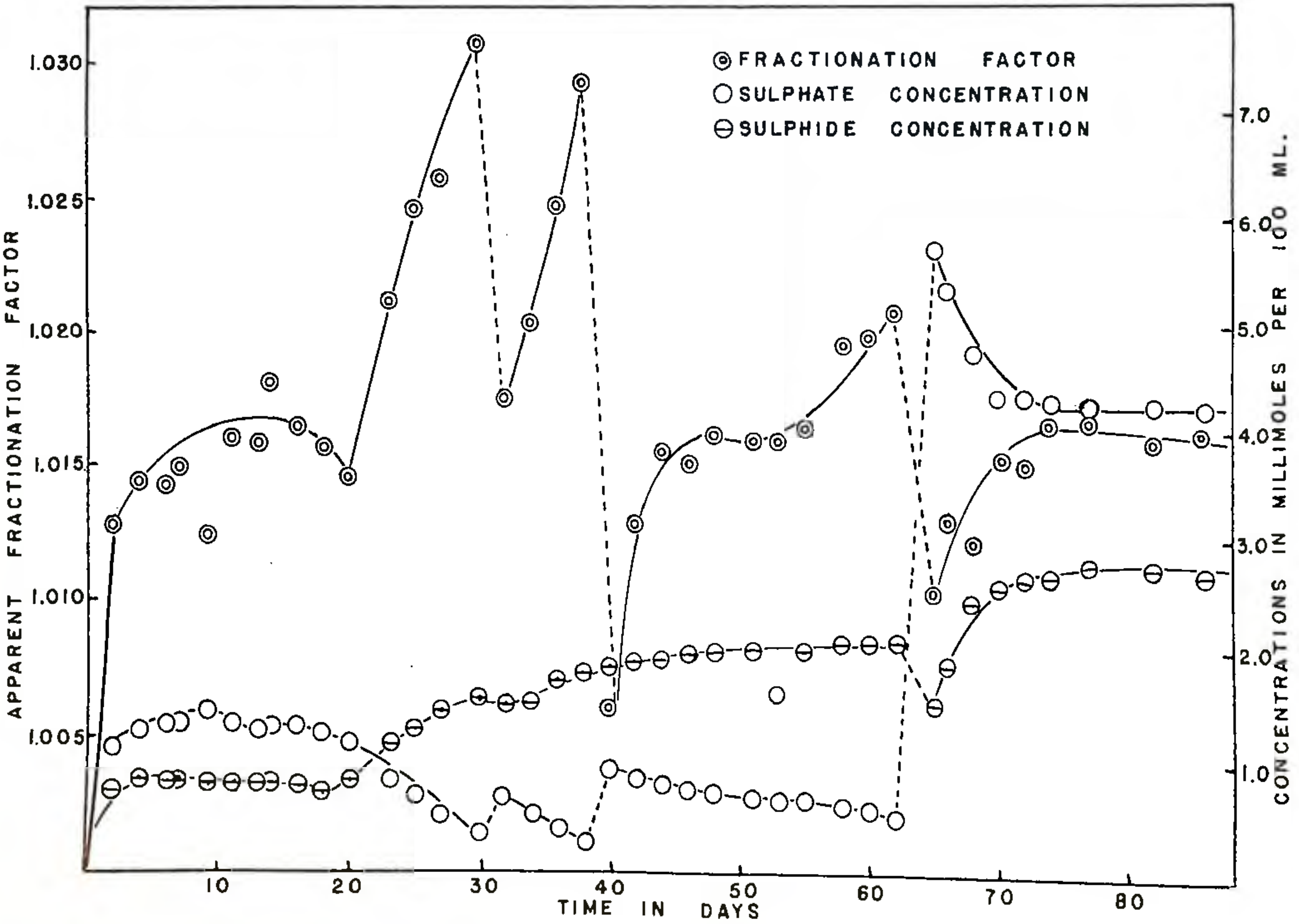


FIGURE 5 FRACTIONATION OF SULPHUR COMPOUNDS IN MIXED CULTURE C

fractionation factor markedly as shown by the dotted lines in Figure 5. During the next seven days, the same results were observed as in the period immediately previous.

From the 41st to the 56th day, a fairly steady state obtained. The sulphate and sulphide concentrations remained fairly constant while the apparent fractionation factor reached a constant value of 1.016. A further addition of fresh medium on the 64th day, this time with a high concentration of sulphate, again resulted in a period of rapid metabolism during which the sulphate concentration dropped markedly. In this case. however, the apparent fractionation factor did not go above 1.016 compared with the value of 1.030 which was reached in the two previous periods of rapid growth. After the 72nd day the apparent fractionation factor again assumed a nearly constant value of 1.016.

The large rise in the fractionation factor during the first two periods of rapid growth was almost entirely due to the enrichment of the sulphate in S^{34}, as the sulphate concentration decreased. This can be clearly seen from the data in Table XI. From the 20th to the 30th day, the isotope ratio of the sulphate decreased by about 2 per cent, while during the same period this ratio for the sulphide decreased by only 0.5 per cent. Similar results are apparent for the second period from the 32nd to the 38th day.

From the 65th to the 74th day, the sulphate concentration again decreased rapidly during a period of rapid metabolism. In fact, during this period the absolute decrease in concentration was larger than in either of the previous two. The relative decrease, however,

TABLE XI

Data for Mixed Culture B
during three periods of rapid growth

Day	Sulphate		Sulphide		Apparent Fractionation Factor
	Concentration mM/100 ml.	S^{32}/S^{34}	Concentration mM/100 ml.	S^{32}/S^{34}	
Period 1					
20	1.22	21.853	0.88	22.173	1.0146
23	0.89	21.753	1.20	22.212	1.0211
25	0.72	21.570	1.32	22.099	1.0245
27	0.54	21.542	1.52	22.095	1.0257
30	0.37	21.417	1.65	22.075	1.0307
Period 2					
32	0.68	21.716	1.55	22.095	1.0175
34	0.56	21.695	1.58	22.085	1.0203
36	0.42	21.524	1.78	22.056	1.0247
38	0.29	21.390	1.83	22.017	1.0293
Period 3					
65	5.75	21.906	1.52	22.129	1.0102
66	5.37	21.877	1.90	22.158	1.0128
68	4.78	21.877	2.46	22.139	1.0120
70	4.37	21.829	2.57	22.158	1.0151
72	4.38	21.825	2.69	22.148	1.0148
74	4.30	21.786	2.69	22.143	1.0164

was much smaller. Hence the sulphate isotope ratio decreased by only 0.5 per cent.

From these data it appears that the large apparent fractionation factors observed during periods of rapid metabolism were due to the enrichment of the sulphate in S^{34} by a unidirectional reduction process with a large isotope effect. In order to determine the value of the isotope effect which would lead to the observed enrichments of the residual sulphate, certain calculations were made. The equation relating extent of reaction and isotope ratios of initial and unreacted substrate was used. From the data of Table XI, values of log p and log(1 - f) were obtained and plotted as shown in Figure 6. By the method of least squares the best straight line was found to have a slope of 0.0177 indicating that the isotope effect was 1.77 per cent during the three periods of rapid growth. This is virtually the same as the values found during steady state conditions.

The results of these two mixed culture experiments are self-consistent. There is no evidence that isotopic equilibrium is being established between sulphate and sulphide as a result of the sulphur cycle. Moreover, the isotope fractionation which is observed can be explained by a 1.8 per cent isotope effect in the bacterial reduction of sulphate to sulphide.

Previous work (97, 98) has indicated that, under similar conditions, the bacterial reduction of sulphate by D. desulphuricans in pure cultures, gives a kinetic isotope effect of the order of 1 per cent rather than 1.8 per cent. More recently, however, Harrison (23) and Jones and Starkey (101) have shown that this isotope effect

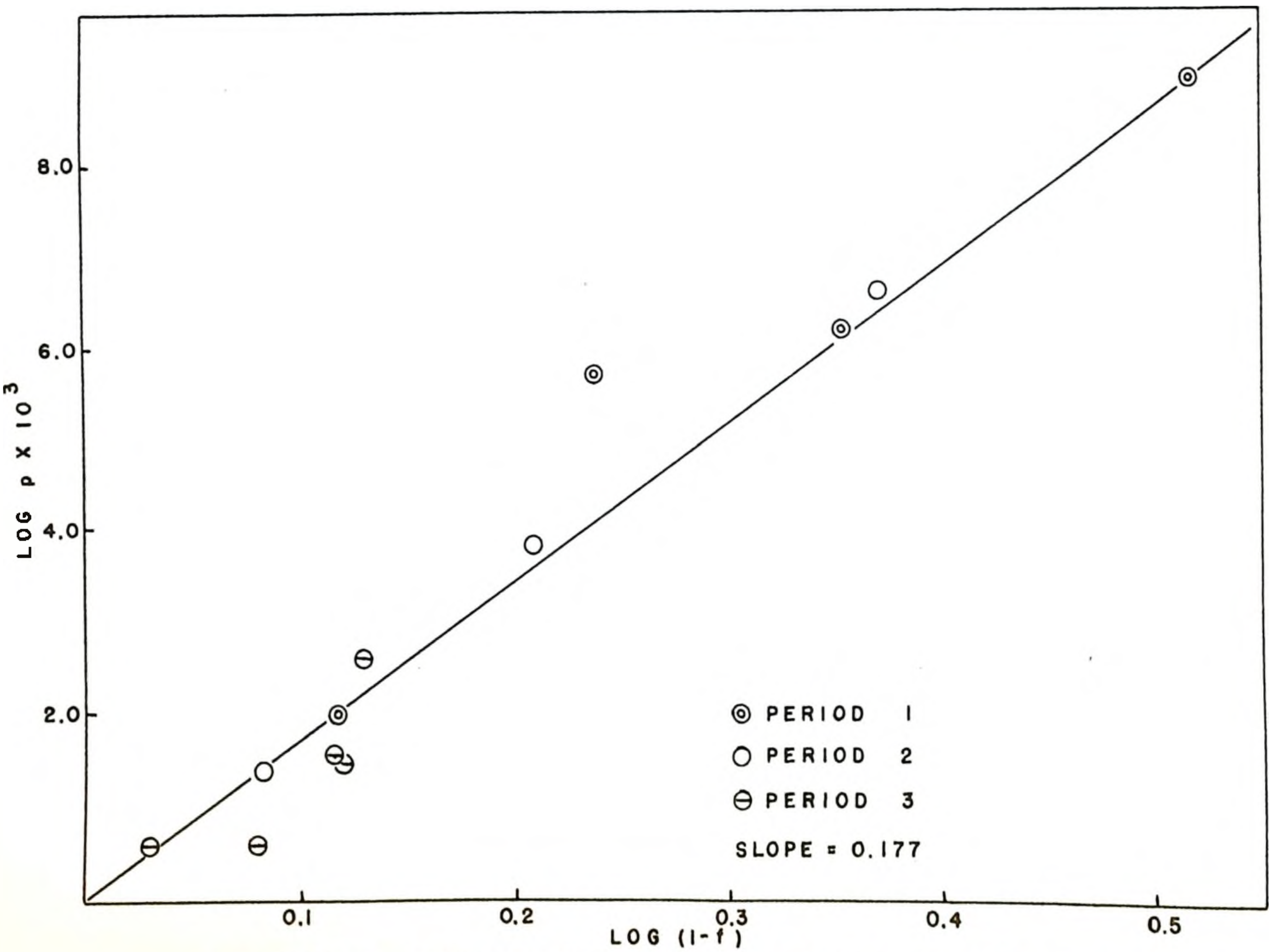


FIGURE 6 ISOTOPE EFFECT FROM MIXED CULTURE C

can vary from 0 to 2.5 per cent, depending on the rate of metabolism. Low metabolic rates lead to high isotope effects, while at high metabolic rates low isotope effects are observed. Because of this relationship, it seemed possible that the *Chromatium* in the mixed cultures were producing a metabolic end-product which would slow the metabolism of the *D. desulphuricans*, thus increasing the kinetic isotope effect in the reduction process to the value of 1.8 per cent which was observed. This possibility has been investigated. Although it seemed unlikely, the alternate possibility that the *D. desulphuricans* were influencing the *Chromatium* in such a way as to lead to an isotope effect in the oxidation part of the cycle was also investigated.

In order to test this latter possibility, "used" cell-free medium from cultures of *D. desulphuricans* was added to resting-cell suspensions of *Chromatium*. Two suspensions with no "used" medium added were kept as controls. Only the first step in the oxidation, that is, from sulphide to internally-stored sulphur, was studied. The results are tabulated in Table XII. Samples 1 and 2 are the controls, while numbers 3, 4 and 5 had "used" medium added. The fractionation factor in each case does not differ significantly from unity. The rates of oxidation, although somewhat erratic, are in the same range as previously found for resting-cell suspensions of *Chromatium* (see Table IX). The not unexpected conclusion from this experiment is that *D. desulphuricans* do not produce a growth factor which influences the metabolism of *Chromatium* in such a way as to produce an isotope effect.

TABLE XII

Bacterial Oxidation of Sulphide to Sulphur
Effect of Growth Factor from D. desulphuricans on
Resting Cell Suspensions of Chromatium

Sulphide Sample No.	Time hours	Oxidation per cent	Optical Density	Rate[a] x 10^6	Fractionation k_1/k_2	Used D. desulphur.Med.
A - 1	24.7	44.7	0.59	1.89	1.0000	No
A - 2	95.2	78.4	0.59	0.69	1.0011	No
A - 3	8.2	8.3	0.59	0.82	0.9947	Yes
A - 4	51.3	13.8	0.59	0.22	0.9922	Yes
A - 5	96.7	68.6	0.59	0.58	0.9990	Yes

[a] Moles hydrogen sulphide oxidized per hour per unit cell population

The alternate possibility that Chromatium produce a factor which influences the metabolism of D. desulphuricans has been tested in a similar but more extensive manner. The first experiment tested for the existence of a growth factor which would influence resting-cell suspensions of D. desulphuricans. The data for this experiment are given in Table XIII. Samples 1(a), 1(b) and 1(c) were from a suspension of the reducing bacteria to which only fresh, unused Chromatium medium had been added, while samples 2(a), 2(b) and 2(c) were from a suspension to which had been added "used" medium. The results for the two suspensions are about identical. In both cases the optical densities remained virtually constant, indicating little or no growth. The rates in both cases increased slightly between the first and second samples, then returned to the initial value between the second and third. The low initial rate can be attributed to a hold-up of hydrogen and sulphide in the medium in the early stages

TABLE XIII

Bacterial Reduction of Sulphate to Sulphide Effect of Growth Factor from *Chromatium* on Resting-Cell Suspensions of *D. desulphuricans*

Sample No.	Optical Density	Rate[a] x 10^6	Fractionation k_1/k_2	*Chromatium* Medium
1(a)	0.19	5.7	1.0118	Fresh
1(b)	0.17	10.0	1.0096	Fresh
1(c)	0.17	6.2	1.0107	Fresh
2(a)	0.19	8.1	1.0094	Used
2(b)	0.20	12.6	1.0078	Used
2(c)	0.19	6.9	1.0091	Used

[a] moles hydrogen sulphide produced per hour per unit cell population

of the run, while the decline towards the end reflects the aging of the suspension. The fractionation factor varies slightly, in both cases being somewhat lower for the second sample. From this experiment apparently no growth factor is produced by *Chromatium* which can influence the metabolism of *D. desulphuricans* in resting-cell suspensions in such a manner as to increase the isotope effect.

The effect of a possible growth factor from *Chromatium* on the metabolism of growing *D. desulphuricans* cultures has also been studied. The results are tabulated in Table XIV. The results from three cultures are reported here. In each case the experiments started with a resting-cell suspension of *D. desulphuricans* which, after the first sample, was

TABLE XIV

Bacterial Reduction of Sulphate to Sulphide Combined Effect of Growth Factor from *Chromatium* and Cell Multiplication on Resting Cell Suspensions of *D. desulphuricans*

Sample No.	Optical Density	Rate[a] x 10^6	Fractionation k_1/k_2	Added Nutrients
3(a)	0.19	4.8	1.0125	No
3(b)	---	15.7	1.0087	NH_4Cl and Yeast Extract after first sample
3(c)	0.24	25.5	1.0069	
3(d)	---	26.6	1.0058	
4(a)	0.19	5.6	1.0098	Fresh medium
4(b)	---	19.8	1.0076	NH_4Cl and Yeast Extract after first sample
4(c)	0.25	24.8	1.0056	
4(d)	---	31.7	1.0058	
5(a)	0.19	7.3	1.0098	Used medium
5(b)	---	26.7	1.0085	NH_4Cl and Yeast Extract after first sample
5(c)	0.29	23.2	1.0075	
5(d)	---	23.5	1.0057	

---

[a] Moles hydrogen sulphide produced per hour per unit cell population

returned to an actively growing state by the addition of ammonium chloride and yeast extract. Initially, Suspension 3 contained no added nutrients; Suspension 4 contained fresh *Chromatium* medium; Suspension 5 contained "used" *Chromatium* medium. The initial samples in each case gave normal rates and fractionation factors for resting-cell suspensions of *D. desulphuricans*. After the addition of the ammonium chloride and yeast extract, active growth resumed, as shown by the increases in optical densities in each case. The rate of metabolism, as indicated by the rate of sulphide production, also rose sharply in all three suspensions. As the rates increased, the fractionation factor decreased in each case. These results are in complete agreement with similar observations on resting-cell suspensions of *D. desulphuricans* made by Harrison (23). These data also indicate that *Chromatium* produce no growth factor which can influence the metabolism of *D. desulphuricans* in growing cultures in such a way as to increase the isotope effect in the reduction step. Thus another explanation must be found for the somewhat high fractionation factor observed in the mixed culture experiments.

Slight differences in technique, particularly with regard to sampling methods, have already been noted. Previous workers always removed the hydrogen sulphide with a stream of nitrogen as it was formed, whereas in the present work total sulphide from a sample of medium was always analyzed. Undoubtedly, sweeping with a stream of nitrogen cannot remove all the hydrogen sulphide from the medium which is at pH 8.5. Hence there is the distinct possibility of an equilibrium being established between $H_2S_{(g)}$ and $HS^-_{(aq)}$. In 1950

Tudge and Thode (19) quoted an unpublished work by Szabo which gave an experimental value of 1.006 for the equilibrium:

$$H_2S^{32}_{(g)} + HS^{34-}_{(aq)} \rightleftharpoons H_2S^{34}_{(g)} + HS^{32-}_{(aq)}$$

at 25°C. This is very close to the difference between the isotope effects obtained in the mixed culture experiments (1.018) and those reported elsewhere (23, 97, 101) for sulphate reduction in pure cultures of reducing bacteria under similar conditions (1.010 to 1.015). Experimental verification that sampling techniques are important has been obtained. During the course of the experiment designated Mixed Culture A, samples of sulphide taken by nitrogen sweep and total removal were found to differ in S^{34} content by as much as 0.3 per cent with the former being more enriched in the heavy isotope.

Verification of the conclusion that this exchange reaction plays a part was obtained by running a continuous culture of *D. desulphuricans* in the same manner as the mixed cultures. The data for this experiment are plotted in Figure 7. The results are similar to those for mixed cultures. By the end of three days the apparent fractionation factor had risen to 1.017, and soon reached a steady value of about 1.016. Clearly, therefore, the apparent fractionation factor of 1.018 obtained in the mixed culture experiments was due entirely to the isotope effect in the reduction step. Furthermore, this fractionation factor in mixed cultures is of a magnitude which is in line with that obtained in pure reducing cultures with similar experimental conditions and sampling techniques.

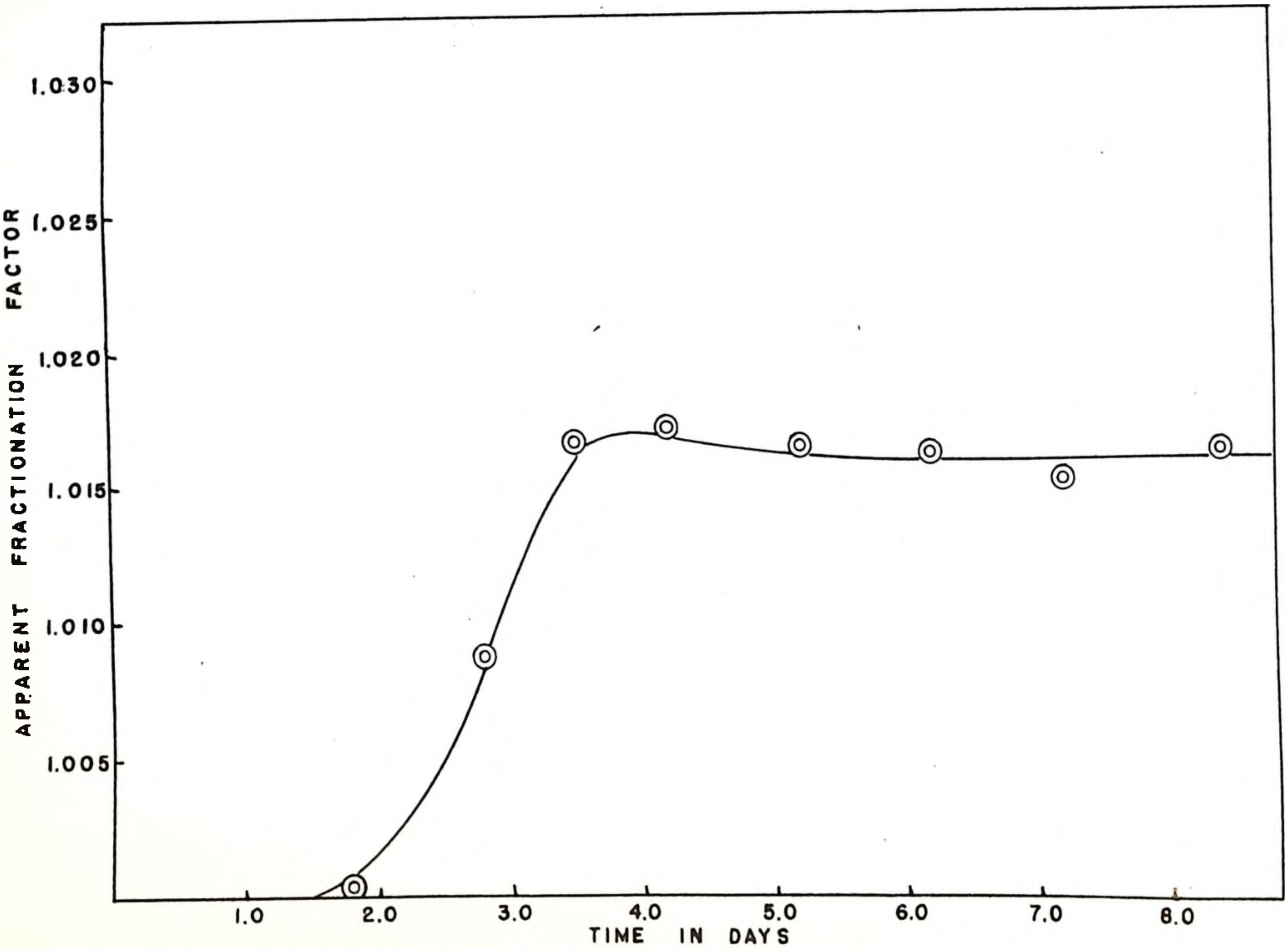


FIGURE 7 CONTINUOUS CULTURE OF <u>DESULPHOVIBRIO</u> <u>DESULPHURICANS</u>

Implications of Biological Processes to Natural Sulphur Isotope Abundances

On the basis of the results obtained in the laboratory with Chromatium and mixed cultures of Chromatium and D. desulphuricans certain conclusions may be reached concerning the role of these bacteria in the naturally occurring sulphur cycle, and particularly concerning the isotope fractionation that occurs therein. Obviously, both types of bacteria are essential to the continued turn-over of sulphur in the cycle. Isotope fractionation, however, would only seem to occur in the reduction process. The net result is an enrichment of S^{34} in the residual sulphate as sulphides are deposited in the sediments of the sea. Thus there is a trend towards an isotope separation which approaches the most favoured distribution of the isotopes between sulphate and sulphide, although none of the steps in the sulphur cycle themselves appear to involve an equilibrium process.

Apparent fractionation factors have been observed in nature, however, which are much higher than the kinetic isotope effect in the bacterial reduction of sulphate. For example, the salt domes of Louisiana and Texas showed an average fractionation of 1.049 between sulphate and sulphide, and 1.039 between sulphate and elemental sulphur (85). These high apparent fractionation factors may, however, be explained on the basis of batch process effects in a reaction involving an isotope effect much smaller than the observed factor.

For example, consider the sulphur-forming lakes in Africa in which both reducing and oxidizing bacteria are active. The source of sulphate may be incoming water or an underlying bed of gypsum. In the absence of oxidizing bacteria, the first hydrogen sulphide produced will be depleted in S^{34} with respect to sulphate. After a few days of biological activity this depletion will be about 1.8 per cent with respect to the source sulphate. In the presence of heavy metals this sulphide will precipitate and be deposited in the sediments, giving rise eventually to sedimentary sulphides. After all the heavy metals have been removed, excess hydrogen sulphide will escape as a gas. Meanwhile, if fresh sulphate is constantly entering the lake, the reaction will continue, with the heavy isotope gradually accumulating in the residual sulphate. This is similar to a distillation process in which the residue becomes more and more enriched in the less volatile compound. If the sulphate in solution reaches a sufficiently high concentration, calcium sulphate will begin to precipitate. Depending upon the extent of reaction, various enrichments of the sulphate in S^{34} will occur. The curve in Figure 8 shows the relationship between per cent enrichment of the residue in the heavy isotope and the extent of reaction for a process involving a 1.8 per cent isotope effect. After 50 per cent reduction, the sulphate in the residue would be 1.2 per cent enriched in S^{34}, giving a total separation of 3.0 per cent between the initial hydrogen sulphide and the residual sulphate. When the reaction has reached 75 per cent completion, the sulphate is enriched in S^{34} by 2.4 per cent and at 90 per cent reaction by 4.1 per cent. In each case the

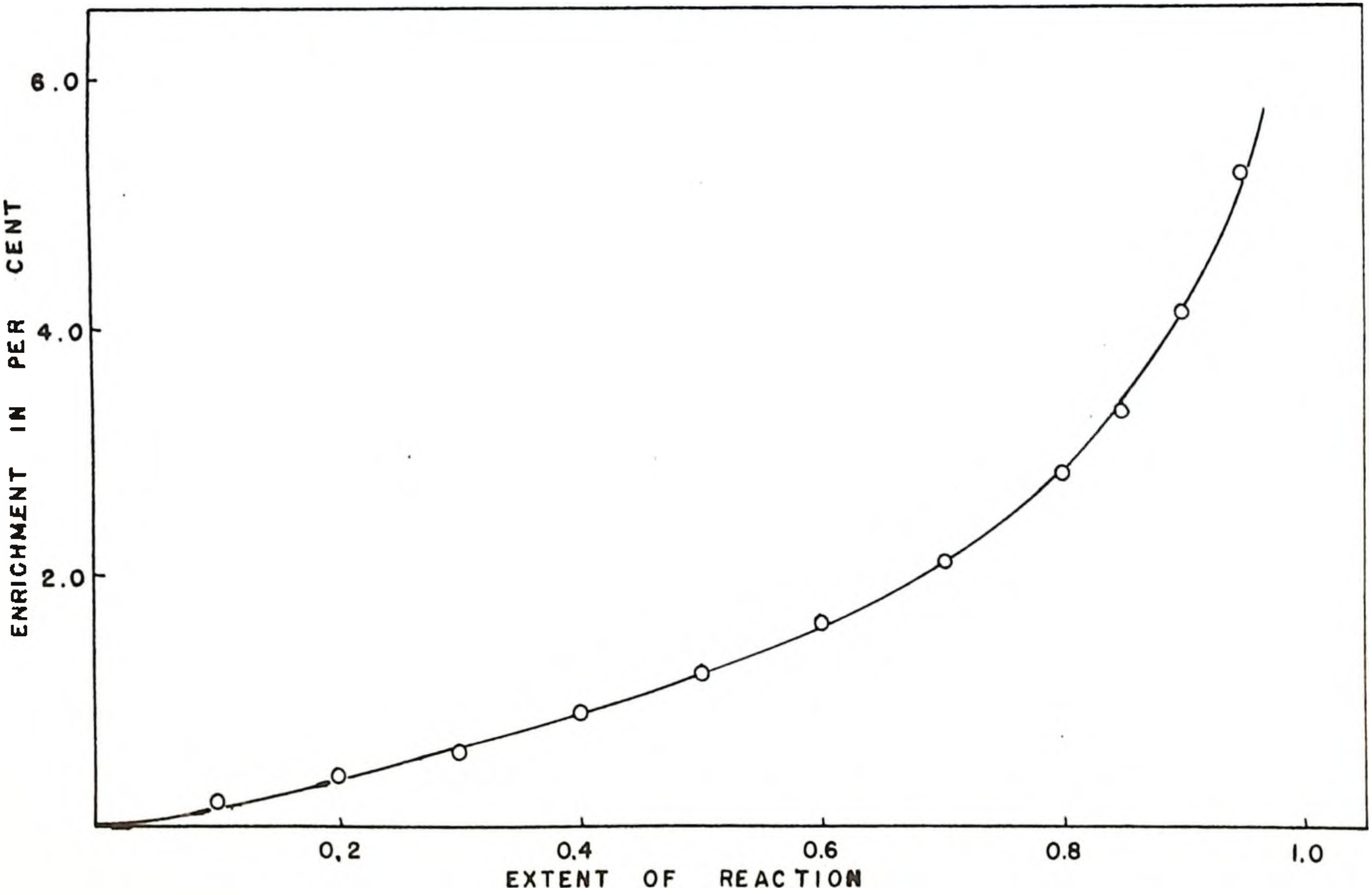


FIGURE 8 ENRICHMENT OF SUBSTRATE IN HEAVY ISOTOPE DURING REACTION WITH A 1.8 % ISOTOPE EFFECT

overall fractionation between sulphate and sulphide or elemental sulphur would be still greater.

Such a batch process can explain the observed fractionation factors for sulphur in the salt domes of Louisiana and Texas. Here the evidence is most convincing that elemental sulphur has been formed by the bacterial reduction of sulphate. The limestone containing large amounts of elemental sulphur has obviously been formed from the gypsum rock, which provided the calcium for the limestone, while the carbon dioxide and water were the oxidation products of the metabolism of the reducing bacteria. The elemental sulphur has also come from the sulphate of the gypsum. The amount of residual sulphate left in the secondary limestone rock varies from one salt dome to another, but in most cases is small, indicating that perhaps as much as 90 per cent of the original sulphate has been reduced. Thus the residual sulphate is considerably enriched in S^{34} as compared with the adjacent gypsum and anhydrite. The sulphur, which would accumulate after the sulphides had all been formed, would tend to have an isotope ratio somewhat closer to that of the sulphate, as is actually found. Thus the isotope ratios of the various sulphur species in the salt domes of Louisiana and Texas are seen to be explicable on the basis of a 1.6 per cent isotope effect in the reduction of sulphate by bacterial action.

In the Cyrenaican lakes, the lower value of the fractionation factor quoted in Table I is probably close to the isotope effect in the reduction step, while the higher value is probably due to a local enrichment of the source sulphate in S^{34} brought about by a batch process effect.

Other results, however, are difficult to explain on the basis of the simple batch process effect just outlined. For instance, many sedimentary sulphides are depleted in S^{34} by as much as 9.5 per cent with respect to meteoritic sulphur from which base level all terrestrial fractionations have presumably taken place. Obviously, a reduction of sulphate with the meteoritic isotope abundances by bacterial action can give a sulphide at most 2.5 per cent depleted in S^{34} on the basis of the studies discussed here and elsewhere. Some other explanation for the high isotope ratios in sedimentary sulphides must be found.

One possibility is that the formation of pyrite from sedimentary sulphide and elemental sulphur involves an isotope effect favouring the light isotope. Harrison (123) has obtained data from recent sediments which indicate that such may be the case. In any event, this aspect of the problem will require further work in order to elucidate the mechanism whereby sulphides with high S^{32}/S^{34} ratios are formed.

SUMMARY

1. A sulphur isotope effect in the oxidation of aqueous sodium sulphite with molecular oxygen has been measured under a variety of conditions. The results are explained theoretically.

2. The isotope exchange reaction

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

has been studied in neutral, aqueous solution and in the presence of resting-cell suspensions of D. desulphuricans. No exchange occurs in the former case, but the reaction does appear to be catalyzed by the enzyme system in the bacteria.

3. The oxidation of sulphite to sulphur and sulphur to sulphate by the photosynthetic autotrophic bacteria Chromatium has been examined for possible sulphur isotope fractionation. None was found.

4. Sulphur isotope fractionation in mixed cultures of Chromatium and D. desulphuricans has been examined. Evidence is presented which indicates that only the reduction of sulphate to sulphide involves an appreciable isotope effect.

5. The implications of biological processes in the natural fractionation of the sulphur isotopes are discussed.

APPENDIX

Calculations of partition function ratios for sulphite ion, sulphate ion and sulphur trioxide have been made by means of equation (9) given in the theoretical section of this thesis. The equation is

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

The vibrational frequencies for molecules containing both S^{32} and S^{34} have been obtained from Harrison (23) for sulphite ion and Tudge and Thode (19) for sulphate ion and sulphur trioxide. G(u) values were taken from the table of Bigeleisen and Mayer (18). The following constants were used in the calculation:

$$\frac{hc}{kT} = 4.454 \times 10^{-3} \quad \text{at } 50^\circ\text{C.}$$

$$\frac{hc}{kT} = 3.857 \times 10^{-3} \quad \text{at } 100^\circ\text{C.}$$

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

The calculation of Q_2'/Q_1' for sulphite ion at 50°C. and 100°C. are assembled in Tables XV and XVI.

TABLE XV

Calculation of C_2'/C_1' for $SO_3^=$ at 50°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	4.2513	0.0557	0.27923	0.01555
2	613.6	616.6	3.0	2.7330	0.0134	0.20367	0.00273
3	923.5	930.0	6.5	4.1133	0.0290	0.27352	0.00793
3	923.5	930.0	6.5	4.1133	0.0290	0.27352	0.00793
4	456.3	468.3	12.0	2.0324	0.0534	0.15877	0.00848
4	456.3	468.3	12.0	2.0324	0.0534	0.15877	0.00848

$$\sum_i G(u_{2_i})\cdot\Delta u_i = 0.05110$$

$C_2'/C_1' = 1.051$ at 50°C.

TABLE XVI

Calculation of C_2'/C_1' for SO at 100°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	3.6815	0.0482	0.25420	0.01225
2	613.6	616.6	3.0	2.3667	0.0116	0.18096	0.00210
3	923.5	930.0	6.5	3.5619	0.0251	0.24846	0.00624
3	923.5	930.0	6.5	3.5619	0.0251	0.24846	0.00624
4	456.3	468.3	12.0	1.7599	0.0463	0.13960	0.00646
4	456.3	468.3	12.0	1.7599	0.0463	0.13960	0.00646

$$\sum_i G(u_{2_i})\cdot\Delta u_i = 0.03975$$

$C_2'/C_1' = 1.040$ at 100 C.

II. Calculation of C_2'/C_1' for SO_3

The calculation of C_2'/C_1' for sulphur trioxide at 50°C. and 100 C. are assembled in Tables XVII and XVIII.

TABLE XVII

Calculation of C_2'/C_1' for SO_3 at 50°C.

i	ν_{2_i}	ν_{1_i}	$\Delta\nu_i$	u_{2_i}	Δu_i	$G(u_{2_i})$	$G(u_{2_i})\cdot\Delta u_i$
1	1069	1069	0	---	---	---	---
2	640.40	652	11.60	2.8523	0.0517	0.21064	0.01089
3	1310.65	1330	19.35	5.8376	0.0862	0.33165	0.02859
3	1310.65	1330	19.35	5.8376	0.0862	0.33165	0.02859
4	530.04	532	1.96	2.3608	0.0087	0.18059	0.00157
4	530.04	532	1.96	2.3608	0.0087	0.18059	0.00157

$$\sum_i G(u_{2_i})\cdot\Delta u_i = 0.07121$$

$C_2'/C_1' = 1.071$ at 50°C.

TABLE XVIII

Calculation of C_2'/C_1' for SO_3 at 100°C.

i	ν_{2_i}	ν_{1_i}	$\Delta\nu_i$	u_{2_i}	Δu_i	$G(u_{2_i})$	$G(u_{2_i})\cdot\Delta u_i$
1	1069	1069	0	---	---	---	---
2	640.40	652	11.60	2.4700	0.0447	0.18754	0.00838
3	1310.65	1330	19.35	5.0552	0.0746	0.30861	0.02302
3	1310.65	1330	19.35	5.0552	0.0746	0.30861	0.02302
4	530.04	532	1.96	2.0458	0.0076	0.15967	0.00121
4	530.04	532	1.96	2.0458	0.0076	0.15967	0.00121

$$\sum_i G(u_{2_i})\cdot\Delta u_i = 0.07121$$

$C_2'/C_1' = 1.071$ at 50°C.

III. Calculation of C_2'/C_1' for $SO_4^=$

The calculations of C_2'/C_1 for sulphate ion at 50°C. and 100°C. are assembled in Tables XIX and XX.

TABLE XIX

Calculation of C_2'/C_1' for $SO_4^=$ at 50°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	---	---	---	---
2	451	451	0	---	---	---	---
3	1097.56	1113.60	16.04	4.8885	0.0714	0.30306	0.02164
3	1097.56	1113.60	16.04	4.8885	0.0714	0.30306	0.02164
3	1097.56	1113.60	16.04	4.8885	0.0714	0.30306	0.02164
4	615.55	618.90	3.35	2.7417	0.0149	0.20406	0.00304
4	615.55	618.90	3.35	2.7417	0.0149	0.20406	0.00304
4	615.55	618.90	3.35	2.7417	0.0149	0.20406	0.00304

$$\sum_i G(u_{2_i})\cdot\Delta u_i = 0.0704$$

$$C_2'/C_1' = 1.074 \text{ at } 50°C.$$

TABLE XX

Calculation of C_2'/C_1' for $SO_4^=$ at 100°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				
2	451	451	0				
3	1097.56	1113.60	16.04	4.2333	0.0619	0.27834	0.01723
3	1097.56	1113.60	16.04	4.2333	0.0619	0.27834	0.01723
3	1097.56	1113.60	16.04	4.2333	0.0619	0.27834	0.01723
4	615.55	618.90	3.35	2.3742	0.0129	0.18117	0.01723
4	615.55	618.90	3.35	2.3742	0.0129	0.18117	0.01723
4	615.55	618.90	3.35	2.3742	0.0129	0.18117	0.01723

$$\sum_i G(u_{2_i})\cdot\Delta u_i = 0.05871$$

$$C_2'/C_1' = 1.059 \text{ at } 100 C.$$

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