THE FRACTIONATION OF SULPHUR ISOTOPES IN THE PLANT METABOLISM OF SULFATES

By

Michiko Midge Ishii, B. Sc.,

A Thesis

Submitted to the Faculty of Arts and Science

in Partial Fulfilment of the Requirements

for the Degree

Master of Science

McMaster University,

October, 1953

MASTER OF SCIENCE (1953)
(Chemistry)

McMASTER UNIVERSITY
Hamilton, Ontario

TITLE: A Study of the Fractionation of Sulphur Isotopes in the Plant Metabolism of Sulphates

AUTHOR: Michiko Midge Ishii, B.Sc. (McMaster University)

SUPERVISOR: Professor H. G. Thode

NUMBER OF PAGES: 40

SCOPE AND CONTENTS:

The isotopic fractionation of sulphur in the plant metabolism of sulphate by chlorella was investigated; and for this purpose an apparatus was devised for growing chlorella under sterile conditions. A green alga from the shores of Lake Erie, and mustard plants from the field were also investigated. No isotopic fractionation was found in the plant metabolism of sulphate either in the laboratory or in nature. A new method for the reduction of sulphate to hydrogen sulphide was also developed.

ACKNOWLEDGEMENTS

The author wishes to thank Dr. H. G. Thode and Dr. H. Kleerekoper for their kind aid, advice and direction in this work. We wish to thank Messrs. J. E. Warren and R. K. Wanless for the mass spectrometric analyses.

The financial assistance of the National Research Council of Canada and the Research Council of Ontario is gratefully acknowledged.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

FRACTIONATION OF SULPHUR ISOTOPES

IN PLANT METABOLISM

INTRODUCTION

The theory of isotope fractionation in equilibrium and unidirectional chemical processes is now fairly well understood. The measurement of isotope fractionation that occurs in chemical reactions therefore gives information concerning the nature and mechanism of the process. Isotope fractionation studies are of particular interest in geochemistry since processes which occurred in the geological past may be traced and information concerning the age and origin of rocks and minerals may be obtained.

In this connection, sulphur isotope studies have been particularly fruitful. Previous investigations show that marked variations in the abundance of the sulphur isotopes do occur (1-10). It is important to determine geochemical and biological processes responsible for the sulphur isotope distribution found. Undoubtedly, the sulphur cycle in nature plays an important part (6,7,9,10). The major aim of this work has been to determine the isotope fractionation which occurs in part of this cycle; namely, in the plant metabolism of sulphate both in the laboratory and in nature.

Historical

Isotopes were first discovered in 1910 by Soddy (11) and Fajans (12) independently. This discovery was an outcome of the study of the radioactive disintegration series. It was found that some of the elements of the uranium and thorium series as well as the end product, lead, had the same chemical and physical properties although they differed in mass and radioactive properties. Soddy concluded that

stable elements may also exist as a mixture of two or more species, and named these species of the same element "isotopes" from the Greek word meaning "same place" in the Periodic Table.

J. J. Thompson (13) proved Soddy's conclusion that stable elements may exist as a mixture of isotopes by designing and building the first mass spectrograph, an instrument used to separate a beam of charged particles according to mass by means of crossed electric and magnetic fields. He showed that neon was a mixture of two isotopes, masses 20 and 22. Later, Aston (14) and Dempster (15) identified and measured the abundances of isotopes of many elements, using the mass spectrograph.

Although there was a variation in the isotopic abundance of elements formed by nuclear processes, no variation in the stable isotopes of the lighter elements was found. Also, the rare heavy isotopes of the lighter elements escaped detection. This was due to the lack of sensitivity of the mass spectrographs available. However, in 1929, the heavy isotopes of oxygen (16) and later, nitrogen (17) and carbon (18) were discovered through the study of fine structure in molecular spectra. In 1931, Urey, Brickwedde and Murphy (19) discovered deuterium.

In 1933, Urey and Rittenberg (20), using statistical mechanics, showed that there were differences in the chemical and physical properties of light and heavy hydrogen and then confirmed it experimentally. Other elements like lithium, boron, carbon, nitrogen and oxygen were studied by calculating equilibrium constants for isotopic exchange

reactions (21) and carrying out experimental tests for confirmation. Most of this work was performed by Urey and his co-workers.

Thus, shown that isotopes differed somewhat in their chemical properties, isotope fractionation in nature and the laboratory was expected. However, due to the lack of high precision in the mass spectrometers used by Urey in 1932, the relative abundance studies of hydrogen, nitrogen and oxygen showed no variation beyond the limits of experimental error (22,23). However, Emeleus et al. (24) reported a variation in the isotopic constitution of hydrogen through accurate density measurements of water from various sources. The differences in density may have been due also to variations in the oxygen isotopes. Therefore, Dole and his associates (25-30) carried out an extensive investigation of the isotopic constitution of oxygen in air, water, and minerals through density studies and found variations up to four per cent. Later, Smith and Thode (31) through mass spectrometric study found agreement with Dole's work.

Urey and his associates (32-35) have carried out extensive investigations of the O^{18} exchange between carbonate ions in solution and water, and have related the O^{18} abundance of the fossils to the temperature of deposition, setting up a paleo-temperature scale. Marine organisms lay down their carbonaceous shells in equilibrium with the sea water and so their O^{18} content can be related to the temperature of the sea at the time of formation through the temperature coefficient for the exchange equilibrium of O^{18} between calcium carbonate and water.

Baertschi and Silverman (36-38) have made mass spectrometer

investigations of the isotopic constitution of oxygen in silicate rocks (igneous, sedimentary and meteoritic) and have found variations up to 2.0 per cent in the O^{16}/O^{18} ratio. They found that sedimentary rocks were richer in heavy oxygen than igneous rocks, thus providing a criterion whereby real igneous rocks may be distinguished from meta-sedimentary rocks.

Nier and his co-workers (39,40) reported a five per cent variation in the abundance of carbon isotopes. A preferential enrichment of C^{13} was found in inorganic compounds while a depletion was found in organic material. Their results have been confirmed by Mars of Sweden (41) and Trofimov of Russia (42). Wickman (43,44) has recently found on investigating the C^{12}/C^{13} ratio of 150 plants that there were no characteristic differences between the plants grown in different environments. Marine plants have a low C^{12}/C^{13} ratio indicating little fractionation with respect to sea water carbonates, while tropical rain forest trees have a high ratio indicating large isotope fractionation, the total variation being two per cent. Fresh water plants and other terrestrial plants have intermediate values.

Schoenheimer and Rittenberg (45) reported a 0.008 per cent excess of N^{15} in amino acids as compared to atmospheric nitrogen. Thode et al. (46) found a 3.5 per cent variation in the isotope ratio of boron. Graham, Macnamara, Crocker and MacFarlane (47) showed that in germanium the Ge^{70}/Ge^{76} ratios vary up to 0.7 ± 0.01 per cent, while Edwards (48) reported a variation of 0.5 per cent in the Cl^{35}/Cl^{37} ratio of samples of halite from two different salt deposits.

Variations in the Isotopic Constitution of Sulphur

During the past several years Thode and co-workers carried out extensive investigations of the isotopic constitution of terrestrial and meteoritic sulphur (1-10). The results of these investigations are summarized in a graph in Figure I where the S^{32}/S^{34} ratio is reported. The ratios of S^{32}/S^{33} and S^{32}/S^{36} vary correspondingly in ratios according to their masses.

It is seen that, depending on the source of the sulphur, the S^{34} concentration varies by as much as nine per cent. In general, sulphates are enriched in S^{34} whereas sulphides and hydrogen sulphide samples are depleted in the heavy isotope. Pyrite minerals have a S^{34} concentration which varies over a wide range, embracing the mean isotope abundance. Meteoritic sulphur has been found to be virtually constant. Investigations of a number of meteorites collected from different parts of the earth and involving both iron and nickel and stony meteorites have given results which indicate little or no variation in the abundances of the sulphur isotopes from meteoritic origin. The range is midway in the range of values for terrestrial sulphur, suggesting that it is the primordial value and that fractionation has occurred since in biological and geological processes, thereby spreading out the ratios above and below the base value. It indicates that meteoritic sulphur has not been subjected to the same fractionation processes as has sulphur of terrestrial origin.

It is seen from the graph that the isotopic composition of igneous sulphur has a slightly greater range than meteoritic sulphur

FIG I

DISTRIBUTION OF S^{34} IN TERRESTRIAL AND METEORITIC SULFU

is lower than meteoritic sulphur and overlaps it. The close agreement between the S^{32}/S^{34} ratios for the sulphur in igneous rocks and meteorites is a further evidence of the theory that meteorites and the earth had a common origin. H. C. Urey has also found that the isotopic compositions of oxygen of igneous and meteoritic origin are identical (49).

Native sulphur has shown a 4.5 per cent variation in the S^{32}/S^{34} ratio. Sulphur of organic origin has been found to cover this entire range, whereas samples of volcanic origin cover an area one-third of the organic, in the region of higher S^{34} concentration.

Like carbon, in which the carbonates are enriched in C^{13} (39,40), sulphates are enriched in S^{34}. Sea water sulphates have been found to be comparatively constant, having a range of 0.4 per cent, while in gypsum deposits sulphates have a variation of 2.5 per cent. Thode, Macnamara and Collins (2) reported the results of a comparison of isotopic abundance data for sulphate, sulphur and hydrogen sulphide present together in well water.

TABLE I

A comparison of isotope abundance data for $SO_4^=$, S^o, and H_2S present together in well water

Sample	Dorchester, Ont.	Tillsonburg, Ont.	Port Ryerse, Ont.	Port Stanley Ont.
	S^{32}/S^{34} ratio			
H_2S	22.705±0.010	22.180±0.010	22.400±0.010	22.215±0.010
$SO_4^=$	21.735±0.010	21.715±0.010	21.650±0.010	21.585±0.010
S^o	22.705±0.010	22.285±0.010	----	----

The hydrogen sulphide and free sulphur present in the same solutions were found to contain from 2-5 per cent less S^{34} than the sulphates. The free sulphur formed from these solutions by air oxidation of hydrogen sulphide had about the same isotopic content as the hydrogen sulphide in the two cases studied.

With regard to pyrite sulphur, a widespread variation in S^{32}/S^{34} ratio was discovered, although, in general, the S^{34} was depleted compared to sulphates. Thode, Macnamara and Collins (2) have shown that there is no correlation between deposition age and isotopic content of the minerals, nor between the isotopic content and crystallizing temperature, although there is a correlation within single samples.

Theoretical

The theory of isotope fractionation in equilibrium processes is now fairly well understood. Urey (50), using statistical mechanics, calculated equilibrium constants for many isotopic reactions involving the light elements. Tudge and Thode (7) extended these calculations to isotopic reactions of sulphur. In general, for the reaction

$$aA_1 + bB_2 \rightleftharpoons aA_2 + bB_1$$

where A and B are molecules which have some one element as a common constituent and subscripts 1 and 2 indicate that the molecule contains either the light or heavy molecule, the equilibrium constant K is given by

$$K = \left[\frac{Q_{A_2}}{Q_{A_1}}\right] \Big/ \left[\frac{Q_{B_2}}{Q_{B_1}}\right]^{b} \cdot e^{-\frac{aE_{oA_2} + bE_{oB_1} - aE_{oA_1} - bE_{oB_2}}{RT}} \qquad (2)$$

where the Q's are partition functions for the different molecules. Instead of taking E_o as the "zero point energy", one can take E_o as the bottom of the "potential energy curve" for the molecule. Since the potential energy curves are practically identical for isotopic molecules, then E_{oA_2} equals E_{oA_1}, and E_{oB_2} equals E_{oB_1}. Thus, the exponential term in the above equation becomes unity and

$$K = \left[\frac{Q'_{A_2}}{Q'_{A_1}}\right] \Big/ \left[\frac{Q'_{B_2}}{Q'_{B_1}}\right]^{b} \qquad (3)$$

Urey (50) devised a method by which the partition function ratio for isotopic molecules is easily obtained from a knowledge of vibrational frequencies alone. The equilibrium constants for many possible exchange reactions of sulphur isotopes calculated by Tudge and Thode (7) varied from 1.000 to 1.096, indicating that considerable fractionation of sulphur isotopes can be expected in equilibrium processes.

Isotopes may also be fractionated due to differences in reaction rates of isotopic atoms and molecules. These differences in reaction rates are small and are of the order of one to ten per cent (51). Bigeleisen has derived a formula for the ratio of the rate constants based on the application of the transition state method. If the reactions between A_1, B, C, --- to give P_1, and A_2, B, C, --- to

give P_2 where A_1 and A_2 are isotopic molecules involving elements other than hydrogens are considered, (52), then the ratio of the rate constants is

$$\frac{k_1}{k_2} = \frac{K_1}{K_2} \frac{C_1^{\neq}}{C_2^{\neq}} \frac{C_{A_2}}{C_{A_1}} \left[\frac{m_2^{\neq}}{m_1^{\neq}}\right] \qquad (4)$$

where K is the transmission coefficient, C is the concentration of the activated complex, $m^{\neq}$ is the effective mass of the complex along the coordinate of decomposition. The ratios of the concentrations of the individual molecules can be replaced by the corresponding ratios of the partition functions and so,

$$\frac{k_1}{k_2} = \frac{K_1}{K_2} \frac{Q_1^{\neq}}{Q_2^{\neq}} \frac{Q_{A_2}}{Q_{A_1}} \left[\frac{m_2^{\neq}}{m_1^{\neq}}\right]^{\frac{1}{2}} \qquad (5)$$

Bigeleisen and Mayer (53) have developed from this the following equation

$$\frac{k_1}{k_2} = \frac{S_1 S_2^{\neq}}{S_2 S_1^{\neq}} \left[\frac{m_2^{\neq}}{m_1^{\neq}}\right]^{\frac{1}{2}} 1 + \sum_i^{3n-6} G(u_i)\Delta u_i - \sum_i^{3n'-6} G(u_i^{\neq})\Delta u_i^{\neq} \qquad (6)$$

where S is a symmetry number, G(u) is a free energy function, 3n-6 is the number of vibrational modes in the molecule, and u_i is equal to $hc(w_1 - w_2)/kT$. By definition, the subscripts 1 and 2 refer to the light and heavy molecules, respectively. The superscript, $^{\neq}$, refers to a property of the transition state. The factor involving the S's is a simple statistical one which arises from the fact that if there were two or more identical atoms of the isotope in question in the molecule there will be a corresponding increase in the probability of one of them reacting. The factor $(m_2^{\neq}/m_1^{\neq})^{\frac{1}{2}}$ gives the ratio of the number of light and

heavy "activated complexes" which decomposes per unit time. The quantity in the square brackets gives a quantitative description of the effect of the differences in zero point energies of the light and heavy molecules in the normal and transition states.

It is therefore possible to calculate the isotope fractionation expected for any process, provided the mechanism is known and the appropriate partition functions can be calculated. In general, there is remarkable agreement between theory and experiment.

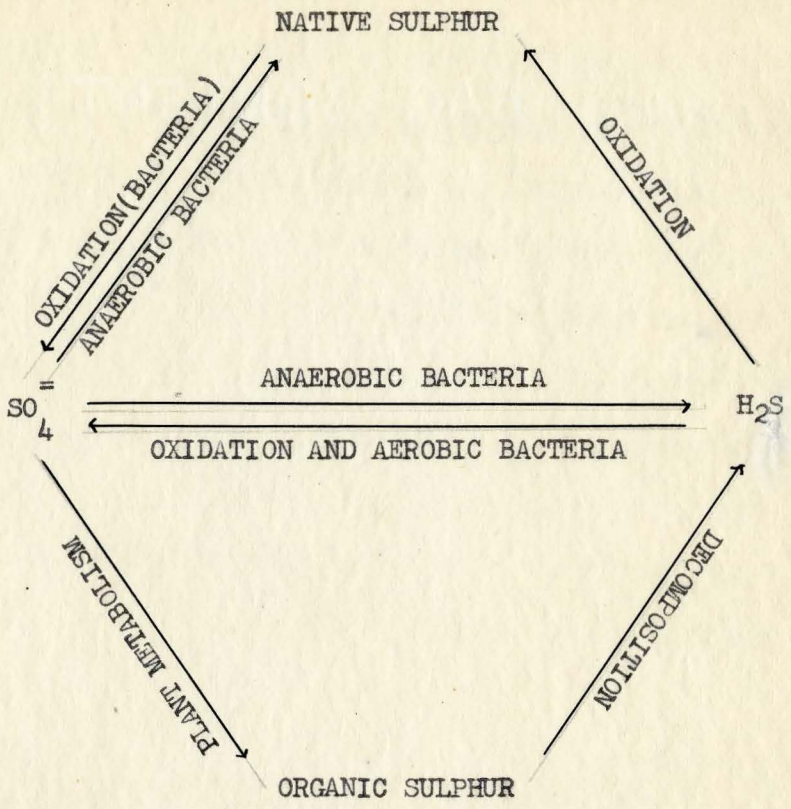
Natural Processes and the Sulphur Cycle

It is clear that the isotopes of sulphur differ in their chemical properties and are fractionated in geological and biological processes. The exchange reaction

$$H_2S^{34}(g) + S^{32}O_4^{=}(soln) \rightleftharpoons H_2S^{32}(g) + S^{34}O_4^{=}(soln)$$

must be considered in these processes. The equilibrium constant has been found to be 1.074 at 25°C; thus the S^{34} is favoured in the sulphate by 7.4 per cent.

A great deal of energy is, however, required to reduce sulphate ion and sulphates will not exchange their sulphur with hydrogen sulphide under normal conditions so that some mechanism is required. It has been suggested (6,9,10) that the biological sulphur cycle in nature which is illustrated below provides this necessary mechanism.



It may be through this sulphur cycle that variations in S^{34} are produced.

Thode, Kleerekoper, and McElcheran (5) have studied the reduction of sulphates to hydrogen sulphide by reducing bacteria, Vibrio desulfuricans, under controlled laboratory conditions where the hydrogen sulphide is swept out by nitrogen. The hydrogen sulphide formed was found to be depleted in S^{34} by about one per cent at $25^{\circ}C$. Macnamara and Thode (6) have studied the reducing action of bacteria in the natural environment. They reported a 3.2 per cent fractionation in sulphur

isotopes between the sulphur and sulphate in an African lake. In this lake the native sulphur is formed by the bacterial reduction of sulphate to hydrogen sulphide (Vibrio desulfuricans) and finally oxidized by purple bacteria to native sulphur. Although this value is only about half that expected if isotopic equilibrium were obtained, natural processes are usually complicated and one can hardly expect perfect equilibrium conditions. In the laboratory experiment, only reduction was involved. The per cent fractionation suggests that it is a unidirectional rather than an equilibrium process. If we assume that the rate-controlling step involves the breaking of a sulphur-oxygen bond, then the separation factor can be calculated, using Equation (6)

$$\frac{k_1}{k_2} = \frac{S_1 S_2^{\neq}}{S_2 S_1^{\neq}} \left[\frac{m_2^*}{m_1^*}\right]^{\frac{1}{2}} \left[1 + \sum_i^{3n-6} G(u_i)\Delta u_i - \sum_i^{3n'-6} G(u_i^{\neq})\Delta u_i^{\neq}\right]$$

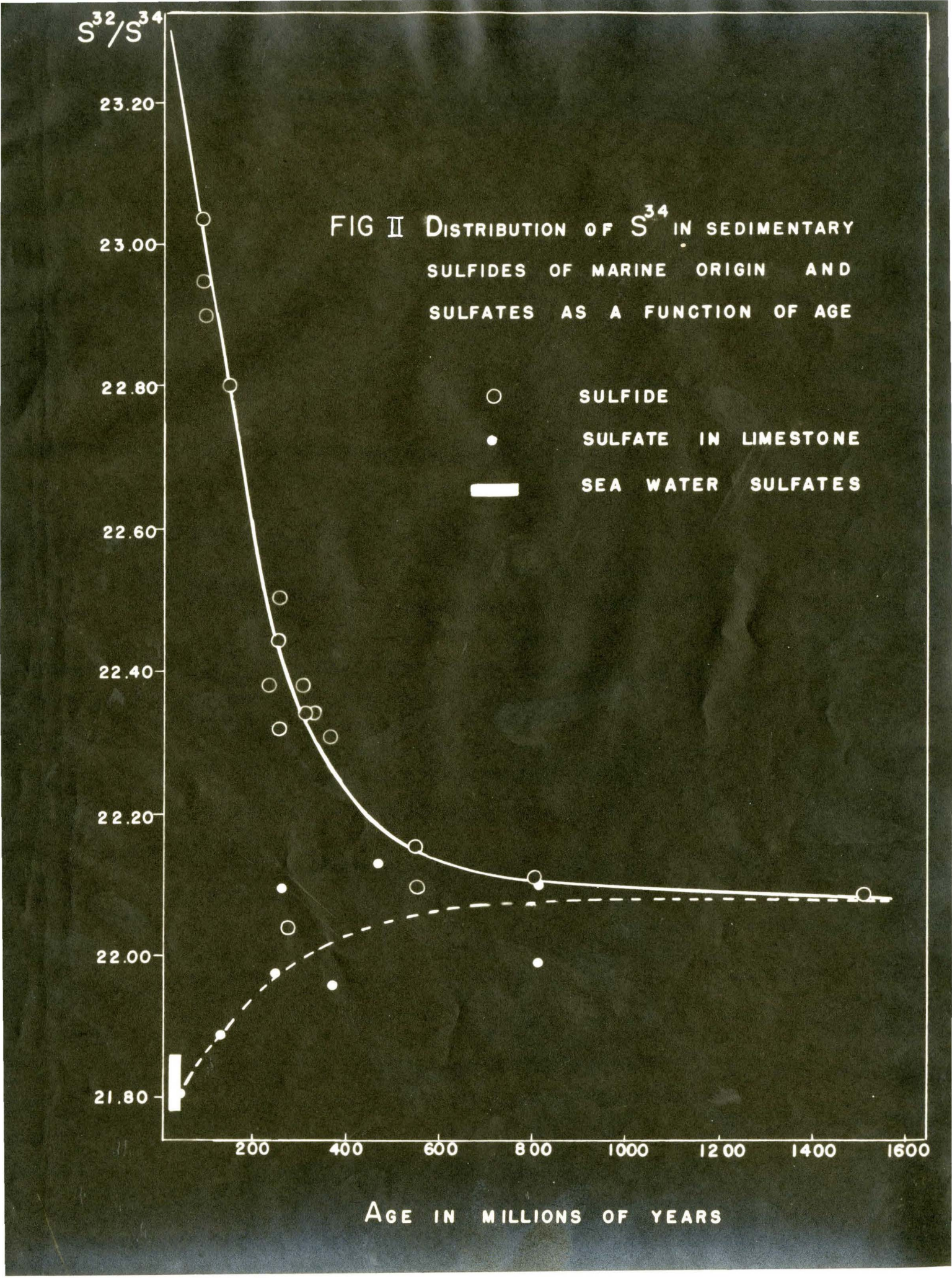
We may assume that the only essential difference between the free energy function of the normal and transition states arises from the vibration frequency of the bond ruptured in the transition state (52). If the force constant of this bond is set equal to zero in the transition state and all other frequencies are assumed to remain the same; also, that the factor involving the S's equals one, then

$$\frac{k_1}{k_2} = \left[\frac{m_2^*}{m_1^*}\right]^{\frac{1}{2}} \left[1 + G(u_i)\Delta u_i\right]$$

The sulphate ion has four vibrational frequencies (7). For $S^{32}O_4^{=}$ the values for w are 980, 451(2), 1113.60(3), and 618.90(3), where the numbers in the brackets represent the degree of degeneracy of a frequency.

For $S^{34}O_4^=$, the corresponding frequencies are 980, 451(2), 1098.56(3) and 615.55(3). Since the first two frequencies are identical for $S^{32}O_4^=$ and $S^{34}O_4^=$, they must not involve the sulphur atom. We may assume that the ruptured bond is the one in which the difference between the two frequencies is the greater; that is, where the frequencies are 1113.60 and 1097.56 for $S^{32}O_4^=$ and $S^{34}O_4^=$, respectively. Then, considering the reduced mass across the sulphur-oxygen bond, the ratio of the reaction rate is found to be 1.03 at 25°C. This is approximately the value found for reactions in nature.

The sulphur isotope content of marine sulphide and sulphate deposits, as found in limestone and shales, was investigated (10). When the isotopic ratios for sedimentary sulphides and sulphates were plotted against geological age, a rather striking correlation was found, as shown in Figure II. Sedimentary sulphides of marine origin are formed from the reaction of iron silicates and the hydrogen sulphite procuded in the bacterial reduction of sulphate. It appears that little or no fractionation occurred between sulphate and sulphide up until 700-800 million years ago, the isotopic content being at "base levels" or, the same value as that for meteoritic and igneous rock sulphur. From that time forward the sulphur isotopes became fractionated at an ever-increasing rate and for the more recent samples the spread is about seven per cent. These results strongly suggest that life involving the sulphur isotopes became abundant about 800 million years ago when isotopic exchange between sulphate and sulphide began, the exchange process becoming more and more rapid as this type


S^{32}/S^{34}
23.20
23.00
22.80
22.60
22.40
22.20
22.00
21.80
200
400
600
800
1000
1200
1400
1600
Age in millions of years
FIG II Distribution of S^{34} in sedimentary sulfides of marine origin and sulfates as a function of age
SULFIDE
SULFATE IN LIMESTONE
SEA WATER SULFATES

of organism became more and more abundant.

There is considerable evidence (54) that living organisms appeared on the earth before this time (that is, 1.4×10^9 years ago). H. C. Urey (58) has recently outlined the chemical history of the formation of the earth based largely on thermodynamic considerations where the early primitive atmosphere was a reducing one consisting of methane, ammonia, hydrogen and water. From this atmosphere, through photochemical reactions, organic compounds were formed which dissolved in the sea and provided conditions favourable for the origin of life. The first primitive living organisms received their supply of free energy from these organic compounds. Later, as these compounds became depleted, and the atmosphere more oxidizing through photosynthesis of water and the loss of hydrogen from the planet, living organisms began to develop which could utilize the free energy available from the oxidation of hydrogen sulphide and sulphur (10). Thus, it may well be that the first living organism appeared 1.4×10^9 years ago although life involving the sulphur isotopes became abundant 800 million years ago.

Certain steps in the sulphur cycle have been studied under controlled laboratory conditions to determine the isotope fractionation. Bacterial processes were studied by McElcheran (59) with Vibrio desulfuricans the bacteria which reduces sulphate directly to hydrogen sulphide, Thiobacillus thio-oxidans which oxidizes sulphur to sulphate and Thio-Thioparus which oxidizes thiosulphate to sulphate. The study of the reduction of sulphate to hydrogen sulphide by anaerobic bacteria has been carried out more extensively recently. Preliminary study of the

plant metabolism of sulphates to produce organic sulphur has been carried out by Rowat (60) on mustard plants. In the work reported in this thesis, chlorella grown at different temperatures, mustard plants from the field and sea-weed from Lake Erie were investigated.

In the plant metabolism of sulphate, the sulphate ions diffuse through the membranes of the plant cells (55). The sulphate is then converted to organic sulphur, mainly in the form of plant protein. The proteins of plants contain from 0.003 to 7.2 per cent sulphur (56) and on the average about 0.6 per cent. According to Rankama (54) the total yield of photosynthesis is approximately 15×10^{10} tons of carbon annually. Now, if the average amount of carbon is 52 per cent and sulphur is 0.6 per cent in plant proteins, the total yield of sulphur in photosynthesis is 1.8×10^{9} tons. The mass of the crust of the earth is 0.043×10^{27} grams (57) and the average sulphur content in igneous rock is 0.052 per cent. Assuming that the earth's crust is composed of only igneous rock, the amount of sulphur is 2.5×10^{16} tons. Thus, at the most, one part in 14,000,000 of the sulphur in the earth's crust takes part in photosynthesis annually. The actual ratio is even smaller, since we have neglected here the amount of sulphur in the oceans, sedimentary rocks and Biosphere, which is roughly eight times that in the earth's crust (57).

All the sulphur which is turned into plant material through photosynthesis is reduced further to hydrogen sulphide by bacteria. Also, the bacteria oxidizes and reduced material in the seas, rocks and sediments so that the bacteria must deal with a much greater amount of

sulphur than plants. Thus, it appears that bacterial processes are more responsible for fractionation of sulphur isotopes than plant metabolism is.

EXPERIMENTAL

A. Aqueous Culture of Chlorella

Apparatus for Plant Culture:- The chlorella was grown in thirty-two litres of nutrient solution contained in four 9-litre jars (Figure III,F) equipped with the following:

M- Outlet tube containing cotton batting and glass wool

X- Inlet tube ending in porous porcelain bubbler

Y- Funnel for introducing original sample of chlorella

Z- Tube equipped with gum rubber connection and screw clamp for removing chlorella and introducing new nutrient between successive runs

The entire system was sterilized by heating in an autoclave at a pressure of two atmospheres.

The jars were placed in a constant temperature bath equipped with a mercury and toluene regulator and relay system. The bath was stirred well with air bubbles and cooled with tap water running through coiled copper tubing. The temperature could be readily regulated within 0.2°.

Nutrient solution was sterilized in a jar (Figure IV) and transferred to jar F via Z. To prevent contamination, the ends of the tubing were immersed in alcohol and the surrounding air heated with a flame.

The apparatus used for the culture of chlorella shown in Figure III was such that the nutrient solution was sterile at all times. Streams of air and carbon dioxide were passed through mercury bubblers A. The mixture containing air and carbon dioxide in the ratio of approximately 5:1 was then sterilized and saturated with water

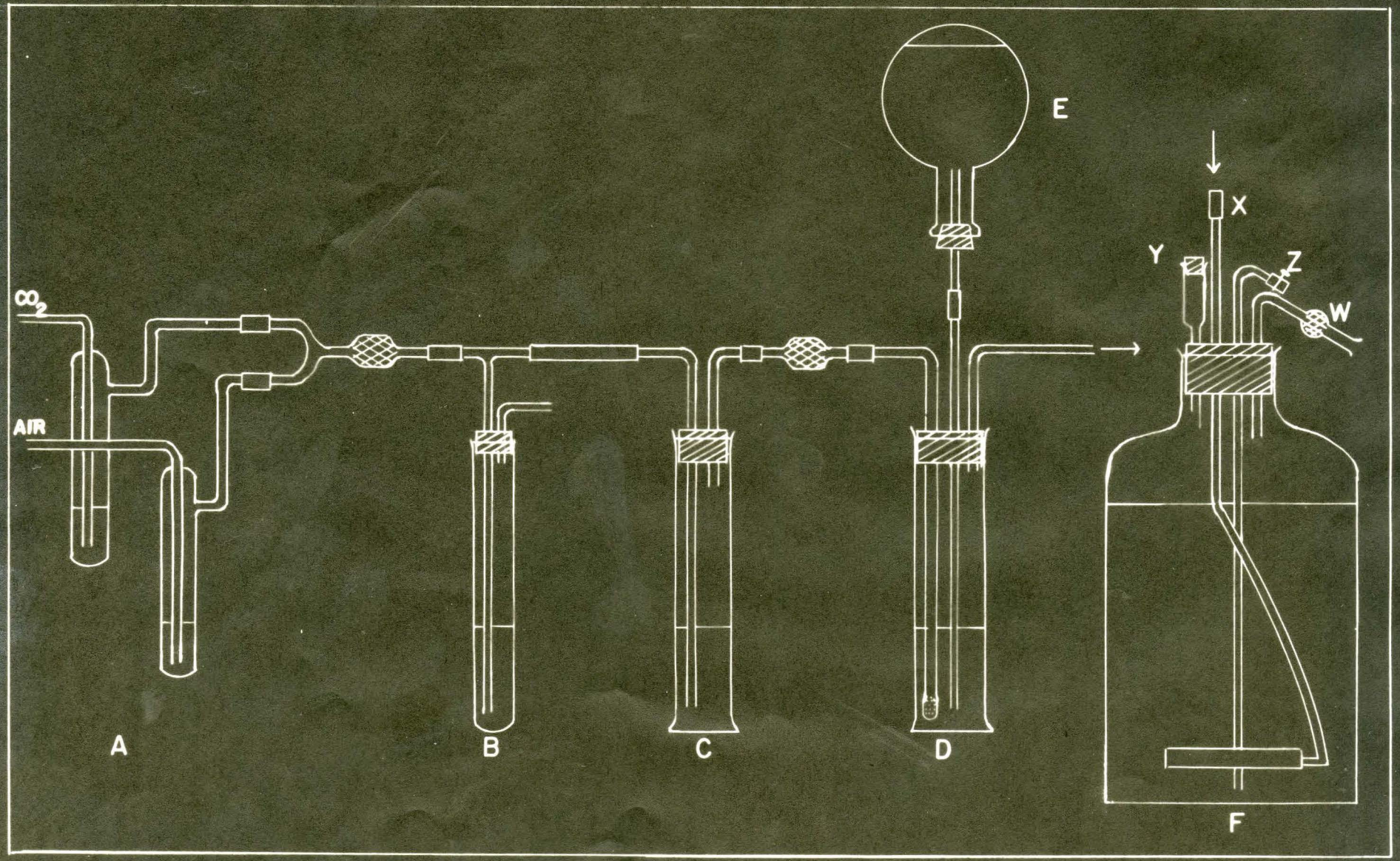


FIG III APPARATUS FOR PLANT CULTURE

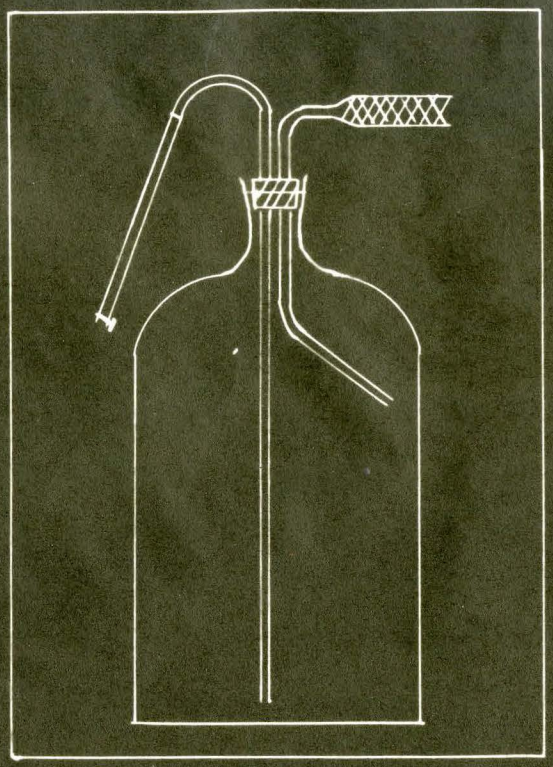


FIG IV NUTRIENT JAR

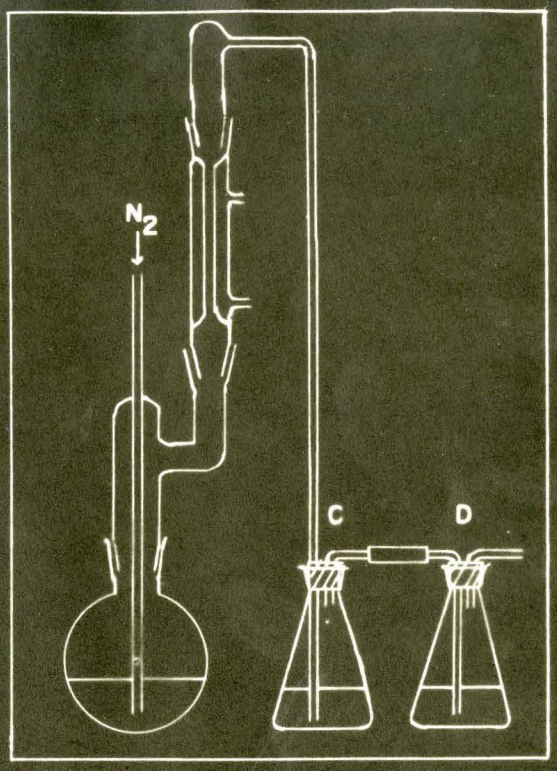


FIG V REDUCTION APPARATUS

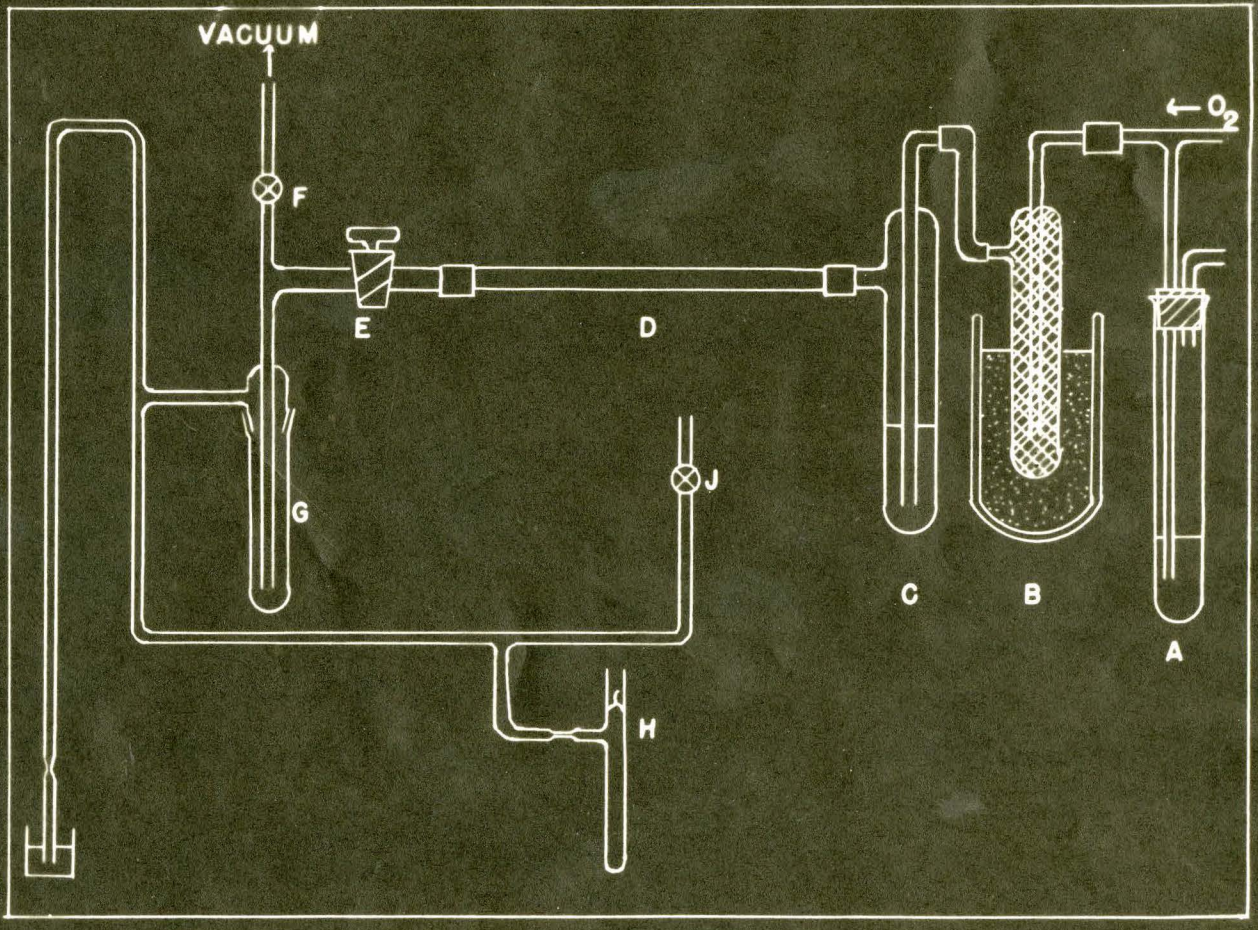


FIG VI SILVER SULFIDE COMBUSTION TRAIN

by passing through the following system: glass wool filter to prevent mercury from bubbling over, concentrated sulphuric acid in hydrometer jar C, a second glass wool filter to prevent the sulphuric acid from splashing over, sterilized ion-free water D. The water evaporating from D was automatically replaced with water from reservoir E. The pressure was regulated by means of a mercury valve B. This mixture of air and carbon dioxide was passed into the nutrient solutions through X. The contents of the jars were kept stirred by the air coming through the porcelain bubblers.

The chlorella was illuminated by four 15W daylight fluorescent lamps, two 40W lamps in series, and one 2000W lamp. These were placed approximately six inches from the jars.

Preparation of the Nutrient Solution:- The water used in the culture solution was prepared by passing distilled water through a column containing a mixture of cation and anion exchange resins. The cation resin, Amberlite IR-120, had been converted to the hydrogen cycle by the action of four per cent hydrochloric acid. The anion resin, Amberlite IRA-400, had been converted to the hydroxyl cycle with four per cent sodium hydroxide.

The nutrient solution was made up as in Table II.

TABLE II

Nutrient Solution for Chlorella

Compound	Concentration
KH_2PO_4	0.009 M
KNO_3	0.012 M
$MgSO_4$	0.010 M
$Fe_2(SO_4)_3$	1.33×10^{-4} M
Na citrate	5.60×10^{-4} M

Two stock solutions were made at concentrations of one hundred times that of the final solution, one consisting of the potassium nitrate and potassium dihydrogen phosphate, and the other of the remaining three compounds, since otherwise, at this concentration ferric phosphate would have precipitated out. The solution was then sterilized by heating in an autoclave at a pressure of two atmospheres for approximately thirty minutes. After it had been allowed to cool, the nutrient solution was added to the jars through Z.

Preparation of Plant Material:- The chlorella were left to grow for several weeks. The length of time depended on the temperature at which the algae were being grown, the period of time required decreasing with increasing temperature. However, at temperatures above 25°C they did not multiply, and at higher temperatures died.

When sufficient chlorella had been produced to give a dense green suspension, the solution was removed by closing W, opening Z

and allowing the air inside to force the solution out. It was then passed through a continuous centrifuge (Foerst, 15,000 RPM) at the rate of one litre per six minutes. The chlorella was then washed with ion-free water at 0°C and recentrifuged. The chlorella was then "freeze-dried," using an oil pump and collecting the water in a liquid air trap, while the algae remained frozen. The completely dried chlorella which was dull green was then set aside for analysis.

B. Analysis of Green Plant Material

The Separation of Sulphate from Protein Sulphur Compounds:-

The procedure followed was basically the one used by Rowat (60) with a few modifications. The methods outlined by Balks and Wehrmann (61) and Bertrand and Silberstein (62) were used. Sulphate was separated from the protein sulphur by extraction with hydrochloric acid and precipitation with barium chloride (61). The remaining protein sulphur was oxidized to sulphate by digestion, first with a mixture of sodium hypobromite and hydrogen peroxide (61) and then, fuming nitric acid (62). Then the mixture was fused with sodium carbonate, dissolved, and the protein sulphur obtained as barium sulphate. The detailed procedure was as follows:

Four grams of dried plant material, 200 ml. of water, and 10 ml. of 20 per cent hydrochloric acid was evaporated over a

sand-bath to a volume of approximately 50 ml; then 200 ml. of 20 per cent hydrochloric acid was added and the evaporation carried out nearly to dryness. The dark thick mixture was then transferred to a Whatman extraction thimble in a Soxhlet and the filtrate diluted to 250 ml. The filtrate was then used as the extractant for an eighteen-hour extraction. The hot solution was then neutralized carefully with 10 per cent sodium hydroxide, and 10 ml. of concentrated hydrochloric acid was added. The solution was heated and 15 ml. of 10 per cent barium chloride added. The barium sulphate obtained was then collected on a Whatman #42 filter paper. This barium sulphate may represent the sulphate originally in the cells or those chemically bound to some other compound which had been cleaved in the digestion procedure.

The filtrate from the above precipitation was neutralized with sodium hydroxide and an 8-10 ml. excess of 10 per cent sodium hydroxide was added. Solid sodium carbonate was then added to precipitate the excess barium ions as barium carbonate. The pale-brown voluminous gelatinous precipitate was filtered and washed thoroughly. To this filtrate 12 ml. of 30 per cent hydrogen peroxide and 8 ml. of sodium hypobromite solution was added. The solution which had been a very dark brown turned a pale orange-brown. The solution was evaporated almost to dryness over a sand-bath. Concentrated hydrochloric acid was cautiously added to the mixture, until the solution was neutral, and the solution was then evaporated to dryness. Then, 25 ml. of

fuming nitric acid was cautiously added to the residue to which had been added the plant residue from the Soxhlet extraction. The mixture was heated over a sand-bath, several ml. more fuming nitric acid was added, and then evaporated to a smaller volume. The mixture was transferred to a porcelain casserole, and evaporated further. Ten per cent sodium carbonate was then carefully added, with stirring, until no effervescence occurred. An equal volume of sodium carbonate to that used was added. Evaporation was continued over a sand-bath, and then over a Meker burner, to dryness. The black mixture gradually formed a voluminous grey-black crust which then slowly melted, producing a colourless liquid. After cooling, the melt was dissolved in water, transferred to a beaker and concentrated hydrochloric acid added until the solution was slightly acidic. The white silica which precipitated was quickly removed by filtration and barium chloride was added to the hot filtrate. The barium sulphate obtained in this way represented the protein sulphur in the plant material.

The Reduction of Barium Sulphate:- In all the work previously carried out in this laboratory on the investigation of the isotopic distribution of sulphur, the reduction of barium sulphate to sulphide was carried out using carbon (2), iron (63), zinc (60) or magnesium (64).

In this investigation a much more efficient and simple reduction procedure was used. A reducing mixture used by Pepkowitz and Shirley (65) originally described by Luke (66) was used. This mixture was

prepared by mixing 500 gm. of 50 per cent hydriodic acid, 480 ml. of 12 M hydrochloric acid and 144 ml. of 50 per cent hypophosphorous acid. This mixture was heated for approximately thirty minutes in order to reduce any sulphate contamination in the reagents and to boil away the hydrogen sulphide produced from it. The pale yellow solution was cooled and stored in a brown bottle to avoid oxidation of the hydriodic acid.

The barium sulphate was reduced in the apparatus shown in Figure V. The sample and approximately 75 ml. of reducing mixture was added to the boiling flask. Nitrogen was gently bubbled through the mixture while the flask was heated with a low flame. The hydrogen sulphide formed was bubbled through distilled water C to absorb the hydrogen chloride fumes which may have been formed at higher temperature, and through a solution of cadmium acetate D, made up by mixing 12.5 gm. of $Cd(OAc)_2 \cdot 2H_2O$, 100 ml. of 12 M acetic acid and 400 ml. of water. When no more hydrogen sulphide was produced as tested with lead acetate paper, D was removed, and the cadmium sulphide converted to silver sulphide by adding 0.1 N silver nitrate to the flask. The gelatinous yellow cadmium sulphide precipitate was converted immediately to the heavier black silver sulphide precipitate. The precipitate was collected in a filter and washed with dilute ammonium hydroxide to dissolve any silver chloride present, and washed thoroughly with distilled water. The precipitate was then dried at 110°C overnight.

The Preparation of Sulphur Dioxide:- The apparatus used to prepare sulphur dioxide for mass spectrometric analysis is illustrated in

Figure VI. A was a mercury pressure regulator for the incoming oxygen. The oxygen was purified by passage through activated charcoal in trap B which was surrounded by a mixture of dry ice and acetone. The purified oxygen was passed through a sulphuric acid bubbler C and then through the tube D containing silver sulphide and phosphorous pentoxide separated by glass wool. The phosphorous pentoxide served to remove any water formed in the combustion. While the oxygen was passed through D and out through the two-way stock-cock E, the other part of the system was evacuated. After complete evacuation and flaming of the break-seal sample tube H, stock-cocks J, then F, were closed and E turned slowly to let the oxygen pass through the rest of the system. When the level of the mercury in the manometer had indicated that the line was completely filled with oxygen, stop-cock J was opened so that the oxygen could leave through it, rather than the mercury reservoir. This modification in the vacuum line from that used in previous experiments made it unnecessary to use as large a stream of oxygen as before. With trap G surrounded by liquid air, the sample was then heated with a pure gas brush flame until globules of silver metal remained.

After combustion, stop-cock J was closed and E was turned so that oxygen no longer passed into the system, which was then again evacuated to remove the non-condensable gases. After closing stop-cock F, the liquid air around trap G was removed to allow the sulphur dioxide to fill the line. Usually 200 mg. of silver sulphide was burned. This produced enough gas to give a pressure of approximately

10 cm. The sulphur dioxide was then frozen into the break-seal sample tube with a dry-ice and acetone trap, the system evacuated for a few seconds to remove carbon dioxide and the sample tube sealed off.

While the sulphur dioxide condensed in the trap, C, was evaporating, the used tube D was removed and replaced by another sample and fresh phosphorous pentoxide, and the first part of the system was swept out with oxygen.

C. Mass Spectrometry

The analyses of the sulphur dioxide samples for the isotopic ratios were carried out with a 90-degree mass spectrometer, modified for simultaneous collection and measurement by means of a null method of ion currents due to the isotopic species of sulphur dioxide at masses 64 and 66. The instrument used a magnetic valve system to facilitate the rapid change from one sample to another. The collector assembly was constructed so that only the ion currents of masses 64 and 66 might strike the collector electrodes, thus eliminating any contribution to the ion currents from adjacent masses. Mass 64 is composed of $S^{32}O^{16}O^{16}$, while mass 66 is made up of $S^{32}O^{16}O^{18}$, $S^{34}O^{16}O^{16}$, $S^{32}O^{17}O^{17}$ and $S^{33}O^{16}O^{17}$. The last two species are of such small abundance that their contribution to the 66 ion current may be neglected.

The ion current at mass 64, amplified by a D.C. amplifier, was used as a reference voltage and hence was made the same for both the standard and unknown gas samples by adjusting the gas pressures for

identical mass 64 peak heights on single collection. The put-and-take potentiometer was moved to a balance position on the recorder chart and the sensitivity of the instrument was determined by calibrating the recorder displacement in terms of the percentage difference between two settings of the put-and-take potentiometer. Magnetic sample-valves were used to switch alternately from standard to unknown gases while allowing the recorder chart to run for approximately thirty seconds on each sample.

Since the ion currents obtained from different samples of sulphur dioxide depend on the ratio of the isotopes, any variation in isotopic content from one sample to the other changes the balance point. The percentage difference from the balance position of the standard gas per cm. displacement is calculated in the calibration. Thus, the displacement of the balance position of the unknown from that of the standard can be calculated as the percentage difference between the S^{32}/S^{34} ratio of the standard and unknown gas.

Thus, suppose the displacement is 6.0 cm. and the percentage difference per cm. is 0.2 per cent. Then, the percentage difference of the mass 64/mass 66 ratio of this sample compared to the standard which is 20.236 is $20.236 \times 6.0 \times \frac{0.2}{100}$. This percentage difference is either subtracted or added to the value given to the standard depending on the direction of the displacement. Now,

$$\frac{64}{66} = \frac{S^{32}O^{16}O^{16}}{S^{34}O^{16}O^{16} + S^{32}O^{16}O^{18}} = \frac{1}{2\frac{O^{18}}{O^{16}} + \frac{S^{34}}{S^{32}}}$$

The value of O^{18}/O^{16} in the oxygen used to burn the samples was taken as 0.00208 as previously determined (67).

$$\text{Hence,} \quad \frac{S^{32}}{S^{34}} = \frac{1}{\frac{66}{64} - 0.00416}$$

Thus, by substituting the value for the 66/64 ratio into the above equation, the S^{32}/S^{34} ratio for the sample can be calculated. By this procedure, relative isotopic abundances could be determined with a precision of 0.02 per cent.

RESULTS AND DISCUSSION

Results

The results of the isotope investigation of sulphur in chlorella grown under sterile conditions at four different temperatures are shown in Table III. In column two, three and four, below, S^{32}/S^{34} ratios are reported for organic protein sulphur, plant sulphate and nutrient sulphate, respectively. The plant sulphate is the sulphate obtained after digestion of the chlorella with hydrochloric acid and extraction with a Soxhlet. The sulphate obtained by oxidation of the residue and filtrate after the Soxhlet extraction is considered to be protein sulphur. The value given for the nutrient sulphate is the average of determinations made before, during, and after the growth of the algae.

TABLE III

Isotopic Distribution of Sulphur in Chlorella

Temperature	S^{32}/S^{34} ratio			Fractionation Factor R_3/R_1
	Protein Sulphur R_1	Plant Sulphate R_2	Nutrient Sulphate R_3	
10°C	22.099	22.225		1.002
15°C	22.124	22.232		1.001
20°C	22.132	22.193	22.151 ± 0.008	1.001
25°C	22.143	22.227		1.000

The isotope fractionation factor obtained by comparing nutrient sulphate and the protein sulphur is given in column five. The results obtained show that there is little or no fractionation in the plant metabolism of sulphates by chlorella grown under sterile conditions in the laboratory.

Similar results were obtained with a green alga taken from the shores of Lake Erie and with mustard plants taken from the fields. These results are summarized in Table IV. The same procedures were used to extract protein sulphur and plant sulphate as in the chlorella grown under sterile conditions. In the case of the Lake Erie alga source sulphate is sulphate in the water in which the alga was found; whereas, in the case of the mustard plants, source sulphate refers to sulphate in the soil in which the plants had grown. Alga #1 is that obtained in the summer of 1952, while #2 is that obtained in 1953.

TABLE IV

Isotopic Distribution of Sulphur in Mustard Plants and Lake Erie Alga

Species	S^{32}/S^{34} ratio			Fractionation Factor R_3/R_1
	Protein Sulphur R_1	Plant Sulphate R_2	Source Sulphate R_3	
Lake Erie Alga				
# 1 1952	22.134	22.099	22.139	1.000
# 2 1953	22.104	22.104	22.104	1.000
Mustard Plants				
# 1 Tumbling Mustard	22.158	22.146	---	---
# 2 Wormseed Mustard	---	22.085	22.095	---
# 3 Brassica Kaber	22.222	22.143	22.247	1.001
# 4 Brassica Kaber	22.207	22.153	22.183	0.999

These results show that there is no fractionation of sulphur isotopes in the plant metabolism of sulphates in the natural environment.

With the Lake Erie alga #2, two Carius determinations of the total sulphur were carried out to check the S^{34} content of combined sulphate and protein sulphur. The results obtained were 22.090 and 22.143, giving a value of 22.117 ± 0.027. Although there is a discrepancy of two-tenths of a per cent between the duplicate determinations, the average is within a few hundredths of a per cent of the values obtained separately for protein-bound sulphur and the sulphate in the plants.

Discussion of Experimental Procedure

The analytical procedure used was adopted only after a careful consideration and testing of other methods found in the literature. The leaching of sulphate from plant material with hydrochloric acid according to the method of Balks and Wehrmann was investigated by Stotz (68) and claimed to be quantitative. However, after most of our analyses had been completed, Springer and Quentin (69) claimed that in this procedure some of the organic sulphur was oxidized to sulphate. The percentage oxidized, however, is negligible so we are quite certain that it has no significance in our results.

The extraction of the sulphate from the organic material was virtually complete in eighteen hours. This was proven by carrying out a continuous extraction which was interrupted at three-hour intervals to analyze the amount of sulphate extracted. It was found that in the fifth extraction hardly any sulphate was obtained so that an eighteen-hour extraction should be quantitative.

The method of Bertrand and Silberstein for the oxidation of organic sulphur in plants was proven to be quantitative by Junge (70) by comparison with the Carius method. Thus, there is no isotopic fractionation in this process.

The reduction of the sulphate with the reducing solution of hydriodic acid, hypophosphorous acid and hydrochloric acid was shown to be better than 98 per cent complete. This was accomplished by reducing a weighed amount of sulphate and bubbling the hydrogen sulphide into a solution of cadmium acetate. When hydrogen sulphide was no longer produced, a

known excess of standard silver nitrate was added to the cadmium sulphide and the excess silver nitrate determined by the Volhard method (71) using potassium thiocyanate. The data is given in Table V.

TABLE V

Efficiency of the Reducing Solution

Compound Reduced	Silver Sulphide Produced	
	Theoretical	Experimental
0.1008 gm $Fe_2(SO_4)_3$	0.1873 gm	0.1843 gm
0.2203 gm Na_2SO_4	0.3842 gm	0.3821 gm

Discussion of Results

In the preliminary work carried out by Rowat (60) on the study of the fractionation of sulphur isotopes in the plant metabolism of sulphates, it was reported that no fractionation was found with mustard plants grown under sterile conditions. This is in complete agreement with our work. In a few cases with mustard plants from the field, Rowat reported fractionations from 0.5 - 2.1 per cent. However, he compared the fractionation between plant sulphate and protein sulphur whereas we are comparing source sulphate with protein sulphur. In one case, where the source sulphate's S^{32}/S^{34} ratio is available, the fractionation factor was found to be 0.997 which again agrees with our work. In the case of volatile sulphur, there is some

fractionation, but in our work we were concerned with protein sulphur since volatile sulphur compounds are actually quite rare in the plant kingdom.

In the study of the reduction of sulphates by reducing bacteria (Vibrio desulfuricans), Thode, Kleerekoper and McElcheran (5) found a 1.01 per cent fractionation. The equilibrium constant K=1.074 at 25°C for the isotopic exchange reaction:

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

Thus, in any process in which an isotopic equilibrium is established, a large fractionation will result.

The one per cent fractionation obtained in the reduction of sulphates by bacteria may be due to an isotope effect in some chemical equilibrium process in an enzyme-catalyzed reaction or a kinetic effect where a S^{34} -O bond is broken less readily than a S^{32} -O bond. In the laboratory experiment, the bacteria operated under anaerobic conditions (in a nitrogen atmosphere) and the hydrogen sulphide was swept out. Under these conditions we might expect the reduction to be unidirectional. The absence of significant quantities of intermediates would suggest that the successive steps are very rapid and that the reaction probably goes to completion after the first reduction step. Thus it would seem that we have isotopic fractionation in the first step, whether we have an equilibrium between sulphate and sulphite or a kinetic effect due to the greater probability of rupture of a S^{32}-O bond compared with a S^{34}-O bond.

In the plant metabolism of sulphates, the experiments indicate that there is no fractionation of sulphur isotopes. It appears,

therefore, that we have neither an equilibrium nor a kinetic isotope effect. However, we expect biochemical processes to be universal in nature, and the enzyme-catalyzed steps in sulphate reduction to be the same, so that if there is fractionation in the bacterial reduction of sulphates, a similar fractionation might be expected in plant metabolism. In all probability, the enzyme-catalyzed reactions are similar in the two processes. However, in the initial rate-controlling step, the concentration of sulphate ion may be different in these cases; thus, sulphur isotope fractionation might appear in the first case and not in the other.

Apparently, very little is known about the phenomenon of the diffusion of ions through cell membranes in plants. The governing factors may be relative mass, charge and size. Now, in considering $S^{32}O_4^{=}$ and $S^{34}O_4^{=}$, it is difficult to calculate the effective masses because of insufficient knowledge about the ionic atmospheres. Also, there is no experimental evidence of isotopic fractionation of ions in solution. In the case of the above two ions, there is no difference in charge and a negligible difference in size. Thus, if the diffusion of the ions into the cells is slow enough to be the rate-controlling step, it may be that no fractionation of sulphur isotopes takes place.

Urey (72) reported that some fractionation of the carbon isotopes occurs in plant photosynthesis. Algae and simple plants were found to contain 2.97 per cent less C^{13} than the carbonate of the nutrient in which they grew. Craig (72) found that land plants and tree leaves contained 2.63 per cent less C^{13} than carbonates did, and that petroleum

contained 2.72 per cent less C^{13}, so that the C^{13}/C^{12} ratios of petroleum and terrestrial plants were approximately the same. In some preliminary work carried out in our laboratory on petroleum, it was found that there was no difference in the S^{32}/S^{34} ratio in the organic sulphur in petroleum and sulphate. Thus, the sulphur and carbon results are in agreement inasmuch as they both suggest that petroleum is formed from plant material.

SUMMARY

The procedure for the analysis of green plant material developed by Rowat (60) was modified and improved. A new method for the reduction of sulphate to hydrogen sulphide was adopted and found to be at least 98% complete. A system for the growing of chlorella under sterile conditions was devised. Little or no fractionation was found in the process of sulphate metabolism in plants, either in the laboratory or in nature. In view of the fact that there is enrichment of heavy isotopes in sulphates in nature, this is a very significant result since it appears that, in this particular step of the sulphur cycle, there is no fractionation. This fact may be of significance in the study of the origin of coal and oil.

REFERENCES

1. Thode, H. G. Research 2, 154 (1949).

2. Thode, H. G., Macnamara, J., and Collins, C. B. Can. J. Research B27, 361 (1949).

3. Macnamara, J., and Thode H. G. Phys. Rev. 78, 307 (1950).

4. Szabo, A., Tudge, A. P., Macnamara, J., and Thode, H. G. Science 111, 464 (1950).

5. Thode, H. G., Kleerekoper, H., and McElcheran, D. C. Research 4, 581 (1951).

6. Macnamara, J., and Thode, H. G. Research 4, 582 (1951).

7. Tudge, A. P., and Thode, H. G. Can. J. Research B28, 567 (1951).

8. Macnamara, J., Fleming W., Szabo, A., Thode, H. G. Can. J. of Chem. 30, 73 (1952).

9. Thode, H. G., and Macnamara, J. Mass Spectroscopy in Physics Research, National Bureau of Standards Circular 522 (1953).

10. Thode, H. G., Macnamara, J., Fleming W. H. Geochim. et Cosmochim. Acta 3, 235 (1953)

11. Soddy, F. Chem. Soc. Ann. Rep. 7, 285 (1910).

12. Fajans, K. Phys. Z. 14, 131 (1913).

13. Thomson, J. J. Rays of Positive Electricity, New York (1921).

14. Aston, F. W. Phil. Mag. 38, 707 (1919); 39, 449 (1920); 42, 436 (1921).

15. Dempster, A. J. Phys. Rev. 11, 316 (1918); 18, 415 (1921) 20, 631 (1922).

16. Giaque, W. F., and Johnston, H. L. J. Am. Chem. Soc. 51, 1436, 3528 (1929).

17. Naude, S. M. Phys. Rev. 34, 1498 (1929); 35, 130 (1930); 36, 333 (1930).

18. King, A. S., and Birge, R. T. Nature, Lond. 124, 127 (1929).

19. Urey, H. C., Brickwedde, F. G., and Murphy, G. M. Phys. Rev. 39, 164, 864 (1931).

20. Urey, H. C., and Rittenberg, D. J. Chem. Phys. 1, 137 (1933); J. Amer. Chem. Soc. 56, 1685 (1934).

21. Urey, H. C., and Greiff, L. ibid. 57, 321 (1935).

22. Bradley, C. A., and Urey, H. C. Phys. Rev. 40, 889 (1932).

23. Murphy, G. M., and Urey, H. C. ibid. 41, 141 (1932).

24. Kmeleus, H. J., James, F. W., King, A., Pearson, T. G., Purcell, R. H., and Briscoe, H. V. A. J. Chem. Soc. 1209 (1934).

25. Dole, M., and Slobod, R. L. J. Am. Chem. Soc. 62, 471 (1940).

26. Dole, M. ibid. 57, 2731 (1935).

27. Dole, M. J. Chem. Phys. 4, 268 (1936).

28. Dole, M. Science 83, 434 (1936).

29. Swartout, A. J., and Dole M. J. Am. Chem. Soc. 61, 2025 (1939)

30. Dole, M., and Jenks, G. Science 100, 40 (1940).

31. Smith, S. R., and Thode, H. G. Montreal Report, National Research Council of Canada, MC-57 (1944).

32. Urey, H. C., McKinney, C., McCrea, J., and Epstein, S. Science 108, 489 (1948).

33. McCrea, J. M., J. Chem. Phys. 18, 849 (1950).

34. Urey, H. C., Lowenstam, H., Epstein, S., and McKinney, C. R. Bull. Geol. Soc. Am. 62, 399 (1951).

35. Epstein, S., Bachsbaum, R., Lowenstam, H., and Urey, H. C. Bull. Geol. Soc. Am. 62, 417 (1951).

36. Baertschi, P., Nature 166, 112 (1950).

37. Baertschi, P., and Silverman, S. R. Geochim. et Cosmochim. Acta, 1, 317 (1951).

38. Silverman, S. R. ibid. 2, 26 (1951).

39. Nier, A. O., and Gulbransen, E. A. J. Am. Chem. Soc. 61, 697 (1939).

40. Murphy, E. F., and Nier, A. O. Phys. Rev. 59, 771 (1941).

41. Mars, K. E. J. Geol. 59, 131 (1951).

42. Trofimov, A. V. Doklady. Akad. Nauk. S.S.S.R. 72, 663 (1950); 85, 169 (1952).

43. Wickman, F. E. Geochim. et Cosmochim. Acta. 2, 243 (1952).

44. Wickman, F. E. Nature 169, 1051 (1952).

45. Schoenheimer, R., and Rittenberg, D. J. Biol. Chem. 12, 7285 (1939).

46. Thode, H. G., Macnamara, J., Lossing, F. P., and Collins, C. B. J. Am. Chem. Soc. 70, 3008 (1948).

47. Graham, R. P., Macnamara, J., Crocker, I. H., and MacFarlane, R. B. Can. J. Chem. 29, 89 (1951).

48. Edwards, W. H. M.Sc. Thesis, McMaster University.

49. Urey, H. C. Paper presented at American Chemical Society, New York, September, 1951.

50. Urey, H. C. J. Chem. Soc. 562 (1947).

51. Bigeleisen, J. J. Chem. Phys. 17, 675 (1949).

52. Bigeleisen, J. J. Phys. Chem. 56, 823 (1952).

53. Bigeleisen, J. and Mayer, M. G. J. Chem. Phys. 15, 261 (1947).

54. Rankama, K., and Sahama, T. G. Geochemistry, The University of Chicago Press, 1950.

55. Fuller, H. J. The Plant World, Henry Holt and Company.

56. Miller, E. C. Plant Physiology, McGraw-Hill Book Company, Inc., 1931.

57. Mason, B. Principles of Geochemistry, John Wiley and Sons, Inc., 1952.

58. Urey, H. C. The Planets, Their Origin and Development, Yale University Press, 1952.

59. McElcheran, D. C. M.Sc. Thesis, McMaster University (1951).

60. Rowat, D. L. M.Sc. Thesis, McMaster University (1952).

61. Balks, R., and Wehrmann, O. Bodenkunde u Pflanzernahr, 9-10, 646 (1938).

62. Bertrand, G., and Silberstein, L. Compt. redus. 189, 86 (1929).

63. Szabo, A. M.Sc. Thesis, McMaster University (1950).

64. Wallouch, R. Unpublished work, McMaster University.

65. Pepkowitz, L. P., and Shirley, E. L. Anal. Chem. 23, 1709 (1951).

66. Luke, C. L. ibid. 15, 602 (1943).

67. Thode, H. G., and Smith, S. R. National Research Council of Canada, Montreal Report MC 57 (1944).

68. Stotz, H. Bodenkunde u pflanzernahr, 6 69 (1937).

69. Springer, R., and Quentin, K., Biochem. Zeits. 322, 486 (1952).

70. Junge, H. Bodenkunde u Phlanzernahr, 19, 346 (1940).

71. Kolthoff, I. M., and Sandell, E. B. Textbook of Quantitative Inorganic Analysis, The Macmillen Co.,(1947).

72. Urey, H. C. Science 108, 489 (1948).

73. Craig, H. Geochim. et Cosmochim. Acta 3, 53 (1953).