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THE UNIVERSITY OF ALBERTA

OXYGEN ISOTOPE STUDIES IN SULPHATE

by

(C) Robert N. Hunt

A THESIS

Submitted to the Faculty of Graduate Studies and Research

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Abstract

This thesis attempts for the first time to understand some aspects of oxygen and sulphur isotope fractionation during the reduction of sulphate to sulphide by synergetic pairs of organisms.

The reduction of sulphate by graphite was examined and careful attention to detail has significantly improved the reproducibility and precision of oxygen isotope measurements in sulphate. The author also contributed to the program of evaluating isotopic abundance ratios by the application of a PDP-8 computer to the ion current measurement system of the mass spectrometer.

Oxygen and sulphur isotopic abundance ratio determinations for unreacted sulphate and sulphur data for the H_2S product were made during four synergetic reductions of sulphate by Bacillus 8P and Clostridium Dm3 . Both normal and inverse kinetic isotope effects were observed. Data for the unreacted sulphate yielded a 3.82 ± 0.05 ratio for the δS^{34}: δO^{18} values. No correlation was observed between the H_2S final product and the sulphate data because of a build up of intermediates and the fastidious nature of these organisms. Despite recent evidence that the δO^{18} value of the unreacted sulphate is related to the isotopic composition of the water, it is believed that this approximately 4:1 enrichment ratio has particular significance.

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CHAPTER I OXYGEN ISOTOPE ABUNDANCES IN SULPHATE

1.1 Introduction

Oxygen isotope studies are proving to be one of the most useful tools for studying the environment and the interactions of rocks, minerals, and water over a wide range of geological conditions. Most of the major oxygen-bearing mineral groups have been studied in detail, such as the carbonates, silicates, and oxides. One of the common rock forming mineral groups which has received little attention yet is present in many physical, chemical, biological, and geological processes, is the sulphate group.

Historically several analytical procedures have been developed for the extraction of oxygen from silicates and other oxygen compounds for precise isotopic analysis. Such studies often make it possible to define environments with greater detail than conventional techniques. In particular, the oxygen and sulphur isotope abundance ratios in sulphate reveal information concerning processes in the oxygen and sulphur cycles in the ocean-atmosphere system.

Since the work of SZABO, TUDGE, MACNAMARA and THODE (1950), variations in the S^{34}/S^{32} abundance ratios in sulphates have been routinely measured for over twenty years. It has long been realized that complementary O^{18}/O^{16} abun-

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dance data could assist in the interpretation of sulphate geochemistry and biochemistry. However, it has only been during the past few years that investigators have carried out extensive oxygen isotope analyses.

1.2 Sulphate Ion Oxygen Isotope Exchange

Because sulphates are important primary and secondary minerals in sedimentary rocks, and in addition can be found in igneous and metamorphic rock systems, it became necessary to learn more of the fundamental oxygen isotope behavior. If sulphate is to be recovered from a variety of solutions and mineral assemblages, it is neces- sary to know what exchange takes place under experimental conditions involved in the recovery of sulphate for oxygen isotopic measurements.

The early work of TEIS (1956) indicated that oxygen isotopes exchanged very slowly between dissolved sulphate and water in near neutral solutions at earth surface temp- eratures. For example, the half period for exchange of Na_2SO_4 in neutral solutions was found to be about 70 years, the full period for near equilibrium being reached in about 450 years. HOERING and KENNEDY (1957) pointed out, however, that the exchange between sulphate and water was accelerated considerably in concentrated acid solutions. Under quite adverse chemical conditions, RAFTER (1967) found negligible

alteration to the isotopic composition of sulphate during ex-
traction procedures which would be encountered during routine
laboratory analyses.

Confirming the general observations of TEIS (1956),
LLOYD (1968) found the exchange rates of sulphate and water
were very slow in normal geological environments (97 per
cent exchange of oceanic sulphate with ocean water having a
mean temperature of $4^{\circ}C$ and a pH of 8.2 would require of the
order of 250,000 years).

TEIS (1956) also examined the exchange of sodium
sulphate and carbon dioxide gas. At $900^{\circ}C$, the half period
was found to be 2.2 hours and by extrapolation to ordinary
temperatures (about $20^{\circ}C$), the half period of exchange was
estimated to be slightly more than 10,000 years.

Thus it can be concluded that in most experimental
and naturally occurring procedures involving the sulphate ion,
the oxygen isotopic exchange rates are extremely slow in
sulphate - water and sulphate - CO_2 systems. At earth
surface conditions, it may require about 10^3 to 10^5 years to
approximate near oxygen isotope exchange equilibrium. With
such a slow exchange, one would expect the isotopic values
of the sulphate to reflect a long term average environmental
condition rather than the "instantaneous" environmental con-
ditions. As such, sulphate can be extracted in the labora-
tory without fractionation of the oxygen isotopes.

1.3 Terrestrial Variations of Oxygen Isotope Abundances in Sulphates

The oxygen isotopic abundance variations of sulphate in nature are dependent upon the history of the sulphate ion exchange in the sulphur cycle. HOLSER and KAPLAN (1966) calculated the time of residence for sulphur in the sea to be about 21 million years. This would be equivalent to more than 80 half-times of exchange. Therefore one would probably expect oceanic sulphate to be in isotopic equilibrium with ocean water. But if the oxidation-reduction turnover of sulphate is responsible for preventing the establishment of isotopic equilibrium, then from HOLSER and KAPLAN (1966), about 30 per cent of the sulphate oxygen passes through the sulphur cycle over a time period probably less than 50,000 years. This represents more than one half of the oxygen found in the present atmosphere, and hence suggests that the sulphur cycle could be one of the important factors regulating the oxygen balance in the ocean-atmosphere system.

With the development of a quantitative and reliable method [RAFTER (1967), LONGINELLI and CRAIG (1967), and LLOYD (1967)] for the extraction of oxygen atoms from sulphate for precise isotopic analysis, the complementary O^{18}/O^{16} isotopic ratios opened up unlimited and almost totally unexplored possibilities for the study of oxygen isotope abundance

ratios for sulphate in geochemical and biochemical environments. The purpose of the research turned towards being able to know the range of isotopic composition of the dissolved sulphate, to check the possible existence of some relation between the oxygen isotopic composition of sulphate and that of the waters in which it is found, and to obtain information of the origin of the sulphate and its history.

RAFTER and MIZUTANI (1967a) examined the oxygen isotopic abundance ratios of sulphate and water from Lake Vanda in the Antarctic. The S^{34}/S^{32} ratio in the sulphate was found to increase approximately four times faster than the O^{18}/O^{16} ratio in the sulphate with depth. Also the O^{18}/O^{16} ratio in the sulphate increased at twice the rate as in the water. A problem arose concerning interpretation. The assumption made at that time was that biological fractionation caused such an enrichment. The establishment of such a correlation for sulphur-34 and the oxygen-18 content of these sulphates led to more extensive work in an attempt to understand the variations of oxygen-18 in naturally occurring sulphates.

LONGINELLI (1968) found no relation between the O^{18}/O^{16} ratios of sulphate and water and temperatures in waters from 34 thermal springs in Tuscany (Italy). CORTECCI and LONGINELLI (1968) found a positive correlation existed for oxygen-18 in sulphate and some lake waters. The results

obtained were concluded to be probably caused by several different factors - one of these factors being a wide spread microbiological activity. Later, LONGINELLI and CORTECCI (1969) found, for two rivers, variations almost always in a direction of positive enrichment in the heavy isotopes (the oxygen-18 and sulphur-34). In contrast to RAFTER and MIZUTANI (1967a), a comparison of S^{34}/S^{32} to O^{18}/O^{16} yielded a slope which varied from 0.6 to 0.7 with time.

LLOYD (1968) did attempt some bacterial reductions of sulphate, but encountered difficulties. It was found difficult to control the rate at which the bacterial reduction progressed. Also the medium would become poisoned with excess hydrogen sulphide after a time, and the reduction would stop. Thus none of the experiments went to completion. From the data obtained with both pure cultures and natural mixtures of bacteria, LLOYD (1968) did indicate that there was a preferential consumption of oxygen-16 by the bacteria.

MIZUTANI and RAFTER (1969) attempted to establish the relationship between sulphur-34 and oxygen-18 values in the sulphate employed for the study of the bacterial reduction of sulphate. They also wished to test the relationship between the oxygen-18 values of sulphate and the water in which the sulphate was formed during the bacterial oxida-

tion of sulphur. In the bacterial reduction of sulphate, MIZUTANI and RAFTER (1969) found that at any stage of the reduction, the remaining sulphate was enriched in both oxygen-18 and sulphur-34. The ratio of the sulphur-34 enrichment to the oxygen-18 enrichment was approximately 4:1 . In the bacterial oxidation of sulphur, very little difference was observed between the oxygen-18 value of any remaining sulphate and that of the water in which the sulphate was found. Such a correlation was most probably the result of the hydrogen sulphide formed from the bacterial reduction becoming available for the bacterial oxidation to sulphate.

The oxygen and sulphur isotopic abundance ratios of some gypsums and evaporites were reported by SAKAI (1972). This work discussed the relationship between the two isotopic species in terms of geological ages of samples from Precambrian to the present. A somewhat similar study was performed by LLOYD (1973), where he analyzed interstitial water from cores. The latter study only presented data for the oxygen in the sulphate, but no meaningful conclusions were forthcoming. The isotopic composition with depth was very interesting, but further work is necessary before meaningful conclusions can be made.

1.4 Present Investigation

At the outset of this project, investigations of O^{18}/O^{16} abundance ratio in sulphates left the following not completely understood:

(a) the terrestrial range of oxygen isotop. variations in sulphates,

(b) the extent and rate of exchange of oxygen atoms,

(c) the kinetic isotope effects involved in the production and reduction (chemical and biological) of sulphates, and

(d) the lack of reproducibility in oxygen isotopic abundance data.

In many cases not only isotope effects, but also basic mechanisms involved were not fully understood. For example, the complex processes of sulphate reduction by bacteria are not yet fully comprehended.

In the present investigation, it was decided to pursue two problems. The first was an examination of the reduction of sulphate by graphite with the view of obtaining consistent oxygen isotope abundance data (Chapter II). The second problem was to effect oxygen isotope fractionation during bacterial sulphate reduction and to compare any effects observed to those obtained during the sulphur isotope fractionation (Chapter IV). The resultant effects

found in both cases would be used as an aid in the under-standing of the mechanisms involved in the microbiological reduction of sulphate (Chapter V).

As seen in Section 1.3 , there were independent developments during the course of the present investigation which are related to the problem selected. However, the approaches taken and the emphasis of the present work are markedly different. For example, MIZUTANI and RAFTER (1969) observed isotope effects during microbiological sulphate reduction under experimental conditions quite different from those of the present work. They utilized several flasks of natural mud and sea water. A given flask was opened at a specified time and its contents examined isotopically. The experiments of the present investigation contrasted to those conditions in several ways. Pure isolates of a spec-ific organism were used as opposed to the complex natural mixture of organisms employed by MIZUTANI and RAFTER (1969). The medium used for the present study was very simple in contrast to the unknown complex medium constituting their "mud".

In any one of the experiments undertaken, one large reaction vessel was used (rather than many flasks), gaseous products were constantly flushed and collected, and the liquid contents periodically examined. Thus, the present investigation was able realistically to evaluate chemical and isotopic balances. The experimental conditions

of MIZUTANI and RAFTER (1969) were quite relevant to the terrestrial situation. On the other hand, the present approach was more orientated towards elucidating fundamental microbiological mechanisms involved in the bacterial reduction of sulphate.

CHAPTER II GRAPHITE REDUCTION OF SULPHATE TO CARBON DIOXIDE

2.1 Review

It is relatively simple to extract oxygen atoms from the sulphate ion and react them with graphite. However, it is very difficult to effect 100 per cent conversion to carbon dioxide, and to avoid oxygen isotope exchange with other molecules. Both of these difficulties must be over-come in order to determine the oxygen isotopic composition of sulphates with good reproduceability.

It would appear that the techniques employed for the oxygen extraction from sulphate have paralleled those used for determining the oxygen isotope abundances in silicates and other oxygen bearing compounds.

One of the pioneering efforts in this regard was conducted by MANION, UREY and BLEAKNEY (1934). In their determination of oxygen isotope abundances in silicates, a mixture of the silicates and carbon was reacted with carbon tetrachloride at $1000^{\circ}C$, whereupon the carbon monoxide formed was converted into water by combustion with hydrogen. The oxygen obtained by the electrolysis of the water was then analyzed. Somewhat later, ATEN and HEVESY (1938) reduced sulphate at $900^{\circ}C$ with some finely ground, sub-ignited coal. In this determination, the mixture of CO_2-CO-H_2 which formed

was converted into water, over a nickel catalyst at 350°C. The resultant electrolysis then yielded oxygen which was analyzed.

From that time until recently, the analytical procedures for the extraction of oxygen were all based on one of the following two types of reactions;

(1) the reduction by carbon at high temperatures (1600 to 2000°C) to yield carbon monoxide, and

(2) the oxidation by fluorine or some halogen fluoride to yield molecular oxygen.

The carbon reduction technique was further modified by HALPERIN and TAUBE (1952) in their studies of oxygen isotope abundances in barium sulphate. They used five times as much graphite as sulphate, where this mixture was placed in a platinum crucible and raised to 1000°C by induction heating. About 75 per cent of the oxygen was converted to CO_2 and the remainder to CO. However, only the CO_2 was analyzed. The reduction method was refined by SWANDER (1953) who converted the oxygen of silicate minerals and rocks to CO in a thermal reaction

$$SiO_2 + 3C \rightarrow SiC + 2CO$$
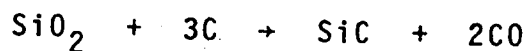
which was quantitative when powdered silica was mixed with graphite and heated in a vacuum to 2000°C. However, when

mixed samples containing alkali metals, alkali earths or aluminum were employed, oxygen yields were accompanied by large isotope fractionations. This method was modified again by CLAYTON and EPSTEIN (1958) to determine the isotopic composition of oxygen in natural oxides of silicon and iron. The carbon monoxide formed was converted to CO_2 using a nickel catalyst and then analyzed in the mass spectrometer. Spectrographically pure graphite was used by VINOGRADOV, DONTSOVA, and CHUPAKHIN (1958) as well as DONTSOVA (1959), to decrease the effects of fractionation previously found. In particular, this improvement now made available a reliable analysis of the alumino-silicates, which had not yet been successfully analyzed by the graphite method of reduction.

A few isotopic analyses of some naturally occurring sulphates were carried out by TEIS (1956) who reduced the sulphate at $900^{\circ}C$ with some finely ground coal, and converted the $CO_2 + CO + H_2$ so formed into water over a nickel catalyst at $350^{\circ}C$, which was then analyzed for oxygen-18. It appears that no further work on the carbon reduction method for the recovery of oxygen as the more stable carbon dioxide was published until 1967.

On the other hand, oxidation techniques were employed by SILVERMAN (1951), and BAERTSCHI and SILVERMAN (1951), to yield molecular oxygen quantitatively from sili-

cate rocks. Two methods developed by these studies were:

(1) the employment of chlorine trifluoride and hydrogen fluoride at 430°C, and

(2) fluorine and hydrogen fluoride at 420°C.

For most rocks and minerals, these were sufficient but for basic and ultra-basic rocks, the yield was about 80 per cent.

TUDGE (1960) later used chlorine pentafluoride for orthophosphate and condensed phosphates. The product, molecular oxygen, was more manageable when converted to CO_2, by the reaction

$$C + O_2 \rightarrow CO_2$$
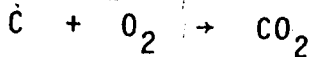
CLAYTON and MAYEDA (1963) as well as LONGINELLI (1965) used bromine pentafluoride for silicates and phosphates, respectively, since it was easier and safer to handle than fluorine. Also it reacts with some minerals which do not react completely with fluorine.

Therefore, by the mid-sixties, the carbon reduction and the fluoride oxidation processes were well established for the quantitative extraction of oxygen from silicates and other oxygen compounds for the determination of O^{18}/O^{16} ratios. These techniques evolved with a preference for the product CO_2 and not CO. This was more suitable, since;

(1) CO is not stable and will disproportionate into CO_2 plus C, whereas CO_2 is stable and only reduces in part to CO in the presence of carbon at a high temperature (above $900^{\circ}C$).

(2) CO_2 is less of a health hazard than carbon monoxide,

(3) CO and N_2 have the same molecular mass range (28) and cannot be separated in the mass spectrometers usually employed in isotope abundance studies, and

(4) CO requires higher temperatures to be the major product of a reduction ($1600-2000^{\circ}C$) whereas CO_2 becomes the major product at lower temperatures (about $1000^{\circ}C$).

LLOYD (1967) ground sulphate samples with spectrographically pure graphite in excess (ten-fold). Then a pellet of the mixture was formed. This was heated in a graphite crucible to $1000^{\circ}C$ by an induction heater with both carbon dioxide and carbon monoxide being the gaseous product. The gas so formed was passed through a nickel catalyst furnace where the carbon monoxide was converted to carbon dioxide, and the total CO_2 product then collected by freezing in liquid nitrogen. LLOYD (1967) also found that the most important factor in obtaining good yields and isotopic reproducibility,

was that the sulphate should be in the form of barium sul-
phate. This is because other sulphates (such as calcium
sulphate) will produce SO_2 and COS in addition to CO and CO_2.

AGGETT, BUNTON, LEWIS, LLEWELLYN, O'CONNOR, CHAR-
MAIN, and ODELL (1965) made use of the catalytic conversion
of carbon monoxide to carbon dioxide through utilization of
the equilibrium

$$2CO \rightleftharpoons CO_2 + C$$
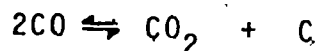
for isotopic analysis of oxygen in organic compounds. They
also pointed out that at low pressures and lo, temperatures
this reaction would normally be too slow, since formation of
CO_2 is favored by low temperature and high pressure. This
difficulty can be best overcome by the use of a high voltage
discharge between two parallel plates. These are most effec-
tive, when used in a vessel partially immersed in liquid
nitrogen so that the CO_2 can be condensed as soon as it is
formed.

LONGINELLI and CRAIG (1967) precipitated sulphate
as barium sulphate and dried it for 3 hours at $120^{\circ}C$ in a
vacuum furnace. The barium sulphate was reduced with excess
spectrographic graphite (ten to fifteen-fold) by induction
heating to $1100^{\circ}C$ in a graphite crucible with lid. Previous
to this, however, the sample had been degassed at a few hun-
dred degrees for fifteen minutes before being reduced at

elevated temperature for 45 minutes. Any CO_2 that was formed was continually condensed, since the heating chamber was connected to a liquid nitrogen trap. The residual CO was converted to CO_2 by sparking with a Tesla coil above a cold trap, using two platinum sheets as electrodes.

RAFTER (1967) also used barium sulphate, however intimately mixed with the pure graphite. This mixture was placed in platinum boats with lids, and initially outgassed at $500^{\circ}C$ *in vacuo,* then heated to $1100^{\circ}C$ in a micro-furnace. As the two products formed, the CO_2 condensed in the first nitrogen cold trap but the CO formed was passed on to a discharge tube with copper plates, immersed in a cold trap to condense the CO_2 formed during the discharge reaction. The two condensed samples were then combined for mass spectrometric analysis.

RAFTER (1967) felt that a lower temperature range (about 900-1050$^{\circ}C$) was adequate for the graphite reduction of sulphates of sodium, potassium, calcium, strontium, and barium. This work realized a higher production of carbon dioxide at these lower temperatures, the reaction being essentially

$$BaSO_4 + 2C \rightarrow BaS + 2CO_2 .$$
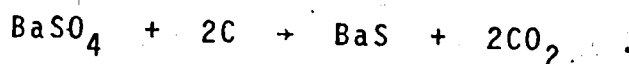
This investigation showed that at elevated temperatures the carbon dioxide-graphite reduction was indeed measurable. After

three hours at $1000^{\circ}C$, 12 per cent of the CO_2 had been reduced to CO. For sulphur isotope studies the recovery of sulphur as BaS is unaltered if there is any carbon monoxide present, but it is vital that all of the sulphate oxygen be recovered as carbon dioxide for oxygen isotope abundance measurements.

RAFTER and MIZUTANI (1967a) further modified the technique of RAFTER (1967) with respect to recovery refinements. They suspected that when the barium sulphate was heated over a gas burner in drying, some of the variability noted ($\pm$ 0.29 $^{\circ}/oo$) was due to the reduction of this sulphate in the gas flame and its reoxidation by the atmospheric oxygen, or possibly an exchange reaction between the carbon dioxide in the gas flame and the sulphate. As a result, drying was thereafter a slow process over an electric hot plate, and only before weighing out portions for analysis. This change in technique led to somewhat more reproducible ($\pm$ 0.18 $^{\circ}/oo$) results. It was this same reduction and subsequent reoxidation by atmospheric oxygen that prompted RAFTER (1967) to avoid filtration of the sulphate on filter paper before its ignition.

If the references are examined in detail, it is found that parameters other than temperature and pressure affect the ratio of the $CO-CO_2$ production. Whereas RAFTER (1967) obtained yields of 16 per cent CO and 75 per cent CO_2 with platinum crucibles, LONGINELLI and CRAIG (1967) found variable

yields from 98 per cent CO_2 with a new crucible, down to about 80 per cent after ten reactions using graphite crucibles. This latter technique reduced the reaction time for the

$$2CO \rightarrow CO_2 + C$$
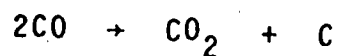
process. LONGINELLI and CRAIG (1967) verified this by heating carbon dioxide of known composition over the graphite crucible until about one half of it was reduced to carbon monoxide. The CO was sparked back to CO_2 and within the precision of the volume readings (to one per cent), all the CO_2 was recovered and the isotopic composition of the oxygen was unchanged ($\pm$ 0.01 ^{o}/oo). Gas chromatographic analysis further showed the final product of the sulphate reduction to be pure carbon dioxide when pure barium sulphate was used. (However, natural barite crystals resulted in the presence of a small amount of sulphur dioxide in the CO_2.)

LONGINELLI (1968) noted a very significant factor, in that standard samples for intercalibration of the measurements are badly needed among the different laboratories. This is basically due to the experimental fact that different techniques of conversion to CO_2 of the CO produced in the reaction between barium sulphate and graphite can cause slight isotopic differences in the final samples.

While the development of oxygen isotope studies in sulphate continued, SAKAI and KROUSE (1971) discussed the reduction technique further. This work pointed out significant memory effects, which were caused by oxygen isotope exchange between the product CO and the hot quartz walls. This effect is negligible when samples possessing a narrow isotopic spread are analyzed (for standard deviations of the order of $\pm$ 0.1 ^{o}/oo). However, with sulphate of widely varying oxygen isotope compositions, the reproducibility of the isotopic determinations can deteriorate to greater than $\pm$ 0.5 ^{o}/oo, despite satisfactory yields. Thus a systematic investigation of the sample preparation was necessary. SAKAI and KROUSE (1971) realized that this was not a problem of mass spectrometry, but rather a lack of precision in duplicating the oxygen isotope abundance ratio of the same sulphate sample. Their long term observations revealed that a tube of quartz, which displayed signs of devitrification after prolonged usage, produced larger memory phenomena than a relatively new tube. NORTHRUP and CLAYTON (1965) had earlier observed an oxygen isotope exchange between carbon dioxide and glass at temperatures above 250°C. This CO_2, SAKAI and KROUSE (1971) noted, should not be an effective participant in exchange phenomena during the conversion. This is apparent, since it is rapidly frozen out of the system by the liquid nitrogen trap. But the other gaseous pro-

duct, carbon monoxide, does remain as a gas phase, and until converted would have a better opportunity to exchange oxygen isotopes with the hot quartz walls. Furthermore, one should not be misled by the amount of carbon monoxide which remains at the end of the heating cycle, since this represents only a fraction of the CO which actually forms during the earlier stages of the reaction. One possible solution involves a minimum of three subsequent conversions to assure the desired reproducibility for a particular sulphate sample (as was done in this thesis). Although the duplication of analyses should always be carried out, often in cases where the material is rare, only one measurement is possible. Thus the solution is really to eliminate memory effects. This is only a possibility if a more efficient CO conversion unit is developed. The simplest and most direct form of internal heating is that of making the platinum boat the location of highest resistance in an electrical circuit. This was done by SAKAI and KROUSE (1971) in conjunction with a water jacket around the quartz tube to greatly reduce oxygen isotope exchange with the quartz wall. This new apparatus showed no signs of memory effects when samples differing in oxygen-18 values by over 22 per mil were analyzed.

The complete procedure for the evaluation of the oxygen and sulphur isotope abundance ratios in the sulphates involves four possible operations. They are:

a. the chemical precipitation of the sulphate in
   the preferred form of barium sulphate,

b. the reduction of the $BaSO_4$ preferably to Ag_2S
   as the final product,

c. the subsequent burning of the Ag_2S to form SO_2,
   for mass spectrometric analysis of the sulphur
   isotope variations, and,

d. the reduction of the $BaSO_4$ to CO_2 for the
   oxygen isotope measurements.

All four of these procedures are reliable in that they cause
no appreciable fractionation of the isotopes. The chief
disadvantage, however, is the requisite time and labor. Such
preparative procedures do constitute a major part of a re-
search program which is focused on the study of sulphur and
oxygen isotopes variations in sulphate. HOLT and ENGELKEMEIN
(1970) described a method by which $BaSO_4$ is rapidly converted
to SO_2 in one operation. Thus the time and effort which is
consumed in the earlier techniques now can be drastically
reduced. Here the barium sulphate is converted to sulphur
dioxide by simply covering the sulphate with pulverized
quartz powder in a fused quartz tube. Then in a vacuum it
is heated to the softening point of quartz ($1400^{\circ}C$), resul-
ting in

$$2BaSO_4 \rightarrow 2BaO + 2SO_2 + O_2 .$$

The SO_2 produced is collected in a cold trap. The oxygen is pumped away and the BaO fuses with the silica surroundings. This method of conversion does not assure a uniform oxygen-18 abundance in the sulphur dioxide, as do previously discussed techniques, but a correction is used for the oxygen-18 interference in the mass spectrometric analysis. HOLT and ENGELKEMEIN (1970) observed no inherent source of interfering impurity associated with this thermal decomposition of pure $BaSO_4$ in the quartz environment.

Although the thermal decomposition of $BaSO_4$ did appear to reduce the sample preparation time and labor, the building of such an apparatus was not justified in view of the non-uniform oxygen-18 abundances encountered with this method. As a result, the proven laborious techniques were continued and the high voltage discharge conversion of CO to CO_2 retained.

2.2 Apparatus Used in the Present Study

Literature describing sulphate reduction processes continually avoids details of the operating procedures. Such methods were impossible to duplicate because of the lack of precision involved in defining seemingly unimportant but controversial steps when one attempts to follow such analyses. As a result, the following description of sulphate to CO_2 conversion will strive to clarify every detail of the method.

The high voltage discharge conversion unit, adopted for the conversion of CO to CO_2, was built into a vacuum system as shown in Figure 2-1. The reduction furnace (F) contained an element (E), made of self-bonded silicon carbide. The "Crusilite" (Norton Company, Worchester, Mass.) heating element was tubular in shape and had a 47 mm I.D. and 55 mm O.D. In the center of the 14" overall length, was a 6" "hot zone" (H). This region of high temperature was formed by cutting a spiral in the element such that the "cold ends" are not spiralled. This action produced a much lower resistance zone at either end of the element, with the center region of highest resistance. There were no mechanical joints so as to ensure no possibility of failure due to mechanical dissimilarities. The "Crusilite" elements were glazed to provide an increased resistance to oxidation. Because of this glaze, excellent temperature control and reproducibility was available - the element has not been replaced after four years of continual use. The glaze prevents rapid aging (at 1100^oC the rate of resistance change was about 10 per cent per thousand hours, or an estimated 25 per cent per thousand hours at 1400^oC).

Temperatures in excess of 1250° were realized when 20 amp at 100 v was applied. The power supply (20 amp, 140 v variac) used was directly wired to the element support sleeves at one end of the doubly spiralled muffle. This type was
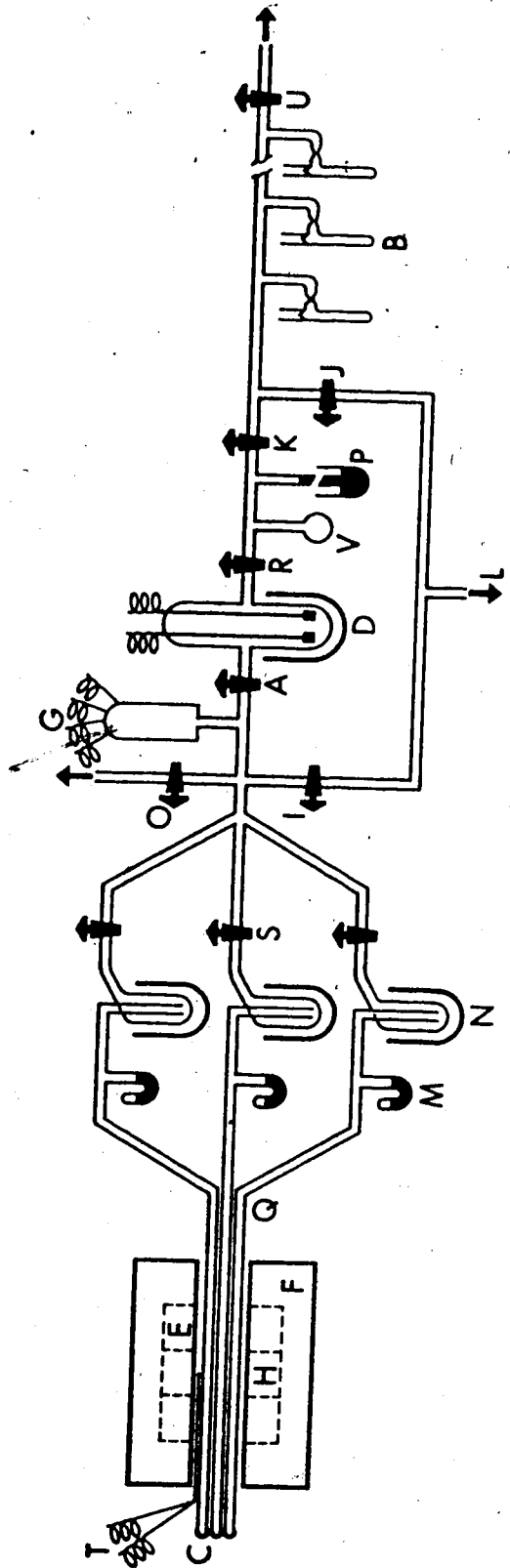
Figure 2-1/ Apparatus for the graphite reduction of sulphate and the CO conversion to CO_2 :

advantageous in that both electrical connections were at the same end, leaving free access to the other end. The element was mounted horizontally, thus allowing the freedom to expand and contract in the firebrick furnace. The element's terminal connectors protruded beyond the furnace face so as to permit good ventilation and prevent overheating.

A Pt-Pt (13 per cent Rd) thermocouple (T) was inserted in the "hot zone" (H) between the inner surface of the element (E) and the quartz tubes (Q) inside. Temperatures higher than $1250^{\circ}C$ were only attempted once, since the quartz combustion tubes (Q) began to soften at these and more elevated temperatures. The three quartz tubes (Q) had ground glass caps (C) through which the specimen could be introduced to and removed from the "hot zone" (H) of the furnace. Temperature control was solely dependent upon the readings from the thermocouple temperature recorder (T).

The quartz tubes (Q) were each monitored qualitatively by a mercury manometer (M) attached to each combustion tube. A liquid nitrogen cold trap (N) was also connected to each chamber so that the carbon dioxide which formed was condensed as soon as it was formed in the respective quartz combustion chambers (Q). With stopcocks (S) closed, each sample in the system could be monitored by the respective pressure manometers (M). One of the three stopcocks (S) was usually

allowed to remain open, thus permitting the CO gas formed in that tube to expand further into the vacuum gauge head (G). As the reaction in the tubes proceeded, the manifold pressure reading could be obtained for one of the three samples being reduced. But thi limitation was minimized by using specimens of about the same size. These manifold readings were most helpful in that they indicated the rate of CO production as well as when the formation had reached completion - no further decrease in the pressure as recorded by the vacuum gauge (G).

As the CO_2 formed, it was frozen down in the liquid nitrogen traps (N) immediately. The CO produced during the reduction still remained between the ground glass caps (C) and the stopcocks (S) - except for the monitored sample. This CO gas was now available for the conversion to carbon dioxide in the high voltage discharge conversion unit (D). With the vacuum gauge (G) turned off, stopcock A was then opened to allow the CO to enter the conversion unit (D). When the reaction

$$2CO \rightarrow CO_2 + C$$
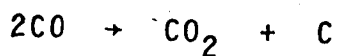
was completed, the vacuum head (G) was turned on to record a quantitative indication of the completeness of the conversion

and also the reduction of the sulphate specimen.

Once the CO to CO_2 conversion was finished, liquid nitrogen was placed around the calibration volume (V). When stopcock R was then opened, the CO_2 from the reduction could be transferred to this calibration volume. If stopcock R was subsequently closed and the cold liquid nitrogen bath removed, a volumetric calculation of the CO_2 gas can be made knowing the recorded manometer pressure (P), and the volume contained between the stopcocks (R and K) including the total volume when these stopcocks were closed. After the CO_2 volume was recorded, the stopcock K was opened allowing the gaseous CO_2 to expand towards the breakseal line (B). With the use of a liquid nitrogen bath, the sample was subsequently removed from the system, and was available for later mass spectrometric analysis. Both stopcocks I and J led to conventional pumping equipment (in this case, a mercury diffusion pump connected to a rotary vacuum pump). These were installed to permit the rapid evacuation of the apparatus.

High voltage for the CO to CO_2 conversion unit was supplied by a 4.5 Kv transformer. The electrodes of the unit were made of platinum, 2 cm by 4 cm, and 0.003 inch in thickness. The ground glass cap, through which the electrode leads passed, was removed when it was considered desirable to clean the platinum electrodes of accumulated carbon. It was found

necessary to cease sparking when a pressure reading was required to monitor progress of the reaction. Otherwise, the gaseous ionization excited the current carrying conductors of the pressure gauge, resulting in erroneous pressures.

Breakseals (B) were attached to the calibration volume (V) for the removal of the carbon dioxide produced by the graphite reduction of sulphate. To replace them, a stopcock (U) was opened to allow the breakseal line pressure to rise to atmospheric pressure. Stopcock (O) permitted the rapid replacement of samples to be reduced in the quartz combustion tubes. Both operations can be completed and the line evacuated with a minimum of disturbance to the rest of the system.

2.3 Method

About 50 mg of $BaSO_4$ (dried overnight at 120°C to expel any water present) was weighed accurately and then transferred to a small agate mortar. Following the same care in weighing and the transferral, about 50 mg of spectrographically pure graphite was weighed (after drying overnight at 900°C *in vacuo*) and transferred to the agate mortar. The specimen and graphite were then intimately mixed and transferred into a previously dried (overnight at 120°C) and weighed platinum boat.

This platinum boat was approximately 4 cm in length, 1 cm in depth, and about 1.5 cm wide. Lids, also platinum, were made for the boats and dried and weighed in the same manner as the boats.

The boat, containing the sample and graphite, was reweighed to obtain the exact weight of the mixture, since about 2 mg were lost during the mixing and transferral processes. The lid was then placed securely on the boat and its contents before another weighing of the sample. The lid always covered completely the boat and its contents, after a suggestion from LONGINELLI (private communication). This procedure gave consistently better yields.

The loss due to the mechanical mixing and transfer was usually of the order of 2 mg, independent of the sample size. This loss was most consistent throughout the entire work of this thesis. That is, as can be seen from Table 2-1, it was entirely the care given to the process that determined the amount of sample and graphite lost.

The boat, plus lid and contents, was then placed in one of the quartz combustion tubes (within the "hot zone") of the reduction furnace. That quartz tube was subsequently evacuated. This was done by first greasing the ground glass cap and then placing it over the quartz tube end after the

Table 2-1   Loss of graphite and sulphate due to
mixing and transfer

Date	Boat	Weight (mg)	Before Mixing (mg)	After Mixing (mg)	Loss (mg)
7/5/70	A	1393	1531	1529	2
	B	1278	1414	1413	3
	C	1428	1562	1562	0
13/5/70	A	1392	1520	1519	1
	B	1278	1410	1409	1
	C	1429	1569	1566	3
15/5/70	A	1392	1530	1531	0
	B	1278	1436	1436	0
	C	1429	1535	1534	1
22/5/70	A	1394	1534	1532	2
	B	1278	1431	1429	2
	C	1430	1539	1539	0

average loss = 1.3 mg

boat had been inserted. Now the appropriate stopcock (S) was opened after closure of stopcocks I and J, to ensure that the reduction chamber was most efficiently evacuated as well as maintaining the remainder of the vacuum system at about 0.001 Torr. Once the first specimen was introduced, the temperature was raised to $500^{o}C$ from the stand-by temperature of 400°C. Thus any water vapor or gases produced or present in the tube were pumped away. Similarly two more specimens were prepared and introduced into the furnace and the evacuated system. The three samples were then left for about 15 minutes to ensure no residual gases were present. It was considered advisable at this stage of the reduction process to close stopcocks I and J to permit observation of the vacuum gauge reading (G) to detect the presence of any residual gas production.

Once no residual gas pressure was detected, dewar flasks containing liquid nitrogen were placed around the three CO_2 traps (N). If a combustion tube was to be monitored, as was always done, then a flask of liquid nitrogen must also be placed around the conversion unit (D). With two of the stopcocks (S) closed and the other open to the tube being monitored, stopcock A was checked to be open and R was now closed. Thus the monitor chamber was open to the vacuum gauge as well as the conversion unit. The furnace was then raised to 1100°C, which required approximately 25 minutes,

and was held at that temperature a further 15 minutes to en-
sure completeness of the reduction. It was turned down to
400°C once the reaction was finished.

As the carbon dioxide formed, it condensed in the
liquid nitrogen traps (N). Any carbon monoxide produced was
registered on the manometers (M). Always one of the three
was the monitor - usually the first introduced into the
quartz tubes. Thus the progress of the reduction (i.e. CO
production) in that reaction tube was observed as the pres-
sure in the quartz combustion chambers rose. When the pres-
sure reached 0.05 Torr, the vacuum gauge was turned off to
prevent erroneous readings (as well as possible damage to
the vacuum tube circuit) when the high voltage discharge (D)
was turned on. As evidenced by the visible gas glow, sparking
was continued until it was ascertained that the CO to CO_2
conversion had proceeded to completion.

The two remaining samples were still contained by
their stopcocks (S), since only one specimen could be con-
verted to carbon dioxide in the discharge chamber at a time.
The monitored pressure usually remained below 0.5 Torr
throughout the experiment. The pressure was seen to drop
markedly as the high voltage discharge enabled the

$$2CO \rightarrow CO_2 + C$$
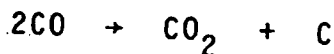
reaction to proceed. Here the discharge was terminated before the vacuum gauge (G) was turned on again, and vice versa, throughout the conversion of gaseous carbon monoxide to the stable carbon dioxide. It required about 15 minutes at elevated temperature, with alternate reading of the pressure, and sparking, until the residual gas was a sufficiently small fraction of the total CO_2 produced. This residual gas was then pumped away.

The liquid nitrogen dewar from the monitor CO_2 trap (N) was removed to allow the CO_2 condensed in the monitored trap (N) to evolve and condense in the discharge chamber, which at this time was still enclosed in a liquid nitrogen bath. Then the vacuum gauge (G) was turned off and the CO to CO_2 conversion unit discharge was engaged once more. This sparking time was extended considerably if the pressure in the system had risen even slightly after the transfer, thereby converting any final traces of carbon monoxide to carbon dioxide. This was easily noted by recording the vacuum head pressure before and after the evolution and subsequent discharge. Now that the reduction to gaseous carbon dioxide was complete, the conversion system (i.e. the monitor combustion tube, the vacuum gauge, and the CO - CO_2 conversion unit) was evacuated to less than 0.001 Torr. This CO_2 produced from the graphite reduction of

sulphate was hence ready for volumetric calibration and its subsequent extraction from the reduction line.

It should be noted that after three hours at $1000^{\circ}C$, about 12 per cent of the CO_2 produced would have been reduced to CO. Such a reduction would have occurred at the hot crucible containing unreacted excess graphite. This was avoided since the CO_2 traps (N), partially immersed in liquid nitrogen, forced the CO_2 to condense as soon as it was formed. (For sulphur isotope studies, the recovery of sulphur as BaS was unaltered if there was any CO present. But it was vital that all of the sulphate oxygen be recovered as CO_2, if the equation

$$BaSO_4 + 2C \rightarrow BaS + 2CO_2$$
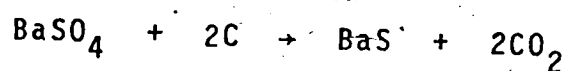
was to be used for the study of oxygen isotopes in sulphate.)

With the conversion system at 0.001 Torr and the CO_2 frozen in liquid nitrogen trap about the discharge chamber, another dewar of liquid nitrogen was placed around the calibration volume (V). Then the CO_2 gas, produced from the graphite reduction of sulphate, was allowed to expand into the calibration colume after closing stopcock K and opening stopcock R. On removal of the cold trap around the conversion unit, the gas evolved into the calibration volume where it was immediately condensed. After the transferral was complete, stopcock R was closed and this cold trap also removed so that the CO_2 could

then expand, thus allowing a calibration of the volume of gas evolved. This manometer reading gave a preliminary check on the completeness of the reduction. A liquid nitrogen flask was placed under a breakseal before the gas was transferred to the breakseal line by the opening of stopcock K. Once the product of the reduction was frozen down in the breakseal (B), stopcock K was closed as a precaution, since small leaks could develop from removal of the breakseal. The breakseal was subsequently removed. The volumetric and breakseal sections were now evacuated and the breakseal line checked, to ensure that no leaks had developed from careless removal or some weakness in the glass after its removal. Once completely sure of no leaks, the stopcock K was opened and the pressure recorded to ensure as perfect a vacuum as possible. The other two samples were then run in exactly the same manner.

After the removal of each CO_2 sample, the temperature of the furnace was noted. Usually after the extraction of the three specimens, the furnace was cool enough to prevent any partial reduction of newly introduced samples. Thus the combustion tubes were opened to the atmosphere gently through stopcocks O and S, after all three specimens were extracted. Once these chambers were at atmospheric pressure, the three groundglass caps (C) were removed and the plati-

num boats were then extracted. Careful removal of these
boats was essential since it was necessary to reweigh them
to obtain another estimate of the completeness of the reac-
tion. This estimate, however, is only valid if most of the
product of the reaction was CO_2, but does give a good indi-
cation of the percentage CO initially in the quartz combus-
tion tubes. That is, it can give the percentage of CO pro-
duced from the reduction of sulphate by graphite. When the
reduced specimens were removed, new samples were then intro-
duced as previously mentioned. If the contents of the old
platinum boats, that is, the BaS, was to be examined for its
sulphur isotope ratios, then the residue from the boats was
leached with warm water and filtered into $AgNO_3$, so that the
resulting silver sulphide could be burned.

2.4 Standards, Correction Factors, and Reproducibility

SILVERMAN (1951) defined the zero of his isotopic
scale to be oxygen from a single sample of sea water, "Hawai-
ian sea water No. RT6". In the published data of CLAYTON and
EPSTEIN (1958) and TAYLOR and EPSTEIN (1962), the standard
was defined to be the oxygen with an O^{18}/O^{16} ratio equal to
0.98473 R_p, where R_p was the O^{18}/O^{16} ratio in SILVERMAN'S
(1951) sample of Potsdam sandstone. If any of the analytical
procedures introduce systematic errors into the measured values,

then normalization to a quartz standard should make the results of different analytical methods agree on the quartz samples. This is however not necessarily true of other minerals and rocks.

The oxygen of mean ocean water is a logical standard for the reporting of oxygen isotope variations in natural materials, including meteoric waters, rocks, and minerals. Such a standard has been proposed by CRAIG (1961) for natural waters and by CLAYTON and CRAIG (1962) for rocks and minerals. A "standard mean ocean water", that is SMOW, was defined to have a

$$O^{18}/O^{16} \text{ (SMOW)} = 1.008 \, O^{18}/O^{16} \text{ (NBS - 1)} ,$$

where NBS - 1 is a water sample distributed by the National Bureau of Standards. This defines an ocean water standard in terms of materials readily available to everyone, rather than in terms of the limited quantity of a particular sample of Potsdam sandstone.

All the oxygen isotope abundance measurements are expressed in parts per thousand with respect to SMOW ("standard mean ocean water") as defined by CRAIG (1961). The usual terminology is used, where δ ("del") is the deviation in parts per thousand ($^O/oo$) of the O^{18}/O^{16} ratio from that of the standard. That is, the per mil isotopic ratio enrichment in a sample relative to the standard, can be expressed as

$$\delta\ O^{18}\ (^o/oo)\ =\ \frac{(O^{18}/O^{16}{}_{sample}\ -\ O^{18}/O^{16}{}_{std})}{(O^{18}/O^{16}{}_{std})}\ \cdot\ \frac{1000}{1}$$

$$=\ (\frac{O^{18}/O^{16}{}_{sample}}{O^{18}/O^{16}{}_{std}}\ -\ 1)\ \cdot\ \frac{1000}{1}\ .$$

CRAIG (1961) introduced the more practical SMOW standard in terms of the PDB standard, where SMOW was defined as

$$\delta\ O^{18}{}_{sample}\ =\ 1.0409\ \delta\ O^{18}{}_{sample}\ -\ 40.92\ ^o/oo\ .$$

wrt SMOW   wrt PDB

The PDB scale referred to the O^{18}/O^{16} ratio in the CO_2 gas evolved from the reaction of H_3PO_4 with PDB carbonate. This Chicago standard for CO_2 was produced from PDB calcium carbonate by reaction with 100 per cent H_3PO_4 at 25.2^oC. (The PDB was a Cretaceous belemnite, from the Peedee formation of South Carolina.)

CRAIG (1957) derived correction factors applicable in the conversion of ion abundance ratio differences to the specific isotope ratio differences. These correction factors, for the mass spectrometric analysis of CO_2, arise because for the analysis of oxygen we measure the mass - 46 beam versus

the combined mass - 44 plus mass - 45 beam. Hence the ratio in terms of the isotopic molecules is:

$$\frac{C^{12}O^{16}O^{18} + C^{13}O^{16}O^{17} + C^{12}O^{17}O^{17}}{C^{12}O^{16}O^{16} + C^{13}O^{16}O^{16} + C^{12}O^{16}O^{17}}$$

whereas the desired ratio, assuming the distribution to be purely a statistical one, is:

$$\frac{C^{12}O^{16}O^{18} + C^{13}O^{16}O^{18}}{C^{12}O^{16}O^{16} + C^{13}O^{16}O^{16}} = \frac{C^{12}O^{16}O^{18}}{C^{12}O^{16}O^{16}}$$

As a result, the measured isotopic ratio for oxygen must be corrected for the other isotopic species. The correction must be such that

$$\delta O^{18} = 1.0014 \, \delta O^{18} - 0.009 \, \delta C^{13} \quad ,$$

which is the correction factor used for oxygen analysis made against the PDB standard. Thus, about 1 per cent of the δC^{13} difference enters into the oxygen correction factor.

Using CRAIG'S (1957) correction factor as well as CRAIG'S (1961) "standard mean ocean water" (SMOW) reference, the isotopic reproducibility was examined for the reduction of sulphate by graphite, yielding CO_2 as the final gaseous product. The volume of oxygen that was recovered as CO_2 was always

greater than 94 per cent of the theoretical value, hence $BaSO_4$ was considered to be quantitatively reduced. Any CO formed in the reduction process was completely converted to CO_2; this was acertained by the volumetric check on reaction completeness.

It was then necessary to check the isotopic reproducibility. A sample of sea water sulphate was selected for the reproducibility tests. These results yielded an average $\delta\ O^{18}$ value of +9.38 $^o/oo$ with respe t o SMOW (Table 2-2). The precision during a mass spectrom t c analysis was typically ± 0.05 $^o/oo$, while the reproducibility over preparations of the same specimen was ± 0.12 $^o/oo$ (the standard deviation). The preparation and reduction reproducibility were better than the ± 0.18 $^o/oo$ achieved by RAFTER and MIZUTANI (1967a). Thus the techniques employed in the present investigation were considered to be satisfactory for studies of oxygen isotope abundance variations in sulphate.

2.5 Investigations on the Graphite Reduction of Sulphate

The graphite reduction of sulphate was considered quantitative and reproducible when the procedures previously outlined were employed. Other workers also achieved reasonable reproducibility but with a wide variety of techniques. These investigators seemed to have their own biases

Table 2-2 Oxygen isotopic composition of sea water sulphate

Specimen Number	Weight of BaSO$_4$ (mg)	CO_2 Volume (cc at NTP) Theo.	Exp.	$\delta\,O^{18}\,(SO_4^=)$ ($^o/oo$) wrt SMOW	Deviation From + 9.38 AVG. ($^o/oo$)
WOS 2000	55	10.5	10.2	9.16 ± 0.02	-0.22
WOS 2001	44	8.4	8.3	9.34 ± 0.09	-0.04
WOS 2003	67	12.8	12.3	9.43 ± 0.07	0.05
WOS 2004	64	12.2	12.1	9.42 ± 0.07	0.04
WOS 2007	66	12.6	12.5	9.38 ± 0.12	0.0
WOS 2008	49	9.4	9.5	9.31 ± 0.03	-0.07
WOS 2009	56	10.7	10.6	9.38 ± 0.01	0.0
WOS 2011	51	9.7	9.6	9.46 ± 0.06	0.08
WOS 2012	43	8.2	8.3	9.17 ± 0.03	-0.21
WOS 2013	38	7.2	6.9	9.27 ± 0.05	-0.11
WOS 2015	57	10.9	10.7	9.53 ± 0.06	0.15
WOS 2017	49	9.4	9.0	9.52 ± 0.08	0.14
WOS 2018	70	13.4	12.7	9.53 ± 0.04	0.15
WOS 2020	44	8.4	8.2	9.39 ± 0.05	0.01
WOS 2021	72	13.7	13.0	9.46 ± 0.02	0.08
				9.38 0.05 *	±0.12 **

Mean $\delta\,O^{18}$ = 9.38^o/oo

* Mean of measurement errors = ± 0.05 o/oo

** Overall deviation (preparation reproducibility and mass spectrometric error) = ± 0.12 ^{o}/oo

as to the choice of crucibles, $CO-CO_2$ conversion units, and C/S ratios. There appeared to be no logical basis for these choices.

For the present investigation it was considered worthwhile to study the effects of varying the C/S ratio, that is, the ratio of the amount of graphite C (mg) to that of sulphate S ($BaSO_4$ in mg)

$$\frac{C}{S} = \frac{\text{mg of graphite before mixing}}{\text{mg of } BaSO_4 \text{ before mixing}} \quad ,$$

since similar studies used markedly different graphite to sulphate ratios (section 2.1). The 15 samples used for the reproducibility study (Table 2-2) all had a C/S ratio of about 1.0, as suggested by RAFTER (1967). On the other hand, nine specimens not listed in Table 2-2 had radically different oxygen isotope compositions. In these samples, the C/S ratio was varied from 0.64 to 3.25. The oxygen isotopic abundance ratios of these variant nine sea water sulphate samples are summarized in Table 2-3 and Figure 2.2. Since all other variables remained constant, the data of Figure 2-2 seemed to imply that the C/S ratio had a significant effect upon the isotopic composition of the CO_2 produced by the reduction of sulphate.

Table 2-3   Oxygen isotope composition of nine
variant sea water sulphates

Specimen Number	Weight BaSO$_4$ (mg)	C/S Ratio	$\delta\,O^{18}\,(SO_4^=)$ (°/oo) wrt S.M.O.W.		Dev From +9.37 (°/oo)
WOS - 2002	56	1.00	10.85	0.07	1.48
WOS - 2005	70	1.00	10.89	0.07	1.52
WOS - 2006	49	1.00	10.91	0.09	1.54
WOS - 2010	77	0.64	8.79	0.08	-0.58
WOS - 2014	52	1.54	10.82	0.03	1.45
WOS - 2016	43	1.96	11.53	0.07	2.16
WOS - 2019	28	3.25	10.02	0.08	0.65
WOS - 2022	44	1.05	9.96	0.03	0.59
WOS - 2023	32	1.09	10.60	0.07	1.23

(NOTE: Samples WOS - 2002, 2005, and 2006 were only evolved carbon dioxide. The carbon monoxide produced in these cases was pumped away to permit a determination of the isotopic composition of the CO_2 product only.)
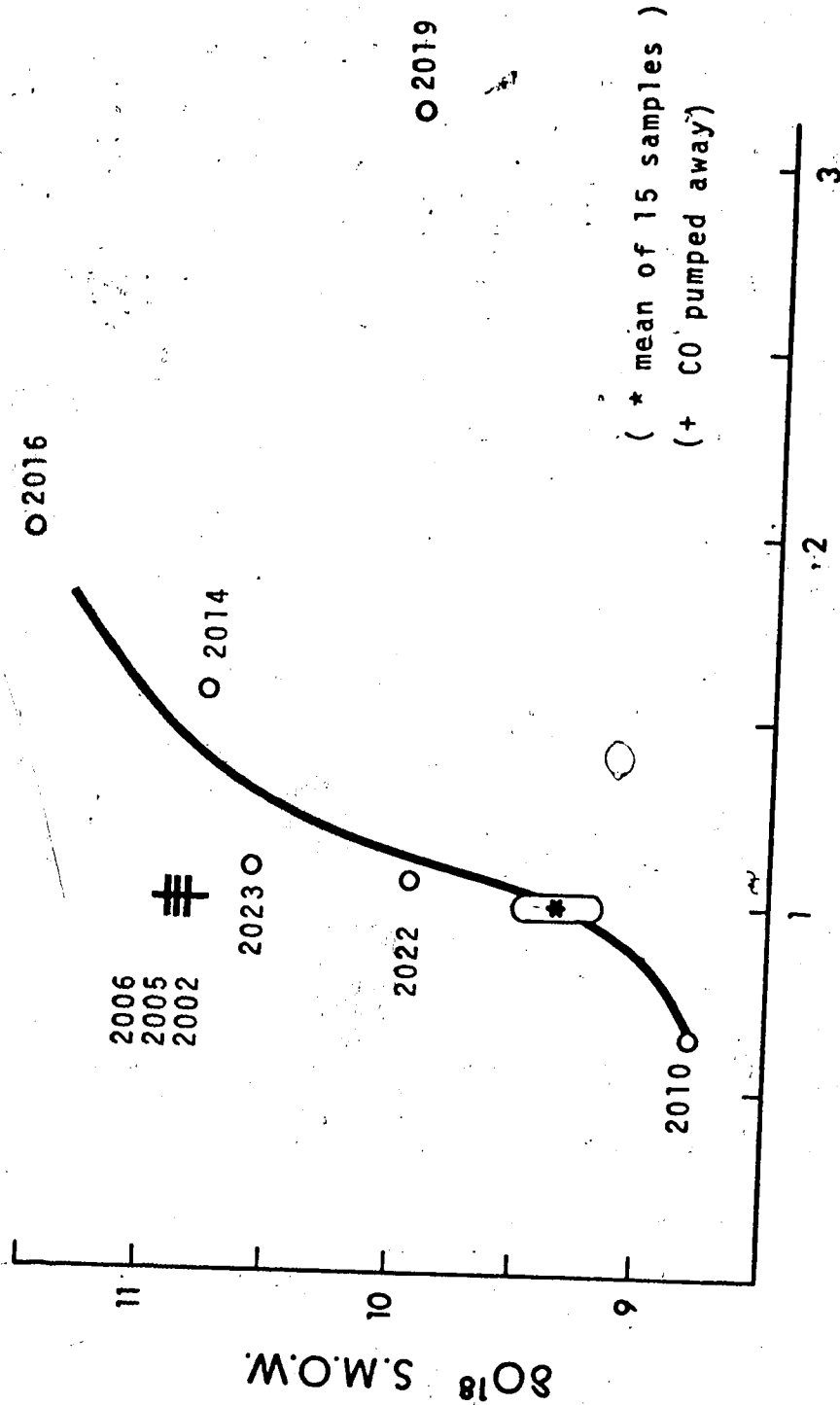
Figure 2-2   Oxygen isotopic composition of nine variant sea
water sulphates.

(* mean of 15 samples)
(+ CO pumped away)

with graphite. It should be noted that three results were not anomalous, since after the CO_2 condensed in the cold traps, the CO product formed at the end of the reduction was pumped away. These three samples, WOS - 2002, WOS - 2005, and WOS - 2006, all had a C/S ratio of about 1.0. These three specimens, used to obtain the oxygen isotope abundance ratio of the evolved CO_2, neglected the CO which formed simultaneously. The δO^{18} value of the CO_2 produced was higher than expected, which means that the CO was enriched in the lighter isotope relative to the CO_2. It was only when the CO was sparked and converted into CO_2 that the lower value of about +9.38 $^o/oo$ was reached. It should be noted that the collected CO_2 in these three cases was heavier by about 1.5 $^o/oo$ (the CO consequently 1.5 $^o/oo$ lighter). With approximately 94 per cent recovery, sparking would have left the remaining unreacted CO heavier by about

$$\frac{94 \ (1.5 \ ^o/oo)}{6} = + 23.5 \ ^o/oo .$$

Thus three of the variant nine samples were processed so as to obtain the oxygen isotope abundance ratio of only the CO_2 produced, ignoring any contribution from the CO evolved. The remaining six were not in any manner designed to be anomalous or at variance with the aims of the reproducibility study. Hence a closer look at these six was mandatory.

Any explanation for such widely varying isotopic abundance ratios of the sea water sulphate samples must consider the relative proportions of carbon and sulphate mixed prior to the reduction process., The C/S ratio was usually kept about 1.0 only as a result of RAFTER's (1967) implication. This relative proportion was in fact stochiometrically more than sufficient, since the reaction

$$BaSO_4 + 2C \rightarrow BaS + 2CO_2$$

required only the ratio described in the above equation. In such a case the C/S ratio stochiometrically necessary was simply

$$\frac{2C}{BaSO_4} = \frac{2(12)}{137 - 96} = \frac{24}{233} = 0.10 \quad .$$

That is, stochiometrically the reaction should have proceeded to completion if there was approximately one part graphite for every ten parts $BaSO_4$ (by weight). Any C/S ratio greater than 0.1 completely satisfied the stochiometric requirements. The question then arose, why were variations of the C/S ratio causing the observed discrepancies in the oxygen isotope abundance ratios, considering the fact that this was about 10 times the stochiometric ratio required? Also, was this then a reaction where stochiometry was applicable?

Before making hasty conclusions concerning the requirements of the primary product of the reduction, it was necessary to also consider the second gaseous product of this reduction. Most probably the CO formed such that

$$BaSO_4 + 4C \rightarrow BaS + 4CO$$
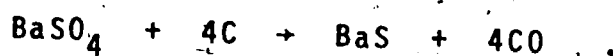
In this case the C/S ratio would be about 0.20. For some combination of the two reactions, which was the observed result for such a reduction of sulphate by graphite, then the reaction could have been of the form

$$BaSO_4 + 3C \rightarrow BaS + 2CO + CO_2 ,$$
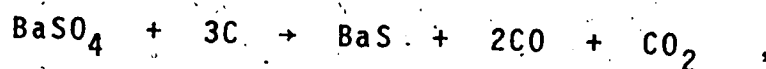
balanced for primarily CO evolution. In the present study, the relative amounts of gaseous product were in the order of about 15 per cent CO and about 85 per cent CO_2. This reaction was approximately

$$13BaSO_4 + 28C \rightarrow 13BaS + 4CO + 24CO_2$$
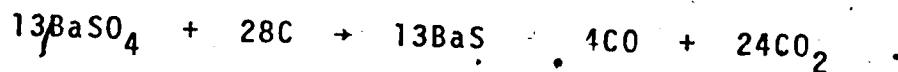
Stochiometrically, the C/S ratio would be only 0.07. That is,

$$\frac{28C}{13BaSO_4} = \frac{14(2C)}{19(BaSO_4)} = \frac{14}{19}(0.10) = 0.07$$

The requirements were relatively unchanged from the previous estimate in which the gaseous final product was just carbon dioxide.

Thus, stochiometric considerations and the known products of the reduction did not seem to explain the experimental isotopic ratio differences. The only conclusion left, was that the reaction proceeded to near completion (that is, greater than 94 per cent reaction) without doing so stochiometrically. This implied some form of mechanical parameters, such as a dependence upon the availability of nearby atoms of graphite. This involved physical contact availability, since the mixing was only a mechanical process, and definitely not a chemical procedure. Here the reduction could have possibly been controlled by the presence or absence of contact carbon atoms which would permit the removal of oxygen atoms from barium sulphate. Neither pellets nor compression of the sulphate and carbon sample, to increase the physical availability of graphite, were investigated. Such procedures would have ensured good physical contact and most probably would have decreased the C/S ratio required to cause a quantitative reduction. Hence compression of the graphite and sulphate after mixing, and the degree of compression, could have altered the C/S ratio necessary to cause the observed variations in the oxygen

abundance ratios.

If mechanical mixing caused the discrepancies recorded in Table 2-3, then an excess of contact graphite was expected to increase the amount of CO formed relative to the CO_2 gaseous product. This interpretation was based on the C/S ratios calculated to yield either a total CO or CO_2 final product. A C/S ratio of 0.10 was found to be stochiometrically sufficient for a CO_2 final product, whereas a ratio of 0.20 was required for CO to be the final product. Thus stochiometrically, more graphite was required to produce a final product of CO than CO_2. Although the reduction did not proceed in a totally stochiometric fashion, the C/S ratio implications were believed applicable for the mechanical mixing of graphite and sulphate. In other words, the graphite reduction of sulphate was both mechanical and stochiometric in nature, with possibly physical contact the most dominant factor. As such, graphite excesses were expected to yield greater quantities of CO. This was confirmed by observation of the sparking time as well as by evaluation of the per cent CO product. This per cent CO formation was calculated using the known weight of the platinum boat and its contents after the reaction had gone to completion. For example,

Sample WOS - 2010   boat plus lid   = 1393.2 mg

BaSO$_4$ =   77.1 mg

C   =   48.5 mg

C/S   =   0.65

total weight before   = 1518.8 mg

after reduction   = 1489.5 mg

reactants   =   29.5 mg

available oxygen for reduction = 77.1 mg $(\frac{64}{233})$ =. 21.16 mg

carbon stochiometrically required = 21.16 mg $(\frac{24}{64})$ =   7.94 mg

stochiometric reactants = 29.10 mg

The measured reactants weighed 29.5 mg while the stochiometric requirements were only 29.1 mg. Hence 0.4 mg of extra carbon was used. Since the total graphite used was 8.34 mg, then the.

$$CO \text{ per cent production} = \frac{(8.34 - 7.94)}{(8.34)} (100\ \%) = 4.8\ \% .$$

Therefore per cent CO formed was about 5 % .

With an excess of graphite available for the reduction of sulphate, a marked increase in CO production was observed (Table 2-4). If a C/S ratio of about 1.0 was employed, the per cent CO was normally in the order of 15 per cent whereas the CO_2 production was about 85 per cent of the

Table 2-4   Per cent CO production for sea
water sulphates reduced with graphite

Specimen Number	C/S Ratio	Dev. From +9.38 °/oo	Per Cent Prod.	Per Cent Reaction
WOS - 2010	0.64	-0.59	5	94
WOS - 2000	0.91	-0.22	8	97
WOS - 2001	1.00	-0.04	13	99
WOS - 2003	1.00	0.05	9	96
WOS - 2004	1.00	0.04	12	99
WOS - 2007	1.00	0.0	21	99
WOS - 2008	1.00	-0.07	14	101
WOS - 2009	1.00	0.0	12	99
WOS - 2011	1.00	0.08	16	99
WOS - 2012	1.00	-0.21	10	101
WOS - 2013	1.00	-0.11	15	96
WOS - 2015	1.00	0.15	23	98
WOS - 2017	1.00	0.14	10	96
WOS - 2018	1.03	0.15	19	95
WOS - 2020	1.03	0.01	15	98
WOS - 2021	1.03	0.08	29	95
WOS - 2022	1.05	0.58	27	97
WOS - 2023	1.09	1.22	49	95
WOS - 2014	1.54	1.44	42	95
WOS - 2016	1.96	2.15	46	94
WOS - 2019	3.25	0.64	63	94

final gaseous product. However, when C/S ratios removed from 1.0 were employed, the relative amounts of CO and CO_2 were much different. The effects of varying the C/S ratio can be seen more clearly when the data of Table 2-4 is plotted as in Figures 2-2 and 2-3. Generally there was an increase in $\delta.O^{18}$ and per cent CO production with increasing C/S ratios (Figures 2-2 and 2-3 respectively). These observations were somewhat dependent upon the per cent reaction obtained during the reduction process (Figure 2-4), when C/S ratios other than 1.0 were employed.

It was demonstrated that if the C/S ratio was greater than 1.0, more than 15 per cent of the gaseous product was CO, with increasing amounts of CO as the C/S ratio rose. Since more CO was present, more $CO \rightarrow CO_2$ conversion was required. It was observed that since lengthy conversions often yielded results which were not quantitative. The variant samples all had relatively low yields (about 94 per cent reaction), and generally large amounts of CO were formed. Although the reductions were quantitative, the O^{18}/O^{16} isotopic ratios did reflect the CO abundance caused by the larger C/S ratios investigated in this study.

More reductions were carried out at C/S ratios less than 1.0, but the majority were not quantitative. (It should be noted that no other investigation ever employed

o
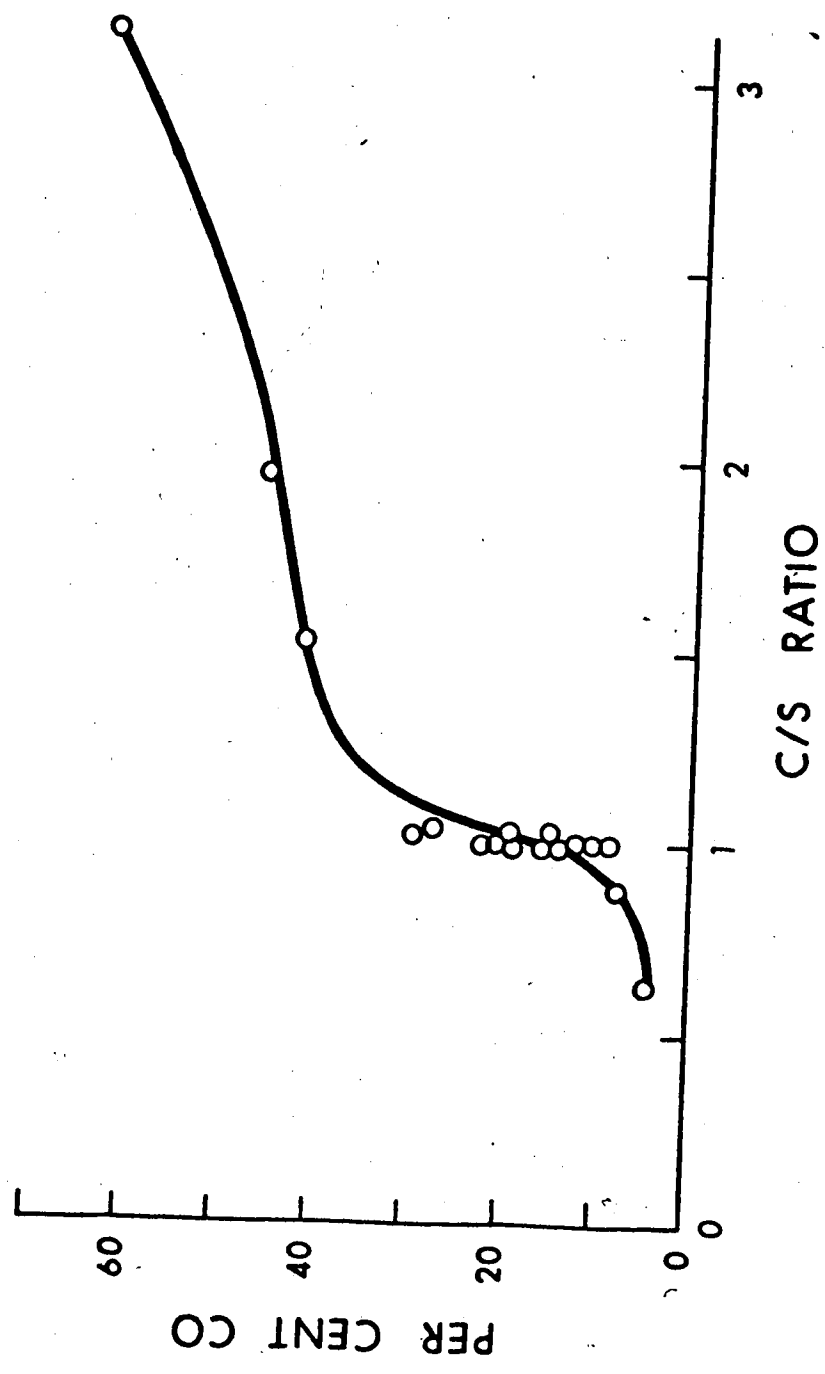
Figure 2-3 Per cent CO production and the c/s ratio.

55
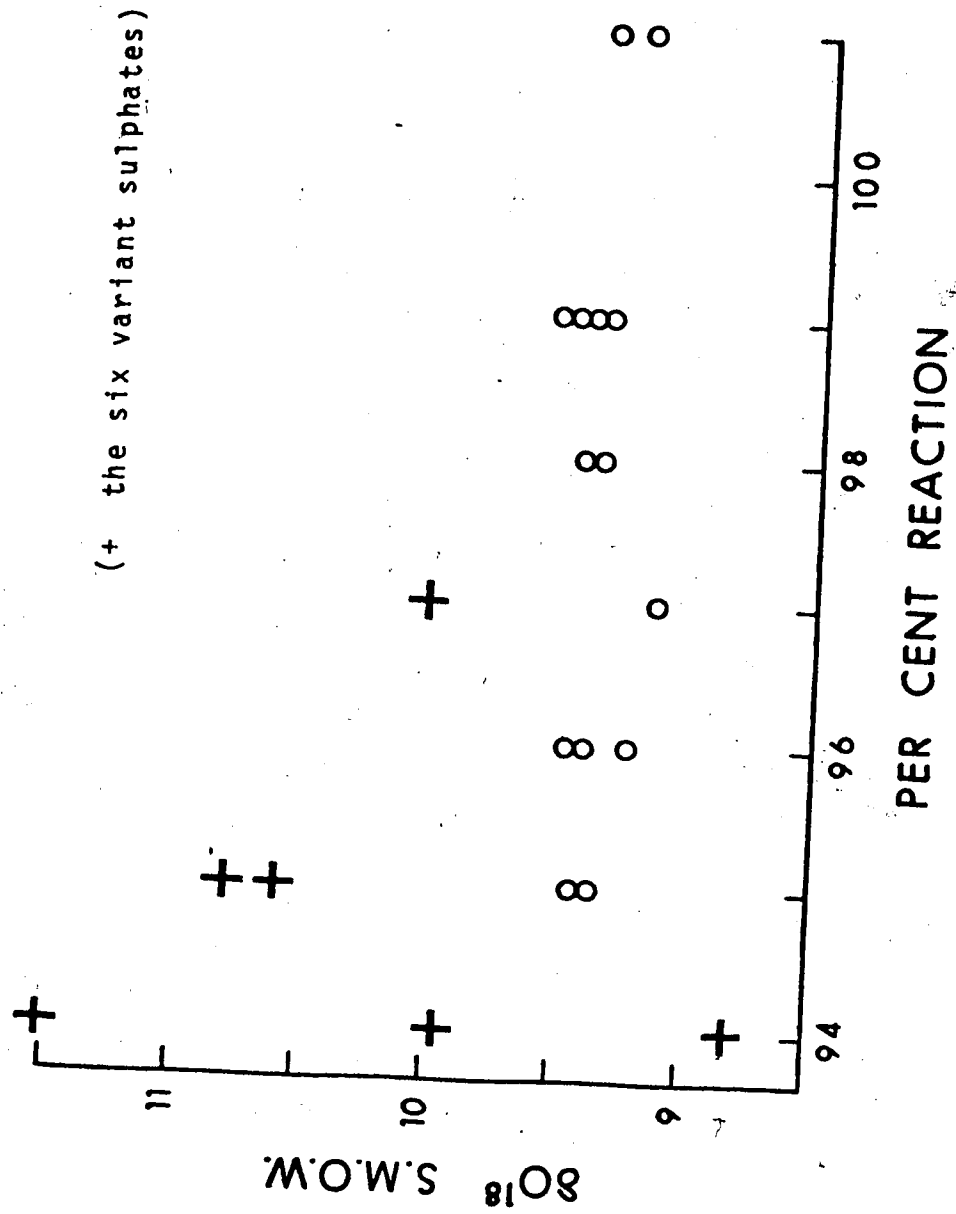
Figure 2-4 $\delta\,O^{18}$ values and the per cent reaction.

a C/S ratio less than unity.) A deficiency of contact graphite atoms was believed to be an explanation for the poor yields. Incorporation of further mixing while the reaction proceeded, or compression of the graphite and sulphate sample before the reduction process, would probably have reduced the numerical value of the optimum C/S ratio, but not the variations in CO formation.

RAFTER (1967) stated that the graphite reduction of sulphate occurred within the temperature range 900 to $1050^{\circ}C$, and observed that graphite reduction was measurable at $960^{\circ}C$. Longinelli (private communication) found that 50 per cent of his CO_2 had been produced at $900^{\circ}C$. Therefore it was concluded that the reduction of sulphate by carbon was well under way by about $900^{\circ}C$. In such a reduction where CO evolution has been shown to be of utmost importance, it was decided to monitor the CO formation as a function of the reduction temperature.

The vacuum gauge used throughout the reduction process, was employed to measure pressure variations as the reaction proceeded. As the reduction began, the decrease in pressure was in reality a measure of gas evolution during the reduction of the sulphate. The evolved gas was considered to be totally CO, since any CO_2 formed was immediately condensed as soon as it was produced. Therefore the vacuum

readings evaluated the qualitative amounts of CO formed.

In those cases where the C/S ratio was such that there was not sufficient contact graphite to ensure that $BaSO_4$ molecule was totally stripped of its oxygen atoms, possibility of other gas evolutions should not be ignored. In addition to CO and CO_2 formation, undesirable products such as SO_2 and some CSO derivatives may have on present. These were for the most part trace products, since the reactions presented (Table 2-4) were all quantitative. Although these undesirable gases were most probably present, they were ignored when considering the recorded pressure readings as evidence of CO formation.

The production of CO was monitored as the reduction temperature rose, for varying C/S ratios. The quality of this data was limited by the vacuum gauge utilized. The results are shown in Figure 2-5. It was felt that the C/S ratio should dictate the production rate of CO. Figure 2-5 suggested that below 960°C, the C/S ratio did not influence the CO production rate since the curves are similar for 5 different C/S ratios varying from 0.63 to 1.86. The $BaSO_4$ reduction, as noted by RAFTER (1967), was well under way by about 960°C. Behavior above this temperature was difficult to monitor because of the large amounts of CO evolved, and the inaccuracy of the meter in this pressure range. It does
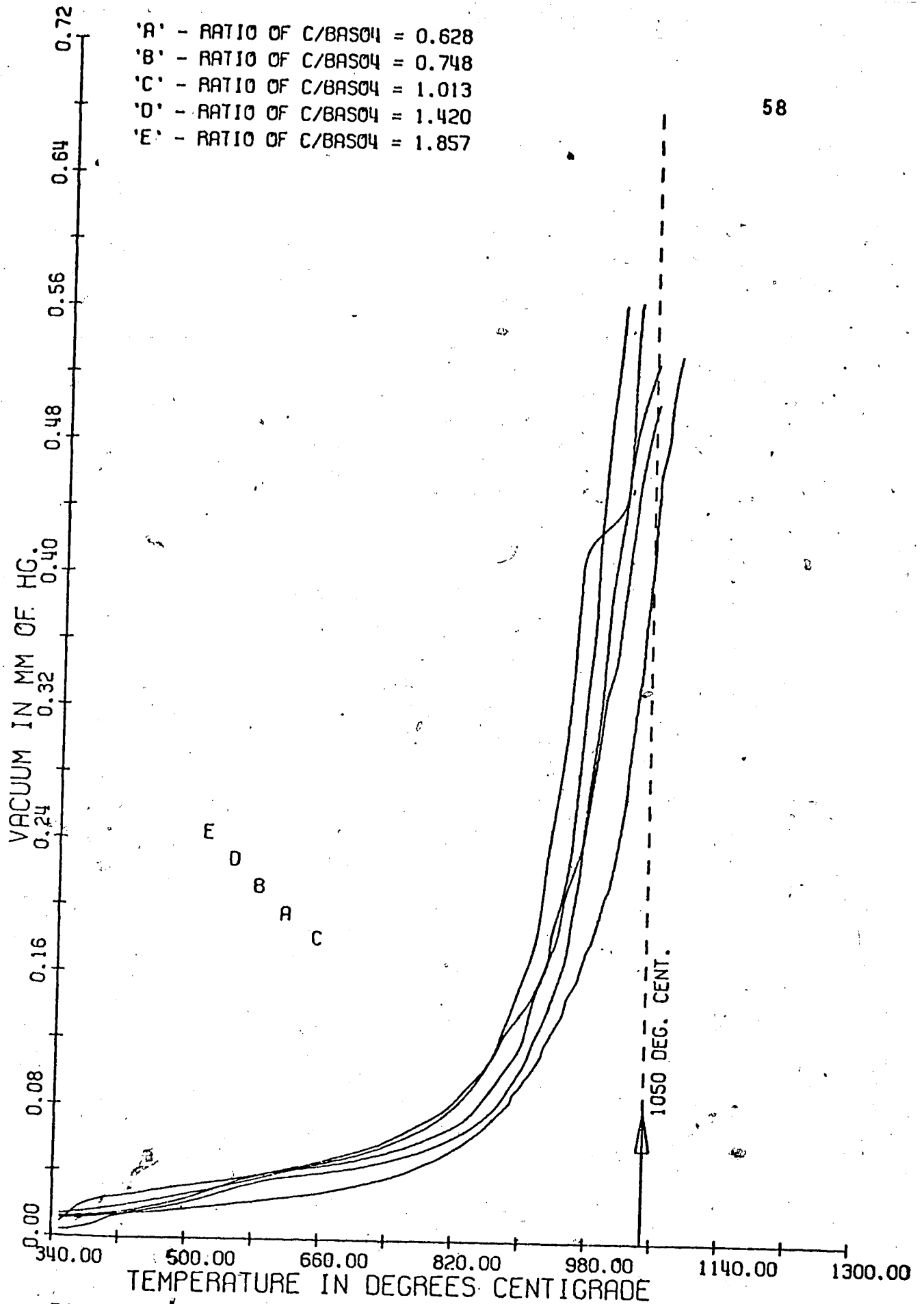
Figure 2-5   CO production and the reduction temperature.

'A' – RATIO OF C/BASO4 = 0.628
'B' – RATIO OF C/BASO4 = 0.748
'C' – RATIO OF C/BASO4 = 1.013
'D' – RATIO OF C/BASO4 = 1.420
'E' – RATIO OF C/BASO4 = 1.857

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follow, that most of the CO production occurred above 960°C.

It should be noted that the samples with a C/S ratio greater than the experimental optimum of 1.0 always indicated more CO present than the others. This was seen to be true from the very beginning of the reductions in Figure 2-5. Even at lower temperatures, where appreciable CO formation was not in evidence, the relative amounts of gas evolution suggested a trend towards more CO when there were excesses graphite available. With a deficiency of optimum contact graphite, production of the undesirable gaseous forms may be an interpretation for the higher pressures recorded in these cases. It was also concluded that out-gassing and drying temperatures for the sample must not exceed 500°C. Here a distinct evolution of CO was present. Such a temperature was never used, nor recommended, especially if excesses of graphite were more than about 8 or 9 times the stochiometric demand.

The relative amounts of CO and CO_2 evolved, were not as constant as expected. RAFTER's (1967) study found $CO-CO_2$ mixtures to contain about 75 per cent CO_2 and 16 per cent CO. His study, as previously mentioned, utilized platinum boats and lids. LONGINELLI and CRAIG (1967) found their mixtures to vary from about 98 per cent CO_2, with a new crucible, down to 80 per cent CO_2 after about 10 reac-

tions with their graphite containers. The mixture of the present study usually was in the order of 85 per cent CO_2 with about 15 per cent CO when the C/S was optimized. With the lower C/S ratios, about 90 per cent of the gaseous product was CO_2, whereas, the higher C/S ratios yielded only about 55 per cent CO_2.

Further mixing of the carbon and sulphate was an impossibility. Compression of the sample could have, as earlier noted, permitted a much reduced C/S ratio, and hence less CO formation. LLOYD (1968) ground his sulphate sample with a tenfold excess of graphite, forming the mixture into a pellet (no information was given on the degree of compression used). From the results of the present study, it was concluded that the most quantitative and reproducible results can only be obtained by sample compression, C/S ratio optimization, and platinum containers.

It is clear that, by working with a C/S ratio of about 1.0, very good reproducibility was obtained as previously found by RAFTER (1967). It may be that if many samples had been run at another C/S ratio, the reproducibility would have been acceptable - although the δO^{18} value may have been different. No doubt further work could have elucidated this phenomenon. It was decided to pursue microbiological isotope fractionation studies using a C/S

ratio of 1.0. This choice was based on the reproducibility obtained with this ratio, and a desire not to deviate significantly from conditions utilized in other laboratories.

It is interesting to note that during the present study, SAKAI and KROUSE (1971) looked at various aspects of the $BaSO_4$ reduction. They found that during the conversion of CO to CO_2, CO^{16} reacted about 1.02 times faster than CO^{18}. (This is in agreement with the data of the present study where the CO formed was pumped away.)

SAKAI and KROUSE (1971) investigated the graphite reduction of sulphate in an attempt to explain why sulphates of widely varying oxygen isotopic compositions were not reproducible. When such samples were analyzed, the isotopic precision   deteriorated to greater than ± 0.5 $^o/oo$, despite satisfactory yields. The possibility of memory effects was investigated. The repeated conversion of samples from the same $BaSO_4$ source, where $BaSO_4$ with significantly differing δO^{18} values had been previously processed, resulted in a very large memory phenomena. With an externally heated quartz tube, these effects were recorded in the order of 2.5 $^o/oo$. Also a tube displaying signs of devitrification after prolonged usage produced larger memory phenomena than relatively new quartz tubes. In fact even new tubes displayed some memory effect, but this was logically related to its process of manufacture.

A possible explanation of this memory phenomena was that pure adsorption had occurred, the product gases being retained by the quartz walls. As pointed out by SAKAI and KROUSE (1971), this was not possible since the yields were consistently greater than 95 per cent. They concluded rather that an exchange had occurred, since the reductions were quantitative. Such an exchange of oxygen atoms was suggested between the hot quartz walls and the gaseous products. It was further noted that NORTHRUP and CLAYTON (1965) reported oxygen isotope exchange between CO_2 and glass at temperatures above $250^\circ C$. However any CO_2 present should not have been a participant in such an exchange phenomena, because during the reduction of sulphate, t was condensed as soon as it was formed. Any CO, however, would have remained in the gaseous phase until converted by the high voltage discharge, thus having a much better opportunity to exchange oxygen isotopes with the hot quartz walls. (Such a phenomenon would have been much more pronounced, had there been larger percentage yields of carbon monoxide.)

Furthermore SAKAI and KROUSE (1971) noted the relative importance of CO formation. The amount of CO which remains at the end of the heating cycle, they stated, was just a fraction of the CO actually produced during the

earlier stages of the reaction. This was based on six ex'   'ents, where it was found that the CO collected during the reduction process corresponded to as much as 50 per cent· of the total oxygen present. Thus in certain stages of the conversion, sufficient CO was available to have participated in the oxygen exchange with the hot quartz walls, there- by producing the noted memory effect.

Such excessive amounts of CO evolved were most probably due to an excessive C/S ratio. That is, there was clearly an excessive amount of graphite present otherwise there would not have been such a large amount of CO formed. No compression technique was employed in their study. However the CO formed must have been subsequently oxidized to CO_2, since the actual and the theoretical yields were consistent.

This memory effect was not applicable to the present reproducibility study, since the $BaSO_4$ samples used were obtained from the same barrel of sea water. When sulphate was used with varying oxygen isotope compositions, inconsistencies were noted and any such samples were re- peatedly reduced until the isotopic reproducibility was con- sidered satisfactory. This was done by successively con- verting a given sample in the same quartz until the δO^{18} values were consistent with the desired reproducibility.

Normally, two reductions, in succession in the same quartz tube, were carried out for each isotopic determination.

Although duplication of analyses should always be an integral part of the isotope abundance ratio determinations, often in cases where the material is rare, only one measurement is possible. To avoid the memory phenomenon, the best solution is to eliminate the memory effect completely. In the present study, such a policy was not available. SAKAI and KROUSE (1971) reduced this memory effect by utilizing the electrical discharge unit throughout the reduction. This permitted the continual removal of CO as the reduction proceeded (this was done as routine procedure in the present study). Although they noted a reduction in the memory phenomena, it was not entirely removed by such a continuous CO conversion. The only solution lay in a more efficient $CO-CO_2$ conversion unit. LLOYD (1967) and LONGINELLI and CRAIG (1967) used induction heating, which was a possible solution. SAKAI and KROUSE (1971) preferred to use an internally heated apparatus for the carbon reduction, but decided to cool the quartz tube walls, thus minimizing the oxygen exchange. A platinum boat was made to be the location of highest resistance in an electrical circuit, thus avoiding external heating. The quartz walls were retained, but became the inner part of a water jacket around the platinum boat.

Thus the quartz walls were kept cool by the water jacket, and eliminated any possibility of oxygen isotope exchange between the CO evolved and the quartz tube. Their apparatus even showed no signs of memory effects when samples differing in δO^{18} values by over 22 $^O/oo$ were analyzed. The present study did not have the advantage of such an apparatus, consequently when varying isotopic composition samples were run, they were done successively in the same quartz combustion tube. Duplication showed up the memory effect, but such widely varying specimens were not a part of the present investigation.

2.6 Summary

1. A method has been developed by RAFTER (1967) for the recovery of oxygen from sulphate for the study of oxygen isotope variations in sulphates.

2. The reproducibility of this method is at present ± 0.1 parts per mil, when the C/S ratio is optimized. Further optimization might be obtained by; (a) compression of the sulphate and graphite sample into a pellet before the reduction, (b) mixing during the reduction process, and (c) the use of platinum boats and lids.

3. This method permits both sulphur and oxygen isotope abundance variations in sulphate on the same sulphate specimen.

4. The graphite to barium sulphate ratio, by weight, does affect the carbon monoxide percentage evolution. The δO^{18} values generally increased with an increasing C/S ratio, as did the CO production.

5. The carbon monoxide formed must be completely converted to carbon dioxide, since it contains a significant portion of the oxygen atoms from the sulphate.

6. Excess graphite was considered necessary for the complete reduction of sulphate. Physical contact might dictate the percentage reaction as well as the relative amount of carbon monoxide formed.

7. Graphite boats produce variable amounts of carbon monoxide, depending upon the age of the containers. Any inherent errors may easily be avoided by the employment of platinum for the container metal.

8. Outgassing and drying temperatures for the sulphate specimen must not exceed 500°C. At such a temperature, distinct carbon monoxide evolution will occur.

9. Carbon dioxide gaseous product is formed below 900°C, whereas carbon monoxide evolution does not occur until about 960°C.

10. Memory effects, due to an exchange phenomenon between CO and the quartz walls, can be successfully eliminated by employing a water jacket as outlined by SAKAI and KROUSE (1971).

11. It may be that differing C/S ratios will yield reproducible and quantitative results, although the δO^{18} value may be somewhat different.

CHAPTER III MASS SPECTROMETRY

3.1 Introduction

With the development of a reproducible and reliable method for the extraction of oxygen atoms from sulphate for precise isotopic analysis, it became necessary to scrutinize the mass spectrometric precision encountered during routine stable isotope measurements.

Once the isotopic reproducibility during the reduction of sulphate by graphite reached about $\pm$ 0.12 ^{o}/oo, it was realized that the precision of the isotopic measurements was typically of the same magnitude ($\pm$ 0 1 ^{o}/oo). Any improvement in the sample preparation reproducibility would have been futile with a mass spectrometric accuracy less than that of the chemical techniques. A possible remedy was concluded to be the recording and evaluation of more mass spectrometric isotopic ratios. Such a solution required more time and labor since a greater number of measurements had to be evaluated. In addition, the routine fluctuations of the source region and the gas pressure would have caused greater variability within a particular analysis if lengthier analyses were performed.

These problems were partially overcome by replacing the original Hewlett-Packard voltmeter and voltage to frequency converter by a Fluke Digital Voltmeter. The digitizing interval

for the Fluke equipment was 18 m sec compared to 1 sec with the Hewlett-Packard integrating voltmeter. With this faster response time, it was decided to couple the Fluke Digital Voltmeter with a computer that could handle large amounts of data in a very short time. This combination was believed to yield a slight increase in the mass spectrometric precision, typically to $\pm$ 0.05 ^{o}/oo, as well as greatly increasing the convenience of tabulating the isotopic abundance ratio measurements The actual application of the PDP-8 computer will be discussed in Section 3.3 ..

3.2 The Mass Spectrometer

The mass spectrometer employed for the isotopic abundance measurements was a 90°, 12 inch radius magnetic analyzer (NIER (1947), McKINNEY, McCREA, EPSTEIN, ALLEN, and UREY (1950)). The O^{18}/O^{16} abundance ratios in sulphates were measured by comparing mass 46 ($C^{12}O^{16}O^{18}$) to mass 44 ($C^{12}O^{16}O^{16}$). The corresponding S^{34}/S^{32} isotopic data was obtained from masses 66 ($S^{34}O^{16}O^{16}$) and 64 ($S^{32}O^{16}O^{16}$).

In the determination of isotopic compositions, single collection often realizes precisions of about 0.1 per cent. The limitation arises primarily from real time fluctuations in the mass spectrometer source region. Such a limitation is usually eliminated by simultaneously collecting ion currents as described by STRAUS (1941), and later modified by numerous workers. Thus the isotopic ratio of a standard and an unknown

sample can be compared improving the mass spectrometric precision to about 0.01 per cent within an analysis.

The standard and unknown gases were introduced into the mass spectrometer through small leaks, designed to reduce the pressure. Any back pressure on these leaks was maintained at a constant level by adjusting the mercury level of the gas reservoirs in the introduction sector. Four magnetic valves were employed to permit alternate measurement of the standard and unknown gas sample ion current. The gas $(CO_2$ or $SO_2)$ was then ionized and accelerated by a potential difference of about 4 kv into the magnetic field. Ion currents corresponding to either masses 44 and 46 for CO_2, or 64 and 66 for SO_2, were collected in Faraday cups and originally amplified by a pair of Cary vibrating reed electrometers. The output voltage from one of the vibrating reed electrometers was conv ted to frequency and used as the time base reference for the Hewlett-Packard voltmeter as described by McCULLOUGH and KROUSE (1965), thus the voltmeter and frequency converter combination was employed as a ratio measuring device. The pressures of the standard and unknown sample as well as the peak shape was monitored by a two pen chart recorder.

This method has since been replaced by a Fluke Digital Voltmeter, as mentioned earlier, with a ratio option. This system was further modified by the interfacing of a

PDP-8 computer to the Fluke voltmeter in order to eliminate tedious data reduction.

3.3 PDP 8 Data Handling

With the rapid influx of data from the faster response Fluke equipment, the precision of mass spectrometric determinations was observed to increase slightly, because of the greater flexibility in averaging time that became available. Thus the introduction of the PDP-8 computer involved integration of the data. It was decided to program the PDP-8 such that it would also evaluate the mean isotopic ratio, as well as the standard deviation obtained during that analysis.

Two samples of the computer output are shown in Figure 3-1 and Figure 3-2. The program was initiated by the operator typing one line of comments. This provided the necessary identification and other pertinent information of sample or standard. Once this line was completed and the RETURN key depressed, the program would type the message INTIME = $2**(\underline{m})$, expecting some single digit to be the reply. This reply was an indication of how many recordings constituted such sub-set mean. That is, the digit typed was the exponent to which the base 2 was raised to acertain how many values were to be taken. Normally 7 was typed for INTIME, where 2^7

```
INTIME=2**7
GMTIME=2**2
FIRST SET MUST BE S !
THIS SET IS S
   0.41945        0.41948       0.41954        0.41947

      0.41949    S

THIS SET IS X
   0.42045        0.42041       0.42044        0.42048

      0.42045    X

THIS SET IS S
   0.41967        0.41073       0.41969        0.41968

      0.41969    S

THIS SET IS X
   0.42057        0.42071       0.42053        0.42064

      0.42061    X

THIS SET IS S
   0.41979        0.41978       0.41977        0.41977

      0.41978    S

THIS SET IS X
   0.42072        0.42063       0.42066        0.42069

      0.42068    X

THIS SET IS S
   0.41989        0.41989       0.41987        0.41986

      0.41988    S

THIS SET IS X
   0.42081        0.42075       0.42126        0.42044

      0.42082    X

   1.00204     1.00200     1.00208     1.00206     1.00202        7
MEAN RATIO     1.00204
STD DEV IS     0.00003
TYPE Y IF DEV SATISFACTORY?               Y
***********************************************************************
```

4:45 PM , 28 / 2 / 72 . SC3 AS X , SAME S .

```
INTIME=2**7
GMTIME=2**2
FIRST SET MUST BE S !
```

Figure 3-1-   Typical PDP-8 output.

```
THIS SET IS S
   0.41824        0.41809        0.41814        0.41821

      0.41817    S

THIS SET IS X
   0.41641        0.41638        0.41611        0.41633

      0.41631    X

THIS SET IS S
   0.41877        0.41871        0.41881        0.41892

      0.41880    S

THIS SET IS X
   0.41683        0.41690        0.41687        0.41682

      0.41686    X

THIS SET IS S
   0.41908        0.41917        0.41913        0.41923

      0.41915    S

THIS SET IS X
   0.41683        0.41712        0.41690        0.41687

      0.41693    X

THIS SET IS S
   0.41904        0.41917        0.41919        0.41910

      0.41913    S

THIS SET IS X
   0.41692        0.41696        0.41695        0.41695

      0.41695    X

   0.99469     0.99471     0.99495     0.99462     0.99472     0.99477

MEAN RATIO      0.99476
STD DEV IS      0.00011
TYPE Y IF DEV SATISFACTORY?        D       DELETE 4
                                           DELETE N

MEAN RATIO      0.99479
STD DEV IS      0.0010
TYPE Y IF DEV SATISFACTORY?        D       DELETE 3
                                           DELETE N

MEAN RATIO      0.99475
STD DEV IS      0.00004
TYPE Y IF DEV SATISFACTORY?        Y
************************************************************************
```

Figure 3-2   PDP-8 output using the delete facility.

readings were algebraically averaged from the Fluke to constitute a sub-set mean. This provided an average over approximately 3 seconds.

The GMTIME = $2^{**}(\underline{n})$ requested was a demand for the number of such sub-sets to be evaluated before the final calculation of the ion current ratio for that partic r gas sample. Each final ratio was hence the mean of 2^n sub-sets of 2^m intervals. This initialization procedure became obligatory for the remainder of the S (standard) and X (unknown) sample determinations. These symbols were also typed in, so as to remind the operator of which sample was to come next. If for some reason it was decided to start over without completing the predetermined number of analyses, then an H (halt) was typed instead of the S or X.

Four isotopic abundance ratios were evaluated for both the standard and unknown gas samples. The final X/S ratio was then calculated by taking the average value of the first two S ratios and dividing this into the first X value. The average of the first two X values was divided by the second value of the S ratio, and so on for the eight S and X ratios. This resulted in six values for X/S which were then averaged. The result of which was printed following the message MEAN RATIO. In addition, the standard deviation was evaluated and presented below the mean ratio. This standard deviation calculation was not rigorous, but rather simply an estimate of the mass spectrometric stability.

If the standard deviation was considered satis-factory, then when prompted by the computer operator who typed Y on the teletype, the program was ready for a new analysis beginning with one line of comments. If however the standard deviation was not considered to be adequate, due to some operator error in running the mass spectrometer or instrumental instabilities, the typing of a D instead would enable the delete subroutine. Thus any of the X/S ratios could be deleted.* The appropriate ratio was then ignored from a re-calculation of the final X/S ratio and the result standard deviation. A standard deviation of under ± 0.1 was con-sidered acceptable, although routinely they were about ± 0.05 °/oo if the INTIME and MTIME were sufficiently large (m about 7 and n as 2).

The actual program permitted the options mentioned while rejecting erroneous demands or mistakes made by the operator. The software was written in assembler (PAL III) language because of the limited storage available in such a small computer and a listing may be found in Appendix A.

The convenience and precision offered by this pro-gram was a definite asset during isotopic abundance ratio studies, and has greatly reduced the monotony of such analyses. The ease of data handling was considered sufficient grounds for the writing of an expanded program which would operate three

(.* The delete facility is designed to permit calculation of the data after some malfunction. It is to be used sparingly.)

mass spectrometers in a similar manner.

This enlarged time-sharing program was written and tested successfully for the simultaneous operation of three mass spectrometers. The length alone prohibits, incorporation of the program into this thesis.

It was envisaged that the PDP-8 and its time-sharing system could successfully be applied to the maintenance of good peak shape, the alteration between the standard and unknown, as well as for control of standard and unknown gas pressures. Such a scheme seemed beyond the scope of a simple PDP-8 time-sharing system. As a result, a T. I. 980 A computer has since been employed to supercede the PDP-8. Although the programming is still in progress, it is obvious that far-more complicated data handling and analysis controls can be successfully employed for routine operations commonly encountered during mass spectrometric analyses. The application of computers in handling mass spectrometric data opened up many possibilities for accurate and simultaneous measurements of isotopic compositions.

CHAPTER IV THE BACTERIAL REDUCTION OF SULPHATE

4.1 Review

The process of sulphate reduction is widespread in nature. Most sulphides in sedimentary rocks and many elementary sulphur deposits were formed by the bacterial reduction of sulphate. For this reason, geologists, bacteriologists, limnologists, and biogeochemists have begun extensive studies on the formation of mineral deposits by microorganisms. Such research was designed to obtain a better understanding of the kind of bacterial reactions involved in different environments. In particular, the present work strove to evaluate the extent of the isotope effect resulting from the biochemical reduction of sulphur bearing substances in nature.

Sulphate reducing bacteria were first described by BEIJERINCK (1895); the first investigation of the sulphur isotope abundance ratios in sulphides and sulphates, was by THODE, MACNAMARA, and COLLINS (1949). This sulphur isotope study dealt with sulphides and sulphates in sedimentary rock. Soon after, the reduction of sulphate by the bacteria *Desulphovibrio desulphuricans* was observed by THODE, KLEEREKOPER, and MCELECHERAN (1951). This was the first demonstration of the ability of microorganisms to enrich one isotope of sulphur in preference to others during metabolic processes. They

showed that this sulphate reducing bacteria was capable of preferential selecting the lighter isotope of sulphur, sulphur-32, during the reduction of sulphate to sulphide. Their conclusion was that the sulphides were generally enriched in the lighter isotope, while the sulphates were enriched in the heavier isotope, sulphur-34.

Wallouch, in some unpublished results quoted by THODE, WANLESS, and WALLOUCH (1954), indicated that isotope fractionation during the bacterial reduction of sulphate was temperature dependent. He found that fractionation increased rapidly with a lowering of the temperature. A 1 per cent depletion of sulphur-34 in the hydrogen sulphide at $25^{\circ}C$ was observed, reaching 2 per cent at $10^{\circ}C$. This temperature coefficient, however, was much too large to be explained on the basis of a simple kinetic or an equilibrium isotope effect. JONES, STARKEY, FEELY and KULP (1956) showed that with *D. desulphuricans*, the rate of reduction and the concentration of sulphate at all concentrations, were controlling factors in determining the degree of fractionation. These properties of bacterial fractionation of the sulphur isotopes during the reduction of sulphate, were used by THODE (1951, 1954), FEELY and KULP (1957), and JENSEN (1958), in their explanations of a number of phenomena occurring naturally. These works illustrated the usefulness of the isotope tech-

nique in the study of certain geological processes.

KAPLAN, RAFTER, and HULSTON (1960) attempted controlled experiments on various stages of the sulphur cycle. Their results were not encouraging. Numerous processes were found that could cause such fractionation of the isotopes, and many natural valence states of sulphur were recorded. With the wide range of environmental conditions studied, their work served to indicate just how generally applicable such investigations were.

JONES and STARKEY (1957) found the degree of fractionation to be increased at reduced temperatures as well as at high sulphate concentrations. It was even suggested that temperature regulated the rate of reduction, thereby controlling the enrichment of the sulphur isotopes and the fractionation observed, the degree of fractionation being dependent on the rate of reduction of the sulphate. JONES and STARKEY (1957) also noted that fractionation was a combination of both chemical and biological reactions. However, it was likely that most natural sulphur formations were biogenic in origin, since temperatures during sulphur deposition were too low for appreciable chemical reduction of sulphate. FEELY and KULP (1957), and HARRISON and THODE (1958), found that the degree of fractionation was inversely proportional to the rate of reduction. That is, greater

isotopic fractionation was observed when the rate of reduction of sulphate was decreased. Similar results were later obtained by KAPLAN, RAFTER, and HULSTON (1950). NAKAI and JENSEN (1960) also reported such observations, however, they used raw cultures. All the previous investigations had used pure cultures of *D. desulphuricans*.

Although attempts were made to interpret these processes in nature in terms of microbiological fractionation of the sulphur isotopes, only HARRISON and THODE (1958) attempted to explain the underlying physiological events. This was partially based on POSTGATE (1952), who established that an intermediate between the sulphate and sulphide was sulphite. Furthermore, the sulphite was observed to be reduced at a faster rate, in the bacterial cell, than the sulphate. HARRISON and THODE (1958) explained this as a reaction mechanism involving two consecutive steps. One step was believed to involve a small isotope effect, with the other producing the large isotope. The first of these steps, the one with the small isotope effect, was suggested to be a result of the uptake of sulphate by the bacteria. The second step was believed to involve the reduction of sulphate to sulphite, the large isotope effect. This large isotope effect would consist of the initial S-O bond breakage occurring in the reduction of sulphate to sulphite.

The observed fractionation was thought to be dependent upon the relative rates of these two steps, that is, the entrance of sulphate into the cell and the breaking of an S-O bond in the reduction to sulphite. HARRISON and THODE (1958) believed these two steps to be competing for control of the rate of reduction. It was of no consequence that the relative rates were closely related to the environment of the bacteria, that is, the temperature, metabolite concentration, and conditions of growth. The question was, which step controlled the reduction rate of the bacteria? Their study concluded that the rate controlling step was the reduction of sulphate to sulphite. This would be expected, since it had been noted by POSTGATE (1952) that sulphite was reduced much more rapidly than sulphate. Any further reduction of sulphite to the end product of a sulphide, could not lead to an isotope effect since the sulphite was reduced as rapidly as it was formed.

KAPLAN and RITTENBERG (1964), unsatisfied with the development of the investigations carried out, re-investigated sulphur isotope fractionation during bacterial sulphate reduction. (They also investigated other important metabolic processes in the sulphur cycle.) Using once more *D. desulphuricans*, KAPLAN and RITTENBERG (1964) studied the effects of temperature and sulphate concentration on the rate of reduction

and the fractionation of the sulphur isotopes. Here the
temperature and sulphate concentration, within the normal
physiological range of these parameters, influenced the
fractionation only in so far as they influenced the reduc-
tion rate. Since reduction requires a gain of electrons,
they also varied the electron donor. The electron donor
previously had been sodium lactate, but now ethanol and
molecular hydrogen were also employed. A marked influence
on the reduction rate was observed by this variation of the
electron donor. Molecular hydrogen was observed to produce
the fastest rate of reduction, all other variables remaining
constant. Meanwhile, the rate with ethanol was about one-
tenth of that observed with molecular hydrogen, whereas lac-
tate was generally about one-half of the hydrogen rate.

With lactate and ethanol as the electron donors,
the isotopic fractionation was noted to have been inversely
proportional to the rate of reduction. This result was in
agreement with HARRISON and THODE's (1958) previous investi-
gation. However, when molecular hydrogen was the electron
donor, the fractionation became directly proportional to the
rate of reduction of the microorganisms. Strain variations
also produced relative changes in the magnitude of the iso-
topic enrichment, but otherwise no differences were observed
due to this modification. It was under such conditions that

the concentration of the metabolite, the temperature and the electron donor were made to permit similar studies the reduction of the sulphite ion. The reduction of sulp was reaffirmed to occur more rapidly than sulphate. The enrichment, however, was usually smaller than during sulphate reduction.

The work of KAPLAN and RITTENBERG (1964) disagreed with the earlier hypothesis of rate controlling steps. Rather, the rate of reduction was always much greater with molecular hydrogen than with either lactate or ethanol as the electron donor. Thus no control of rate occurred during the sulphate reduction, but instead this rate was dependent on the electron donor. It was the availability of electrons at the reduction site, and not penetration of the sulphate, that must have been the rate controlling factor. KAPLAN and RITTENBERG (1964) explained the variety of enrichments found in terms of several possible equilibrium steps prior to a final unidirectional rate controlling step.

KEMP and THODE (1968) undertook a further reexamination of the mechanisms and factors influencing the bacterial reduction of the sulphate ion. Their results hardly differed from those of KAPLAN and RITTENBERG (1964), except that the enrichments and rates with the electron donor ethanol were distinctly different from previous investigations. Although the inverse proportion was still present, the magni-

,tude of the reduction was markedly different. One conclusion made was that the passage of H_2S from the cell was fast and hence not a controlling factor in the reduction of sulphate. Their reason for such a suggestion was that the H_2S formed was toxic. That is, the sulphide product was toxic towards the bacterial cell, thus only a small isotope effect was expected in the liberation of the H_2S gaseous product. KEMP and THODE (1968) suggested that the fractionations observed were due to sequential additions of two isotope effects, for both the sulphate and sulphite reductions.

RAFTER (1967) presented the graphite reduction method, it became logical to pursue the measurement of oxygen isotopes in sulphates. With the oxygen and sulphur isotopic abundance ratios for the sulphate reduction, it would be two tools to "crack the same nut". Hence oxygen isotope effects in sulphates was a complementary process for the under-standing of the bacterial reduction of sulphate. LLOYD (1967) reported that in the microbiological reduction of sulphate, the bacteria preferentially metabolized oxygen-16. Hence the δO^{18} value of the residual sulphate in solution became progressively heavier. This was expected, since the oxygen and sulphur isotopes would have most probably followed similar patterns when the bacteria metabolized the sulphate.

A better understanding of sulphate reduction, involving correlations between the sulphur and oxygen isotopic variations, was the aim of the present investigation. The only work done, in addition to the present study, on the relationship between δO^{18} and δS^{34} values of sulphate undergoing bacterial reduction was that of MIZUTANI and RAFTER (1969). Using wet sulphate-free stream mud, MIZUTANI and RAFTER (1969) found the sulphate enriched in oxygen-18 and sulphur-34 relative to the original sulphate used. The ratio of the enrichment of sulphur-34 to the oxygen-18 enrichment in the sulphate, was found to be approximately 4:1. This isotopic enrichment ratio of the sulphate was observed to be independent of the temperature of the reduction. No attempt was made to explain this ratio of the isotopic enrichments.

The bacterial reduction of sulphate has been partially explained in terms of physiological processes rather than physical-chemical reactions. The dependence of isotope fractionation on environmental conditions suggests a wide range of isotopic fractionation factors for many natural processes. Considering its widespread occurrence in nature, it became imperative to better understand these kinds of reactions. For example, the metabolic product of sulphur bacteria plays an important role in organic and inorganic natural phenomena, such as the changes in oxidation-reduction

potentials, the consumption of oxygen, and the precipitation of sulphide minerals. In particular, the present investigation was designed to better evaluate the extent of the isotope effect, and to explain any correlations observed between the sulphur and oxygen isotopic species.

4.2 Microbiological Techniques

During sulphate, sulphite, and elemental sulphur reductions, hydrogen sulphide gas is the end product. In order to facilitate this H_2S evolution, the addition of nutrient, and the medium extractions, a reaction vessel was employed as shown in Figure 4-1. This reaction vessel was a modification of that described by KROUSE, MCCREADY, HUSAIN, and CAMPBELL, (1967). With such a container, nutrient addition or medium extraction could be accomplished without a loss of evolved H_2S gas.

The modified Erlenmeyer flask rested in a thermostatically controlled temperature bath. This constant temperature bath contained distilled water of such a depth as to completely immerse the medium in the reaction flask, yet not interfering with the operation of the sampling port. The reaction flask contained a porous aerator which permitted the flushing gas to immediately disperse in the medium. The product of the bacterial reduction, the H_2S gas, was swept

Nutrient Addition Port

N₂ →

Sampling Port

Porous Aerator
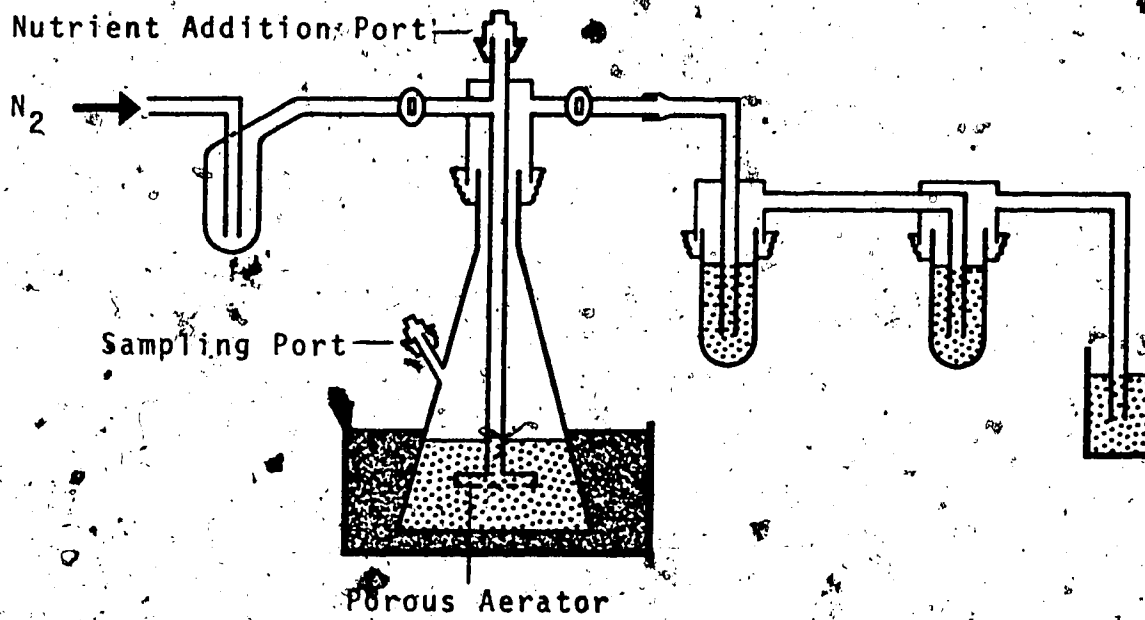
Figure 4-1   Apparatus for the bacterial
reduction of sulphate.

continually from the reaction vessel with deoxygenated nitrogen. This was achieved by bubbling nitrogen through a pyrogallol solution, before it entered the reaction vessel, to remove any traces of oxygen not eliminated during the manufacture of the high purity nitrogen gas. (This oxygen scrubbing solution consisted of 10 parts 20 per cent KOH to 4 parts 40 per cent pyrogal, by volume.)

The H_2S, extracted by the nitrogen stream, was bubbled through two scrubbers containing distilled water. It was then trapped from the nitrogen stream by a cadmium acetate mixture, yielding the precipitate cadmium sulphide. The two distilled water scrubbers were employed to remove any HCl vapor from the nitrogen and H_2S gaseous mixture. If the chlorie gas was not removed, then it would have become a contaminent on passage through the acetate solution. Aqueous silver nitrate was added to the acetate solution bearing the yellow CdS precipitate, once the sulphide sample had been removed. The result was a black precipitate Ag_2S. It was subsequently boiled to remove traces of HCl, as well as to induce precipitate self-adhesion. In any one reduction, several H_2S product fractions were collected over chosen time intervals for kinetic isotope analyses. Data was obtained by simply weighing the Ag_2S, quantitatively prepared from the H_2S fractions.

Aseptic conditions were maintained in the reaction vessel by employing two bacterialogical filters; one for the entrance of the deoxygenated nitrogen, the other for the nitrogen and sulphide gaseous mixture leaving the reaction flask for the sulphide purification system. The reaction vessel consisted of an Erlenmeyer flask with a sampling port, a nutrient addition port, and a control head, all of which were ground glass joints held tight by small retaining springs. The control head permitted the incoming nitrogen gas to escape through the aerator into the medium, then forced it to exit through the bacterial filter leading to the scrubbing system.

The reaction flask, with all the ground glass port caps secured in place, was autoclaved to produce an aseptic container for the reduction process. The medium after preparation was similarily autoclaved to form the sterile nutrient solution necessary for bacterial growth and subsequent reduction of sulphate without contamination from other sources. For the bacterial reduction of sulphate, the nutrient solution was a modification of "Butlin's" medium (BUTLIN, ADAMS, and THOMAS (1949)). This medium was most convenient since the carbon source for the bacteria also acted as the electron donor during the reduction. This base medium contained:

0.5 g KH_2PO_4

1.0 g NH_4Cl

0.1 g $CaCl_2 \cdot 6 H_2O$

1.0 g   Yeast extract

0.002 g $FeCl_2$

3.5 g   60 per cent Na lactate

1000 ml d. d. H_2O

pH was 7.2

The original Butlin's medium (BUTLIN, ADAMS, and THOMAS (1949)) contained some sulphates required for good bacterial growth, but these were omitted and $FeCl_2$ was used so as not to deprive the microorganisms of nutritional requirements, yet at the same time permitting no extraneous sulphate ions to be present in the medium save the known sulphate to be reduced.

The sterile nutrient broth was added aseptically to the autoclaved reaction flask. The inoculum was then added aseptically to the medium in the reaction vessel, and the oxygen free nitrogen gas was then permitted to flush out the air present in the sulphate reduction container. The nitrogen flow rate was adjusted to prevent too vigorous an aeration of the vessel as well as the scrubbing and trapping system. Once the flow was considered satisfactory, the sam-

pling port was opened aseptically and one gram of sterile Na_2SO_4 introduced. The commencement of sulphate reduction usually was observed within about one half an hour after the addition of the sulphate. This was evidenced by the formation of yellow cadmium sulphide precipitate in the sulphide scrubber.

Blank determinations showed that the sulphide contamination from all available sources was negligible. That is, after two days no apparent sulphide was observed in the acetate scrubber. As the reaction proceeded, sulphide samples were removed when considered large enough for isotopic analysis. To determine the rate of sulphate reduction, 90 ml aliquots from the reaction vessel contents were withdrawn at the same time as a sulphide sample was collected. This medium fraction contained sufficient sulphate for a rate determination as well as the measurement of the oxygen and sulphur isotopic composition of the fraction. To avoid further reduction of this sulphate by the microorganisms present in the medium, the 90 ml aliquots were immediately autoclaved after their extraction.

4.3 Chemical Preparation

During the bacterial reduction of sulphate, the modified Butlin's medium provided adequate nutrients for the metabolic processes of the bacteria. One of the by-products

of their metabolism was hydrogen chloride gas. The two
scrubbers contained distilled water in which the HCl
gas readily dissolved. If the chloride had not been re-
moved before the sulphide was precipitated, it would have
dissolved in the acetate solution. Thus when the AgNO$_3$ was
added, the precipitate AgCl would have formed, intimately
mixed with the desired Ag$_2$S product. Its chemical removal
could have been done at a later stage of the proceedings,
but then it would have become somewhat of a health hazard.
The dissolution in water was the fastest and safest method
of removing this contaminant. The distilled water in the
scrubbers was replaced whenever a sulphate and sulphide frac-
tion were withdrawn.

The acetate solution used to precipitate the H$_2$S
as CdS consisted of a mixture of 500 ml of 17N glacial acetic
acid, 62.5 g CdOAc, and 2000 ml of distilled water. The CdS
fraction, when removed from the reduction line, was immediate-
ly washed by 0.1 N AgNO$_3$ to form the more stable Ag$_2$S preci-
pitate. This was then slowly heated to near boiling to induce
any chlorine or other undesirable gases to leave the solution,
and in addition cause the black precipitate to self-adhere.
This usually produced a few conglomerates of sulphide rather
than multiple smaller groupings of Ag$_2$S. As a precaution,
NH$_4$OH was then added, forcing any remaining AgCl precipitate

to become soluble and hence removable. The ammonia vapors made this process quite undesirable, but usually very little, if any, chlorine remained. The resulting Ag_2S precipitate was thoroughly washed at least four times. The fifth washing was used to transfer the sulphide into a 50 ml beaker, then the beaker and its contents were placed in a drying oven at $40^{\circ}C$. After two days the dried Ag_2S precipitate was weighed and then placed in a sealed container until further processing was necessary.

The sulphate and medium fractions extracted from the reaction flask were immediately autoclaved to prevent any further reduction of the sulphate ion. The sterile product was then stored for processing at a later time. Both sulphide and sulphate fractions were withdrawn as the reduction proceeded.   Once no further reduction of sulphate was observed, the process was permitted to continue for three days. When no more CdS had formed, the last remanent fraction of sulphide was removed and processed as described. The remainder of the sulphate and medium in the reaction vessel was autoclaved as a precaution, and stored.

The resulting sulphate and sulphide specimens were later chemically treated as a group, thus eliminating any chemical discrepancies between samples of a sulphate reduction.

A few drops of HCl were added to each sulphate plus medium sample to ensure acid conditions, the necessary environment for $BaSO_4$ but not barium carbonate precipitation. About 40 ml of 0.1N $BaCl_2$ was added to each sulphate solution to form the desired $BaSO_4$ precipitate. This fine white precipitate was thoroughly washed three times to rid the solution of remanent medium. A fourth washing was employed to transfer these precipitates into 50 ml beakers. These sulphate specimens were then dried in an oven at 40^0C to rid the specimen of water so that accurate weighing could be made of the residual sulphate from the bacterial reductions.

The drying of the sulphate precipitate at low temperatures was found to be the only quantitative technique for extraction of $BaSO_4$ from solution. Filter paper, centrifuging, and high temperature drying had all been previously attempted without satisfactory results. The fine precipitate could not be quantitatively extracted from the filter paper, nor from the centrifuge tube. High temperature drying tended to give rise to boiling, which in turn caused the precipitate to disperse in the beaker. No such splattering was present when low temperatures were involved. These dried $BaSO_4$ specimens were then weighed to permit the evaluation of percentage reduction. The resultant $BaSO_4$ samples were then divided into two portions; the

larger portion for oxygen isotopic abundance ratio measurements of the sulphate, and the smaller portion for the determination of the sulphur isotopic composition of the sulphate.

The $BaSO_4$ designated for oxygen isotopic abundance ratio measurements was already in a suitable form for the direct reduction by graphite. These samples were reduced by the carbon reduction method already described. The resultant CO_2 product was then analyzed mass spectrometrically for the oxygen isotopic composition of the unreacted sulphate from the bacterial reductions.

The $BaSO_4$ designated for sulphur isotope studies was not in a convenient form. It was necessary to chemically reduce the sulphate to sulphur dioxide, through a series of chemical reactions (RAFTER (1957), GAVELIN, PARWELL, and RYHAGE (1960), THODE, MACNAMARA, and DUNFORD (1961), and RICKE (1964)). The method of THODE, MACNAMARA, and DUNFORD (1961) was the technique basically adopted for the present study. The $BaSO_4$ designated for sulphur isotope studies was placed in a 200 ml flask as shown in Figure 4-2. This reduction flask was fitted by means of a ground glass joint and retaining springs to the lower end of a reflux container. The addition of a reducing agent to the $BaSO_4$ sample resulted in H_2S evolution. This sulphide gas was forced by a nitrogen
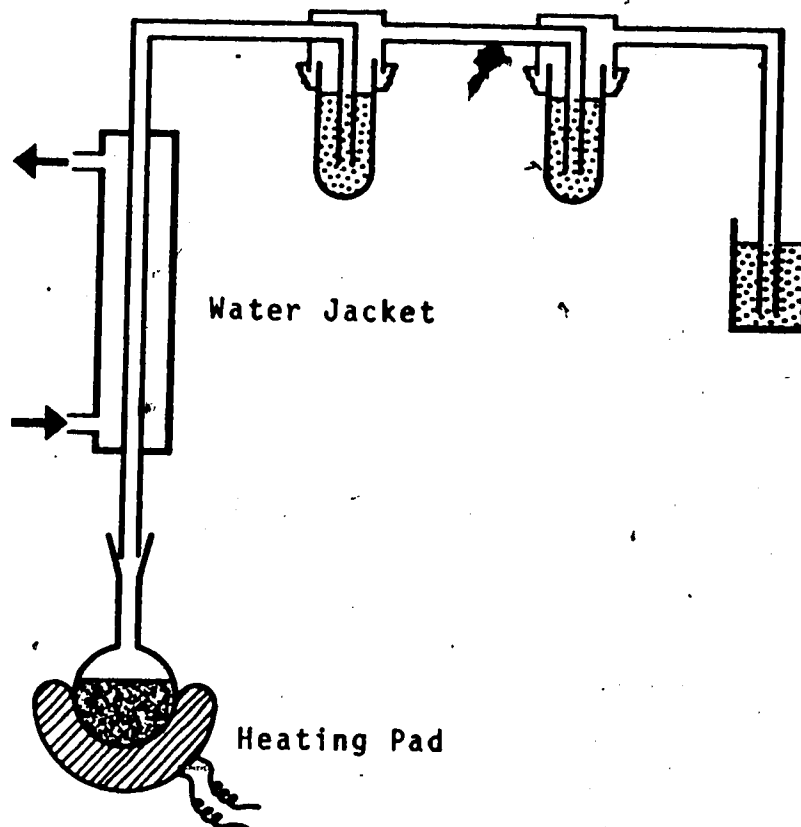
Figure 4-2 Apparatus for the chemical reduction of sulphate.

stream through the water jacket to the distilled water
and acetate scrubbers. The distilled water was employed
to remove any chlorine gas evolved from the reduction
process due to the breakdown of the reducing reagent.
The H_2S was trapped as CdS as outlined previously.
This chemical reduction of sulphate proceeded in a
similar manner to the bacterial reductions except much
faster, since there were heating jackets placed
around the flasks containing the sulphate and reduction
mixture. A reflux condenser was necessary to prevent
the reduction mixture from evaporating. This process
involved a negligible isotope effect because there
was nearly 100% conversion.

The chemical reduction of sulphate was brought
about by means of a very strong reduction mixture
consisting of 500 ml HI, with 816 ml of concentrated
HCl, and 245 ml of 50 per cent H_3PO_2. This was produced
by careful addition of these acids, then the resultant
mixture was boiled for 45 minutes to expel any H_2S
or chlorine gases. Heating jackets were employed to
maintain moderate boiling of the reduction mixture.
The 30 mg sulphate sample usually required about one

hour for its quantitative chemical reduction to sulphide.

The resultant CdS product was precipitated as Ag_2S by the addition of 0.1N $AgNO_3$, boiled, dried, and weighed as previously described in section 4.2. This Ag_2S was then converted to SO_2 so that the sulphur isotopic composition of the unreacted sulphate could be determined. (This process was also used to convert the sulphide product of the bacterial reductions to SO_2 for sulphur isotopic abundance ratio measurements.) The Ag_2S was placed in a quartz boat and burned in O_2 to form SO_2, which could be readily analyzed mass spectrometrically for the sulphur isotopic content. The SO_2 product of the Ag_2S burning was formed following the procedures of RAFTER (1957), HULSTON and SHILTON (1958), and THODE, MONSTER, and DUNFORD (1961). Here the Ag_2S was placed in a quartz boat in a furnace at $1200^{\circ}C$, and purified oxygen gas then passed over the hot silver sulphide. The SO_2 so formed was quantitatively measured after the removal of any CO_2, water vapor, or O_2 contaminants.

Unfortunately this conversion of $BaSO_4$ to SO_2, for the determination of sulphur isotopic abundance ratios in sulphate, involved multiple operations. There was the chemical reduction liberating H_2S, then the tedious double

precipitation, and finally the oxygen burning which liberated the desired SO_2. Each procedure caused no appreciable fractionation of the isotopes, but involved very tedious and time consuming operations. The chief disadvantage of any such technique to convert $BaSO_4$ to SO_2 was the requisite time and labor. Such procedures did consume a major part of the experimental effort in the present research program. Recently, HOLT and ENGELKEMEIR (1970) described a method by which $BaSO_4$ was rapidly converted to SO_2 in one operation. This method is to be recommended for any study where $BaSO_4$ must be converted to SO_2.

HOLT and ENGELKEMEIR (1970) investigated the thermal decomposition of $BaSO_4$. The sulphate was covered with pulverized quartz powder in a fused quartz tube and heated in vacuum to the softening point of quartz. At such a temperature, about $1400^\circ C$, the reaction

$$BaSO_4 \rightarrow BaO + SO_2 + 0.5\ O_2$$

yielded SO_2, which was collected in a cold trap. The O_2 was then pumped away while the BaO fused with the silica surroundings. This method, however, did not assure a uniform oxygen-18 abundance in the SO_2 as did the procedures involving sulphide samples oxidized by oxygen from a common source. A correction

for such an oxygen-18 interference was made from measurements of both the 66/64 and the 50/48 mass ratios.

The yields from such thermal decompositions of $BaSO_4$ were always greater than 98 per cent of the theoretical yield. The chief impurity found was CO_2. The gas produced usually consisted of about 99 per cent SO_2, 0.5 per cent CO_2, and 0.5 per cent other impurities. The other impurities were mainly water and mass 28 materials, that is, N_2 and CO. No SO_3 was detected and only traces of CS_2 and COS were observed. Aside from the obvious advantages of time and labor, samples from about 5 mg to 50 mg were quantitatively converted.

4.4 The Bacterial Reductions

A series of bacterial sulphate reduction experiments were conducted at $24^{\circ}C$ and $30^{\circ}C$, using 1000 mls of the modified Butlin's medium as the culture media and 1 g of Na_2SO_4. Oxygen free nitrogen was swept through the reaction vessels as previously described. The inoculum however, was not just one strain of a sulphate reducing bacteria, such as *D. desulphuricans* employed in previous investigations.

To help elucidate the mechanisms of sulphate reduction to sulphite as well as the further reduction of sulphite to sulphide, the inoculum consisted of two strains of bacteria. One strain, *Bacillus 8P*, was a sulphate reducer

unable to metabolize sulphite; whereas the other strain,
Clostridium Dm 3, was a sulphite reducing bacteria which
would yield the final H_2S product, but was unable to uti-
lize sulphate. Hence the two bacterial strains had dis-
tinctive functions. The final H_2S product was only the
result of a double reduction. Unreacted sulphate indicated
the *Bacillus 8P's* inability to further reduce sulphate,
while an accumulation of intermediate sulphite suggested
the *Clostridium Dm 3's* inability to reduce any more sulphite.
By observing the amount of sulphide, unreacted sulphate, and
sulphite intermediate at various stages of the reduction, an
attempt could then be made to more clearly understand the
mechanisms involved in the bacterial reduction of the sul-
phate ion.

The sterile medium in the aseptic reaction vessel
was inoculated with these two bacterial strains. As the mi-
crobiological reduction proceeded, the deoxygenated nitrogen
forced the H_2S product through the sulphide purification
system. Fractions of the sulphide and medium (containing
the unreacted sulphate and sulphite) were withdrawn through-
out the reduction process. These products were treated as
previously described. This two strain inoculum was used for
the reduction of sulphate on four separate occasions. Two
reductions were carried out at $24^{\circ}C$ and two more at $30^{\circ}C$,
to ascertain temperature dependency of the reduction rate.

The amounts of media, innoculate, and sulphate concentrations were identical in all four cases.

Of the twelve bacterial reduction-of-sulphate experiments investigated during the course of the present study, these four were singled out for a most intense investigation. Most of the other sulphate reductions were long term experiments employing only one bacterial strain for the reduction of sulphate to sulphide. These reductions were considered unsatisfactory at the time because of the apparent lack of completeness of reduction. That is, the reduction of sulphate was usually less than 30 per cent of that believed possible, although most adequate precautions had been taken to ensure that the reducing bacteria had all the nutrients required for a complete reduction of the available sulphate. (No real understanding of the sulphate reduction was believed possible with such poor yields.) The isotopic composition of the sulphate fractions were determined for some of these runs. No appreciable variations in the isotopic abundance ratios were observed as the reaction proceeded. Even when the bacterial reduction of sulphate had completely ceased, the isotopic enrichments were not as substantial as expected.

The concept of two distinctively different strains, with different metabolisms, was the second simplest form of sulphate reduction by microorganisms. With no apparent success with only one strain, the employment of two strains was the most obvious choice. To eliminate any confusion arising from the double reduction, it was decided to use strains which had no common sulphur metabolic processes. With the synergism of the SO_4-SO_3^* reducer unable to reduce sulphite and the SO_3-H_2S^* reducing bacteria incapable of reducing sulphate, the intermediate sulphite observed by POSTGATE (1952) could be independently evaluated.

KAPLAN and RITTENBERG (1964) and KEMP and THODE (1968) concluded that the rate of reduction was inversely proportional to the fractionation of the sulphur isotopes if lactate or ethanol was the electron donor. With such a donor, it became apparent that the rate of reduction should be slow, but how slow became a very important question. A total reduction time of about a week was considered much too short, yet months could have resulted in the possibility of no significant results, as the previous sulphate reductions had shown. The optimum time was considered to be in the order of about six weeks. Then arose the problem of finding both a SO_4-SO_3 and a SO_3-H_2S reducer which were compatible, totally dissimilar in metabolic functions involving sulphur-

(* $SO_4^=$ and $SO_3^=$ for convenience in the remainder of the text are simply SO_4 and SO_3; charge omitted. However, SO_2 and H_2S refer to the gaseous forms.)

bearing ions, yet such that the reduction process would be complete in about six weeks.

The selection and isolation of such a pair of microorganisms was no simple task. Dr. F. Cook personally undertook this tedious task and provided the two strains employed (unpublished work by Dr. F. D. Cook).

During the reduction experiments, fractions of sulphate and sulphide were withdrawn, as recorded in Table 4-1. The total amount of sulphur used was calculated from the amount of Na_2SO_4 added. With a knowledge of H_2S evolved and sulphate removed, the amount of intermediate was determined. (This intermediate was logically the product of the sulphate reduction, and the known material needed by the sulphite reducing bacteria.) The accumulation of this intermediate was evaluated as shown in Figure 4-3. This was calculated from a sulphur mass balance. Also, knowing the concentration of the unreacted sulphate, the medium volume, and the sulphide evolved, the instantaneous sulphur mass balance for a particular sample (n) can be evaluated using

$$((mg\ S)_{BaSO_4} + (mg\ S)_{Ag_2S} + (mg\ S)_{int})_n = ((mg\ S)_{BaSO_4} + (mg\ S)_{int})_{n-1}$$

A sulphur balance was required to obtain the concentration of the sulphite ions, because any oxygen mass balance would have involved oxygen from the medium constituents. This oxygen

Table 4-1   Sulphate and sulphide fractions from four

bacterial reductions of sulphate

Specimen Number	Time (days)	BaSO$_4$ (mg)	Ag$_2$S (mg)	S in BaSO$_4$ (mg/l)	S in Ag$_2$S (mg/l)	Calc. S int. (mg/l)
1 - 0	0	--	--	226	--	--
1 - 1	6	95	130	145	17	64
1 - 2	8	92	65	140	9	60
1 - 3	11	90	109	137	17	46
1 - 4	19	81	83	124	15	44
1 - 5	41	66	40	100	8	60
2 - 0	0	--	--	226	--	--
2 - 1	6	121	131	185	17	24
2 - 2	8	118	85	180	12	17
2 - 3	11	111	119	170	19	8
2 - 4	19	109	61	166	11	1
3 - 0	0	--	--	226	--	--
3 - 1	6	82	97	125	13	88
3 - 2	8	83	87	127	12	74
3 - 3	11	80	80	122	13	66
3 - 4	19	81	68	124	12	52
4 - 0	0	--	--	226	--	--
4 - 1	6	106	90	162	12	52
4 - 2	8	102	79	156	11	47
4 - 3	11	105	68	160	11	32
4 - 4	19	98	69	150	12	30
4 - 5	41	n. d.	28	n. d.	6	n. d.
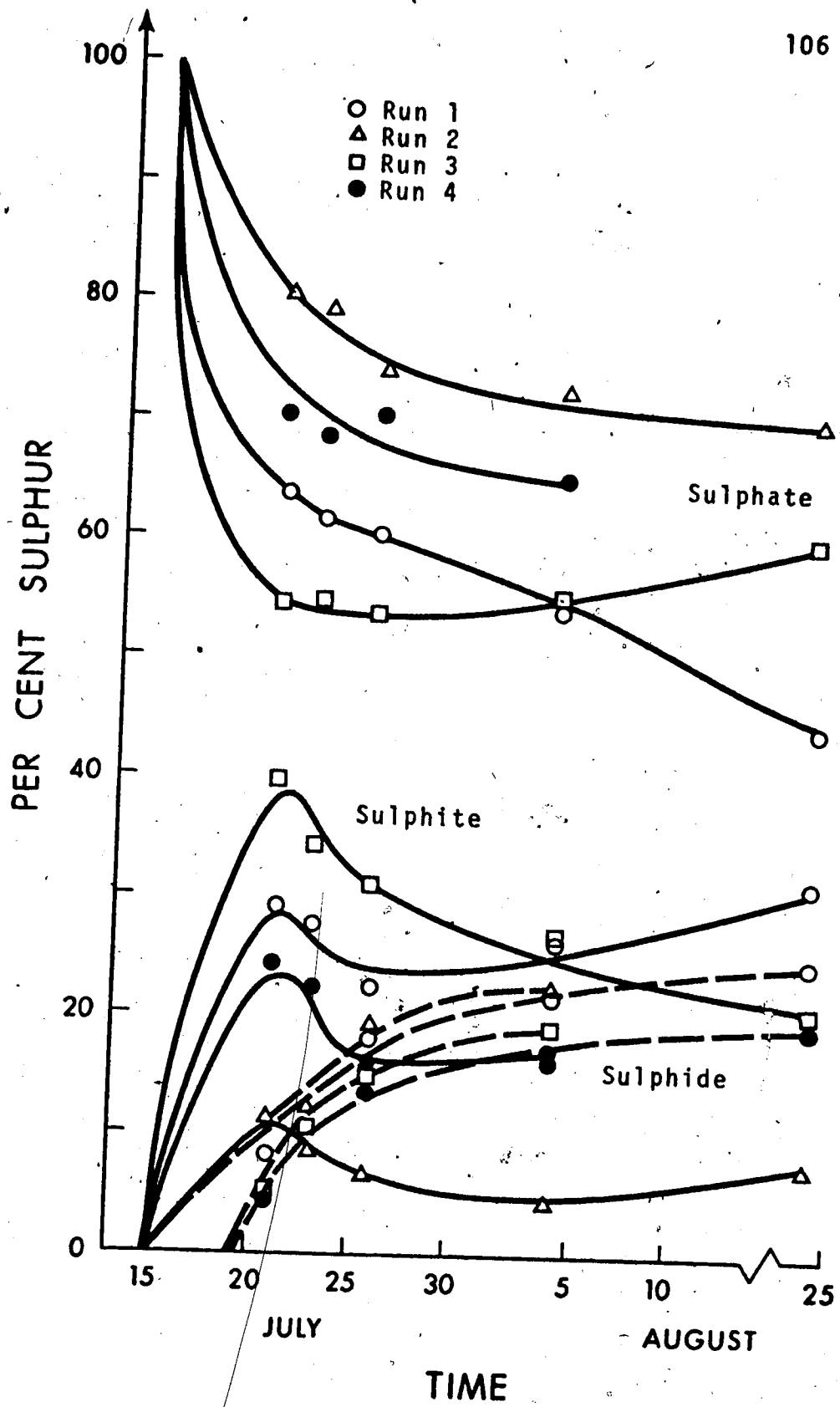
Figure 4-3   Sulphur distribution during the bacterial reduction of sulphate.

was from so many possible sources that isotopic analyses of
the CO_2 evolution were meaningless. Most of the oxygen did
in fact come from the reduction of sulphate, but the proportion
from extraneous materials was sufficient to render fruitful
interpretations impossible.

The mass spectrometric results obtained were
tabulated using the δ notation:

$$\delta = (\frac{R_{sample}}{R_{std}} - 1) \times 1000 \quad ,$$

where R was the S^{34}/S^{32} or the O^{18}/O^{16} abundance ratio of the
sample and standard. The enrichment of both the sulphur
and oxygen species in the unreacted sulphate at any stage
of the reduction was noted in Table 4-2. The unreacted
sulphate was progressively enriched in oxygen-18 and in
sulphur-34 relative to the original sulphate introduced.

To avoid calibration of isotopic standards, one of
the sulphate reduction samples, the original unreacted
sulphate 1-0, was employed as an internal standard for all
four bacterial reduction of sulphate experiments. The
graphite used for the reduction of sulphate to CO_2, for
oxygen isotope studies of the sulphate, was from a common
source. Similarly the oxygen used for the burning of the
Ag_2S to yield SO_2 was also from a common source. Thus the
internal standard used for both oxygen and sulphur isotopic

Table 4-2   Oxygen and sulphur isotopic variations
during the bacterial reduction of sulphate

| Sample Number | Per Cent Reaction | Sulphate ($^o/oo$) | | Sulphide ($^o/oo$) |
		δS^{34} wrt 1 - 0	δO^{18} wrt 1 - 0	δS^{34} wrt 1 - 0
1 - 0	0	0	0	--
1 - 1	35.8	+2.16	+0.58	-13.74
1 - 2	38.1	+2.02	+0.47	-25.70
1 - 3	39.4	+1.98	+0.44	-25.21
1 - 4	45.1	+2.65	+0.66	-24.69
1 - 5	55.8	+3.73	+0.90	-49.27
2 - 0	0	0	0	--
2 - 1	18.1	+1.93	+0.46	-14.15
2 - 2	20.3	+1.33	+0.32	-28.67
2 - 3	24.8	+2.45	+0.69	- 0.38
2 - 4	26.5	+3.84	+1.02	n. d.
3 - 0	0	0	0	--
3 - 1	44.7	+1.11	+0.26	+ 0.66
3 - 2	43.8	+2.16	n. d.	- 6.78
3 - 3	46.0	+1.37	+0.33	- 4.89
3 - 4	45.1	+3.34	+0.86	- 8.99
4 - 0	0	0	0	--
4 - 1	28.3	+1.35	+0.33	- 3.48
4 - 2	31.0	+1.94	+0.●●	-14.06
4 - 3	29.2	+1.15	+0.29	-21.25
4 - 4	33.6	+1.58	+0.45	-27.23
4 - 5	n. d.	n. d.	n. d.	-24.77

$$\text{Per Cent Reaction} = \left(1 - \frac{\text{conc. of sulphate}}{\text{initial conc.}} \right) \cdot 100 \%$$

standards involved no carbon or oxygen corrections.

The apparent non-linearity of the isotopic data took on a new dimension when the per cent H_2S production was considered. Figure 4-4 yielded most intriguing sulphur and oxygen isotopic abundance ratios if the general trend of each reduction was considered. The depletion or enrichment of these isotopic species was totally sympathetic. These variations in isotopic composition became more startling when the relationship between the O^{18}/O^{16} and S^{34}/S^{32} ratios were plotted as figure 4-5. Here the relative enrichments of sulphur-34 to oxygen-18 yielded a ratio of about 4:1. This ratio in the sulphate was observed to have been independent of the temperature of the reduction.

Variations in the isotopic composition of the unreacted sulphate were most probably a result of the metabolic activity of the sulphate reducing bacteria constituting part of the inoculum. However, the remainder of the inoculum, the sulphite reducing bacteria, were capable of producing variations in the isotopic composition of the sulphate. The effects of their metabolism were believed recorded only in the sulphur isotopic composition of the H_2S gas evolved. The isotopic data from the final sulphide product was noted in Table 4-2 and presented as Figure 4-6.
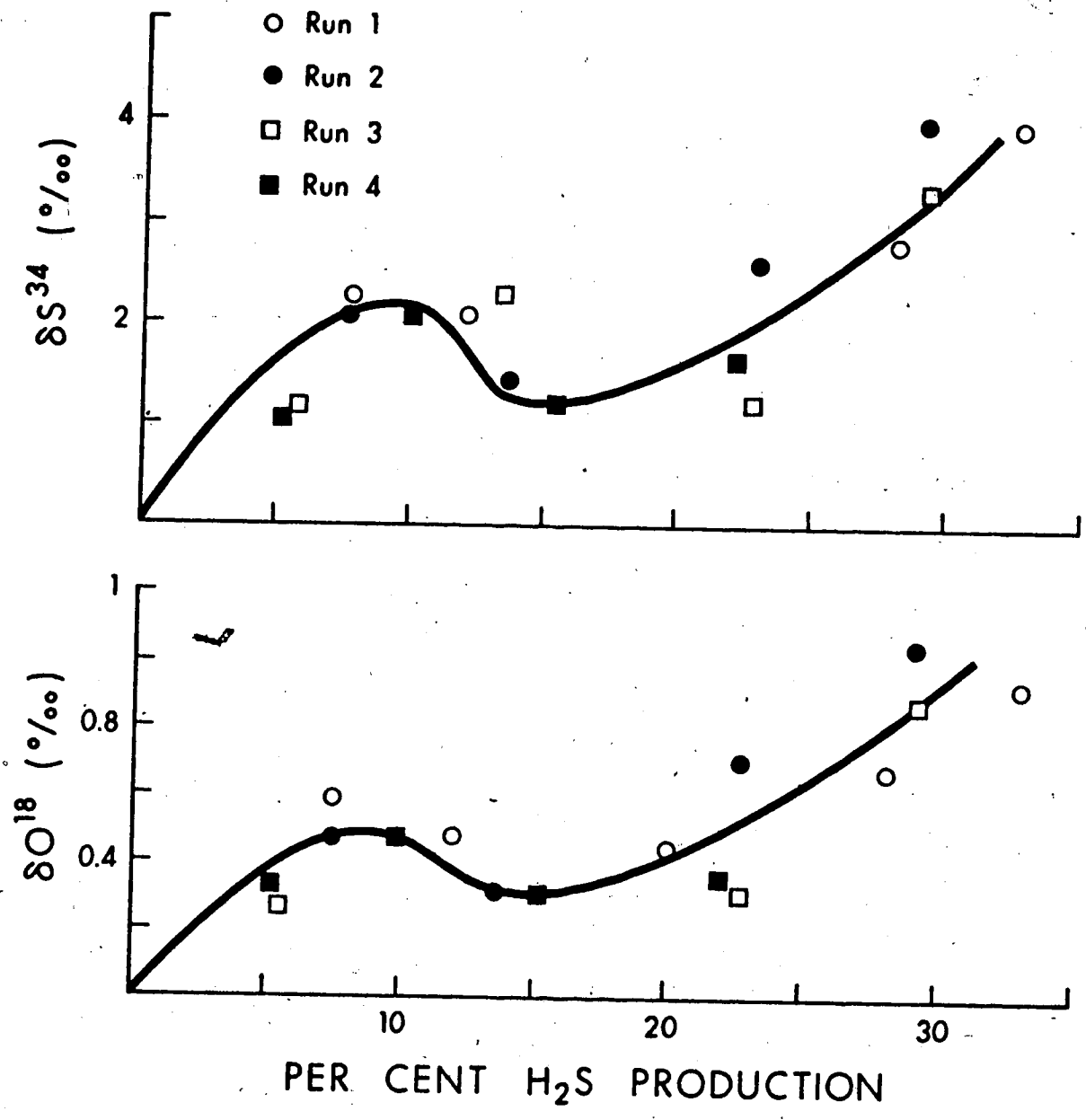
Figure 4-4 δS^{34} and δO^{18} values for unreacted sulphate as a function of the H_2S production.
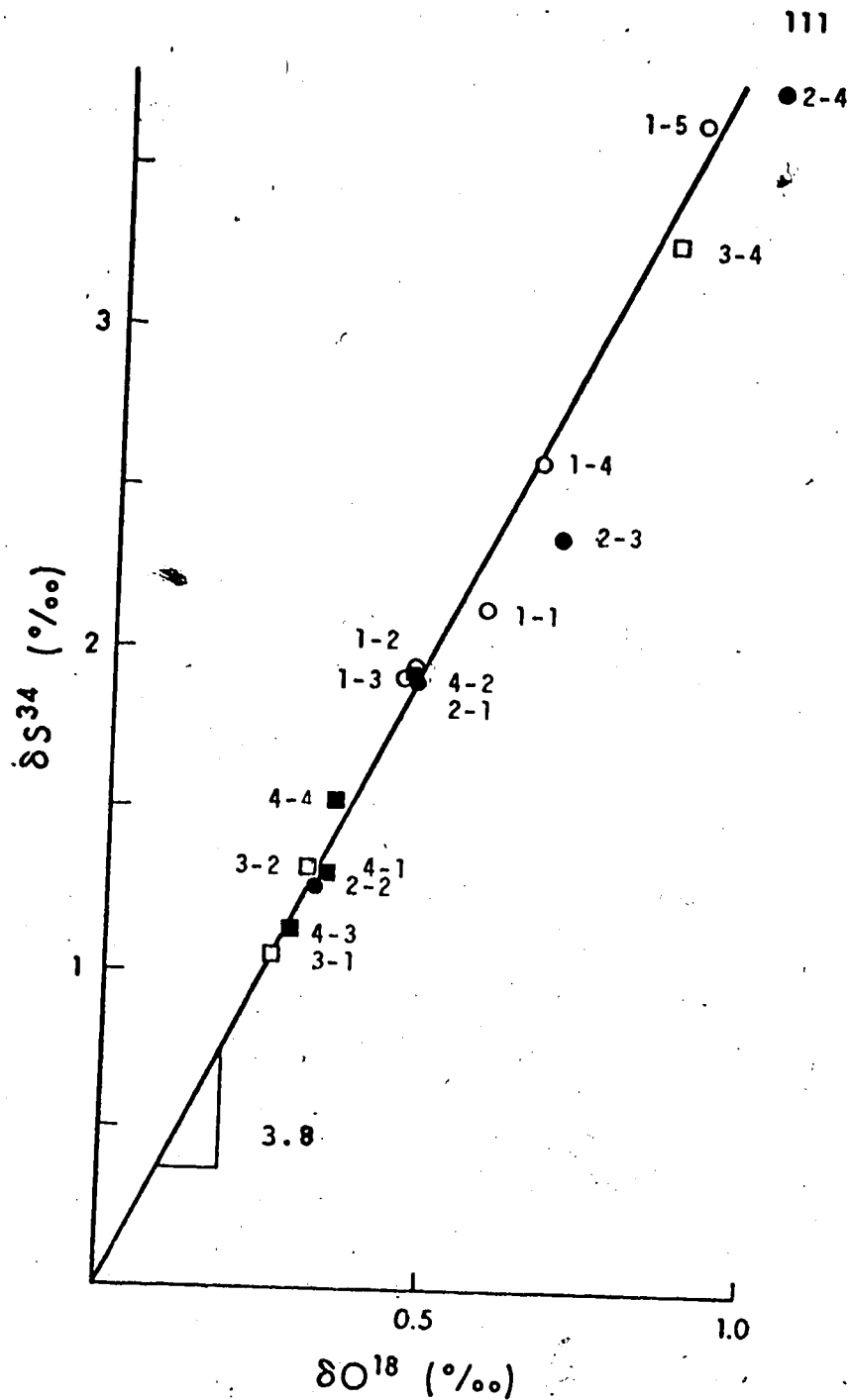
Figure 4-5 Relationship between δ O^{18} and δ S^{34}
values of sulphate during bacterial
sulphate reduction.

N. B. Calculation of the best fitting straight
line yields a slope of 3.82 ± 0.22. Errors of
0.05 °/oo in both coordinates yield a sum of
square residuals of 7.3 with 14 degrees of free-
dom indicating the individual measurement pre-
cision probably better than 0.05 °/oo.
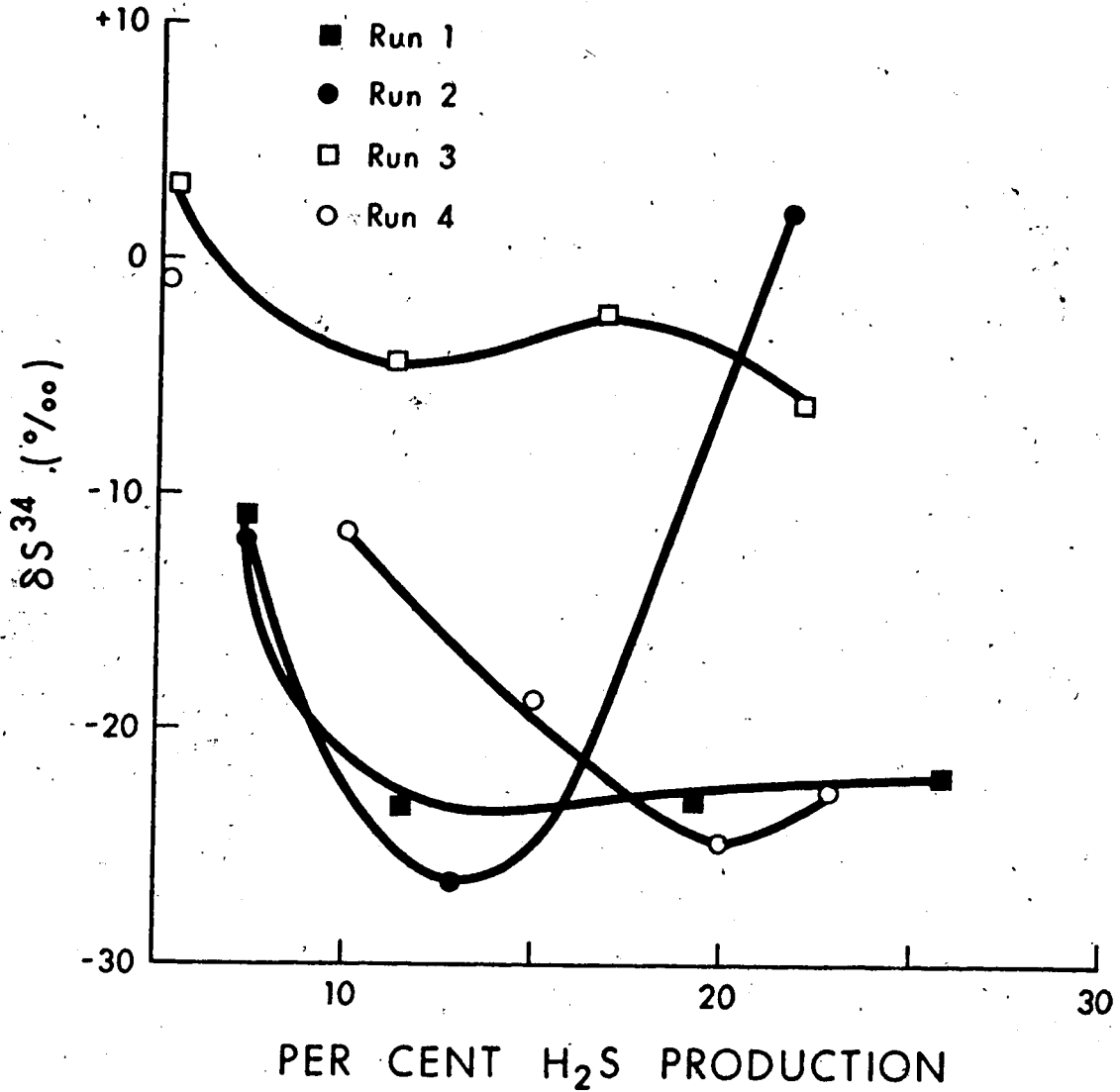
Figure 4-6 δS^{34} values of the H_2S evolved versus the per cent H_2S production.

4.5 Discussion

Since the development of oxygen isotopic abundance ratio measurements in sulphate, no investigators have studied the sulphur and oxygen isotopic correlations observed during the bacterial reduction of sulphate, except MIZUTANI and RAFTER (1969) and the present study. In addition, MIZUTANI and RAFTER (1969) did not conduct their investigation with known microorganisms, but rather with an indeterminate conglomerate of organisms found in stream mud. Hence microbiological conclusions were limited. An analysis of sulphate reduction mechanisms was also impossible with such a complicated medium, in spite of the fact that such conditions are naturally occurring. The understanding of biological metabolisms in such complex enviroments, and in particular the reduction of sulphate, requires intensive investigation if such mechanisms are to be fully understood.

Differences in temperature of the reductions in the present study were observed to alter the fractionation of the isotopes. The first sulphate fraction removed from the reaction flask (Figure 4-4) showed most clearly that the fractionation was greater for reductions 1 and 2 at $24^{\circ}C$, than for runs 3 and 4 at $30^{\circ}C$. (This information was basically masked by the inversions, but witnessed most clearly in runs 1 and 4 with the sulphur isotopic composition of the unreacted sulphate.) This confirmed the observations of earlier investigators. Here a decrease in temperature

caused a decrease in the rate of reduction, which in turn caused an increase in the fractionation. Temperature was only considered important in so far as it modified the rate of reduction.

The metabolic activity of the sulphate reducer became apparent when observing the unreacted sulphate. The relative enrichment ratio was expected, but not the isotopic inversions recorded in both the sulphur and oxygen isotopic data. These variations were thought to have been transferred to the sulphite produced. The sulphite reduction was monitored by observation of the H_2S product (Figure 4-6 shows the sulphur isotopic composition of the sulphite product.) Inversions did appear, but no apparent correlation between the two reduction processes was at first obvious.

A comparison of the relative fractionation of the sulphite and sulphate was thought to be an aid in determining the mechanisms of $SO_4 \rightarrow SO_3 \rightarrow H_2S$ reduction. A plot of the sulphur isotopic variations in the sulphide and unreacted sulphate is shown in Figure 4-7. The same sulphide to sulphate comparison is presented in Figure 4-8 using MIZUTANI and RAFTER's (1969) data. Similar radically varying relative fractionations were observed.

In both MIZUTANI and RAFTER's (1969) study and the present investigation, the slope at any instant was a comparison of the fractionation of sulphur isotopes in the sulphide,
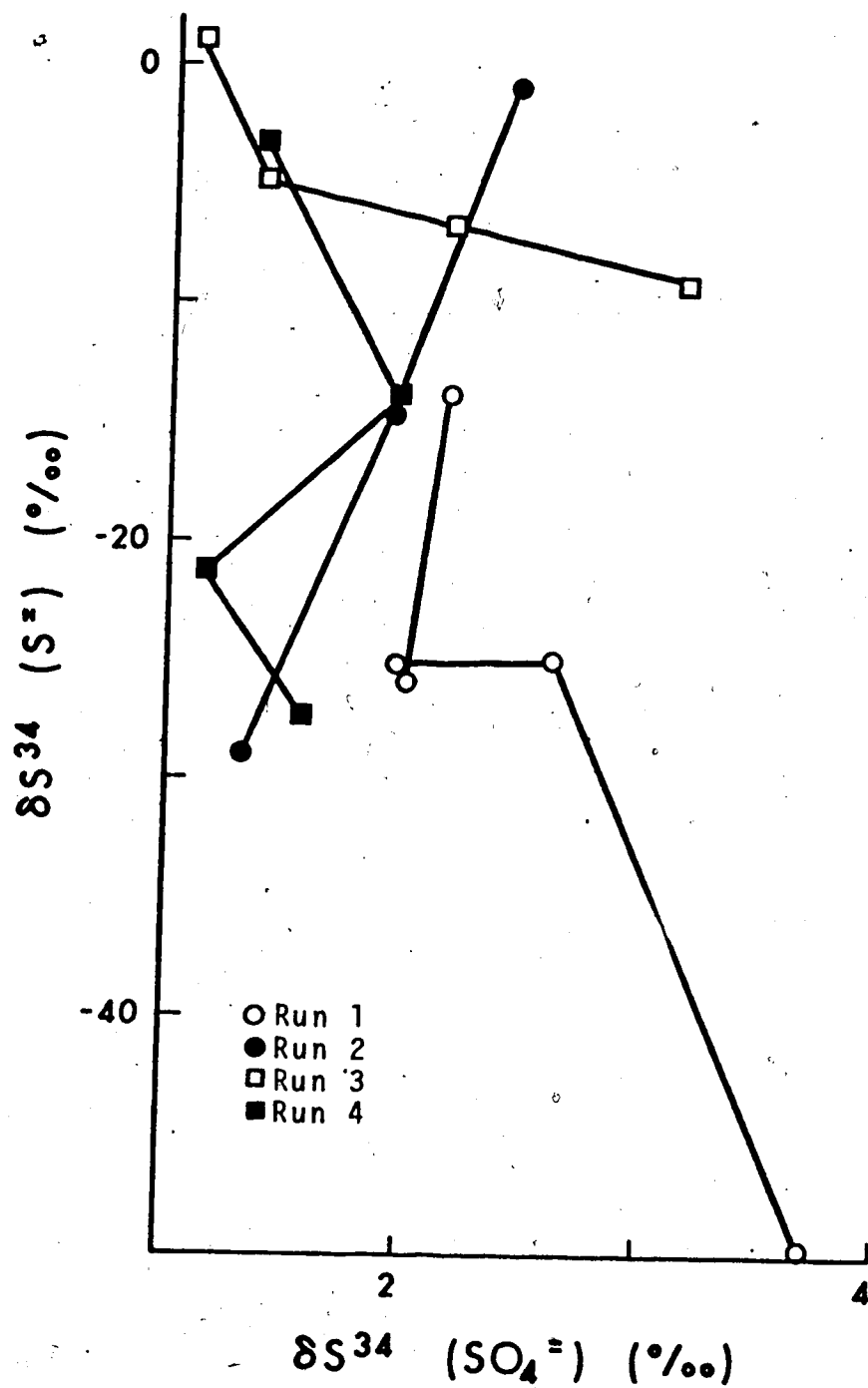
Figure 4-7 δS^{34} of the sulphide versus
the δS^{34} values in sulphate
(present study).
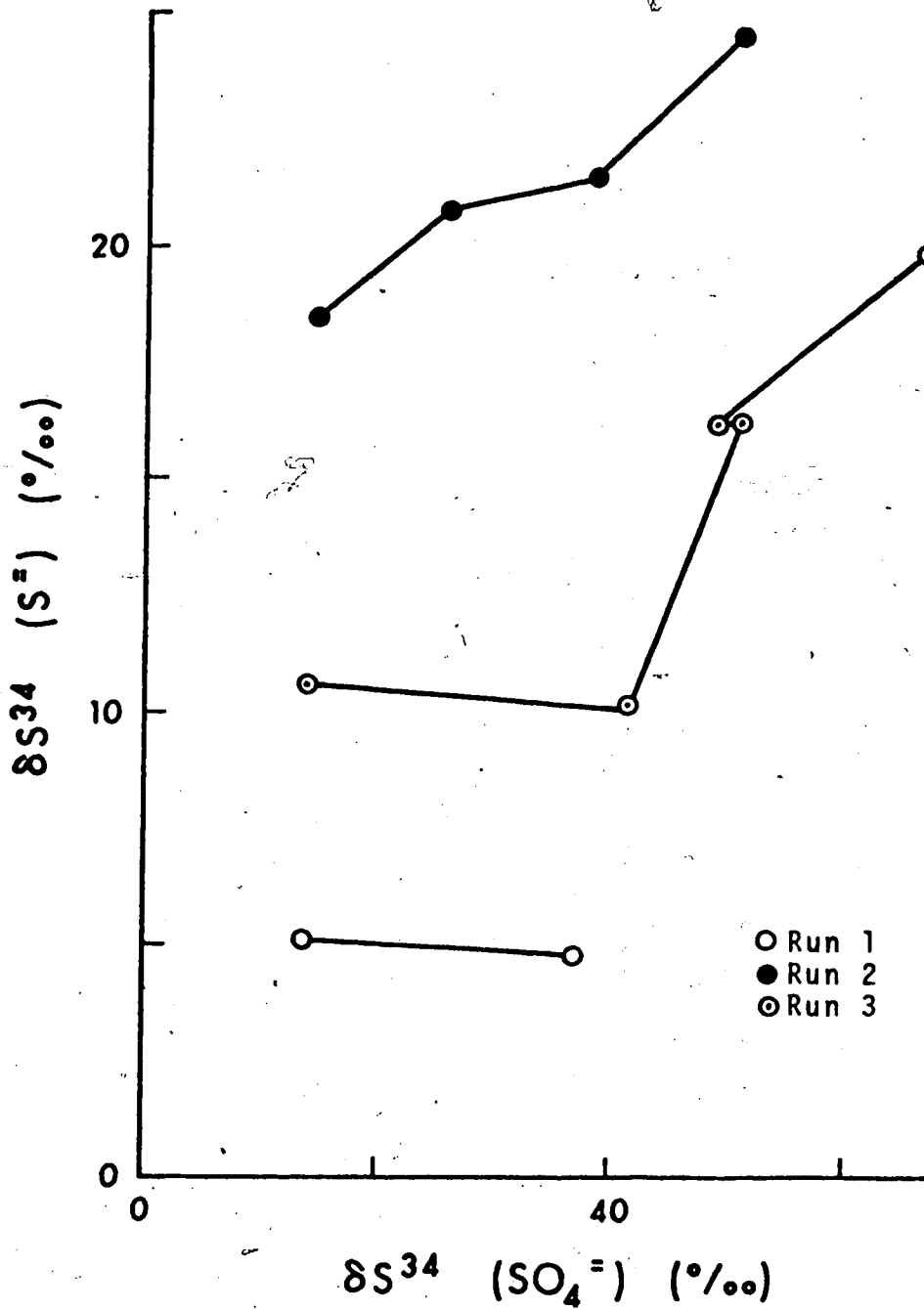
Figure 4-8 δ S^{34} of the sulphide versus the δ S^{34} values in sulphate (Mizutani and Rafter (1969)).

to the fractionation of the sulphur isotopes in the un-
reacted sulphate. The slope variations implied that the
sulphite fractionation and the sulphate fractionation were
not always consistent. (The isotopic inversions of the
present investigation did not appear to cause any change
in the rate proportionality. Runs 2 and 3 compared favorably
to reductions 1 and 2 of MIZUTANI and RAFTER (1969), where
no inversions were observed.)

It has been established (Section 4.1) that the sulphate
fractionation rate is inversely proportional to the rate of
reduction. However, since both studies under consideration
have recorded instances where the sulphite fractionation
rate was both inversely and directly proportional to the
sulphate rate of fractionation, it was concluded that both
inversely and directly proportional reduction rates were
present during the same reduction process (runs 1 and 4 of
figure 4-7, and run 3 of figure 4-8). Hence sulphite was
reduced both faster and slower than sulphate. Thus the rate
controlling step was sulphate and/or sulphite reduction,
depending upon which of the 7 reductions was under consid-
eration. It was hard to conceive of such oscillations in the
rate control during a single bacterial reduction experiment.
Either both sulphate and sulphite reductions were rate con-
trolling and each competing for control of the rate, or else
some other factor controlled the rate of the $SO_4 \rightarrow SO_3 \rightarrow H_2S$

reduction. The complexity of this rate control did seem to become more obvious as the temperature of the reduction increased.

One advantage of employing two strains of bacteria which performed distinctly different metabolic processes, is that the intermediate was known to have been sulphite. The size of this reservoir would definitely elucidate the predominant rate controlling step during a particular sulphate reduction. If the $SO_4 \rightarrow SO_3$ reduction was rate controlling, then there would be no sulphite ions present in the reaction flask once the reduction had ceased. On the other hand, if the $SO_3 \rightarrow H_2S$ reduction was rate controlling, then when the reduction ceased, sulphite would be in solution since it had not been completely reduced by the sulphite reducing bacteria. Thus the double reduction permitted both an isotopic and mass balance, which would yield the amount of the intermediate sulphite present during the reduction, as well as the isotopic composition of this intermediate. These calculations, however, could not be performed on the oxygen data since no reliable oxygen bearing product was liberated, hence only the sulphur isotopes were used for the isotopic and mass balances. At any instant during the bacterial reduction of sulphate, the mass balance yielded

$$100\% = (\% \text{ mg S})_{H_2S} + (\% \text{ mg S})_{\substack{un \\ SO_4}} + (\% \text{ mg S})_{int.} \quad ,$$

where the amount of sulphur in that available system must
equal the amount removed as sulphide, plus the amount
remaining as unreacted sulphate, plus the amount accumulated
as intermediate. The instantaneous isotopic balance yielded

$$(\delta S^{34})_{\substack{total\ S \\ present}} (100\%) = (\delta S^{34})_{H_2S} (\% \ H_2S) + (\delta S)_{\substack{un \\ SO_4}} \cdot$$

$$(\% \text{ un } SO_4) + (\delta S)_{\substack{int \\ SO_3}} (\% \text{ int } SO_3)$$

Here the isotopic composition (^{o}/oo) of the total sulphur
present must be equal to the per cent isotopic compositions
of the sulphide product, the unreacted sulphate, and the
sulphite intermediate. Once the amount of sulphur tied up
as intermediate was determined from the instantaneous mass
balance, then its instantaneous isotopic composition was
evaluated from the isotope balance. These instantaneous
values are recorded in Table 4-3, and reflect the behavior
of the system. (These calculations were only applicable for

Table 4-3   Instantaneous mass and isotopic
balances calculated to determine the
behavior of the sulphite intermediate

Specimen Number	Inst. Mass Balance (% of S available)			Inst. Isotopic Balance (δS^{34}, $^o/oo$)		
	H_2S	un SO_4	int SO_3	H_2S	un SO_4	int SO_3
1 - 1	7.5	64.2	28.3	-13.74	+2.16	-1.26
1 - 2	4.3	67.0	28.7	-25.70	+2.02	+3.00
1 - 3	8.5	68.5	23.0	-25.21	+1.98	+13.46
1 - 4	8.2	67.8	24.0	-24.69	+2.65	+21.49
1 - 5	4.8	59.5	35.7	-49.27	+3.73	+21.61
2 - 1	7.5	81.9	10.6	-14.15	+1.93	-4.90
2 - 2	5.8	86.1	8.1	-28.67	+1.33	-7.81
2 - 3	9.6	86.3	4.1	-0.38	+2.45	-37.51
2 - 4	6.2	93.3	0.5	n. d.	+3.84	n. d.
3 - 1	5.8	55.3	38.9	+0.66	+1.11	-1.68
3 - 2	5.6	59.6	34.8	-6.78	+2.16	-2.73
3 - 3	6.5	60.7	32.8	-4.89	+1.37	-0.48
3 - 4	6.4	66.0	27.6	-8.99	+3.34	-3.29
4 - 1	5.3	71.7	23.0	-3.48	+0.33	-0.23
4 - 2	5.1	72.9	22.0	-14.06	+0.49	+2.50
4 - 3	5.4	78.8	15.8	-21.25	+0.29	+11.89
4 - 4	6.3	78.1	15.6	-27.23	+0.45	+23.32
4 - 5	3.3	n. d.	n. d.	-24.77	n. d.	n. d.

(The δS^{34} value of the initial sulphate was taken as
0 $^o/oo$; the amount of sulphur in the initial sulphate
was = 226 mg, and for these experiments 90 ml aliquots
were withdrawn.)

times when the sulphate and sulphide fractions were withdrawn.) The isotopic composition of the intermediate sulphite was not always observed to lie between that of the unreacted sulphate and the H_2S product, as shown in Figures 4-9, 4-10, 4-11, and 4-12.

Isotope fractionation during the $SO_4 \rightarrow H_2S$ reduction was possible, since there was a large sulphate reservoir available for sulphate reduction to sulphite. The $SO_3 \rightarrow H_2S$ reduction however, had a smaller reservoir. Even with such a limited reservoir, the H_2S should have been isotopically lighter than the sulphite intermediate. As long as sulphite was permitted to accumulate, the sulphide usually has been lighter than the corresponding sulphite. When the isotopic composition of the intermediate remained between the sulphate and sulphide isotopic abundance ratios, it was assumed that there was sufficient sulphite present to have permitted isotope selection by the sulphite reducing bacteria. Such a situation seems to have occurred in the third reduction (Figure 4-11). The sulphate reduction rate was faster than that of the sulphite reduction, hence a sulphite reservoir was present. As the sulphite reservoir increased in size, the $SO_3 \rightarrow H_2S$ reduction preferentially metabolized the lighter sulphur-32. The H_2S product was observed to become isotopically lighter. From Figure 4-11 it appears
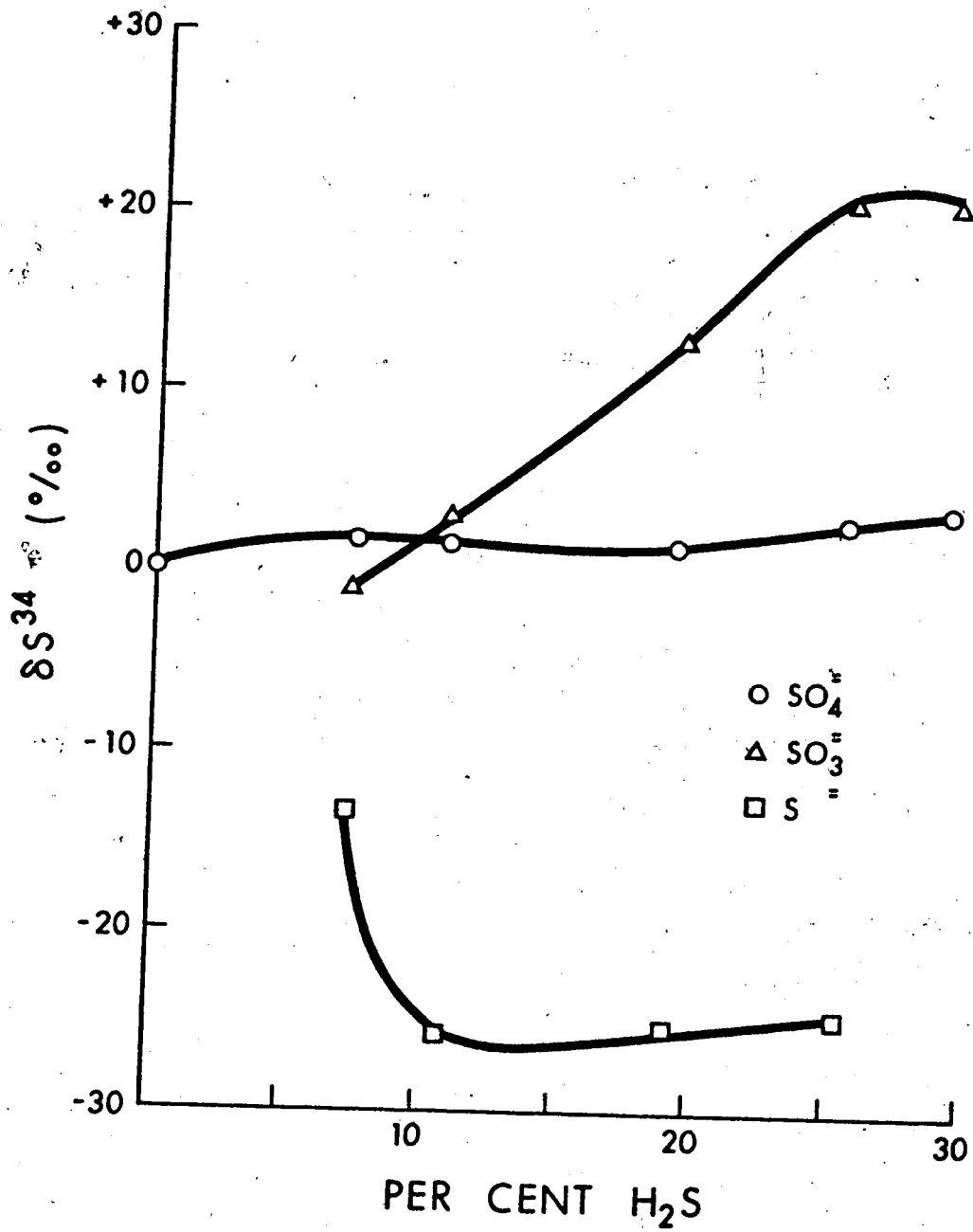
Figure 4-9   Isotope effects during bacterial
reduction No. 1 .
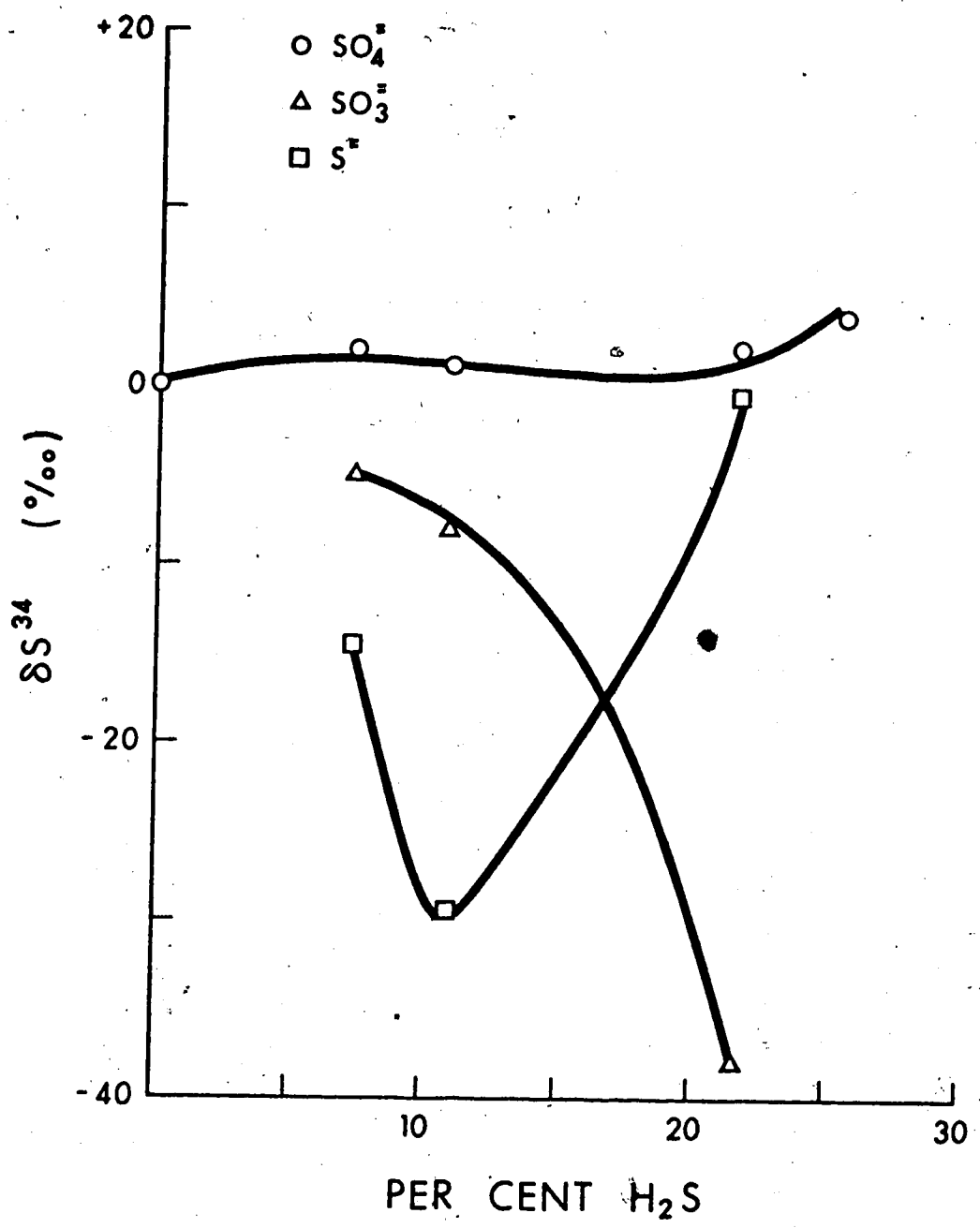
Figure 4-10   Isotope effects during bacterial
reduction No. 2 .
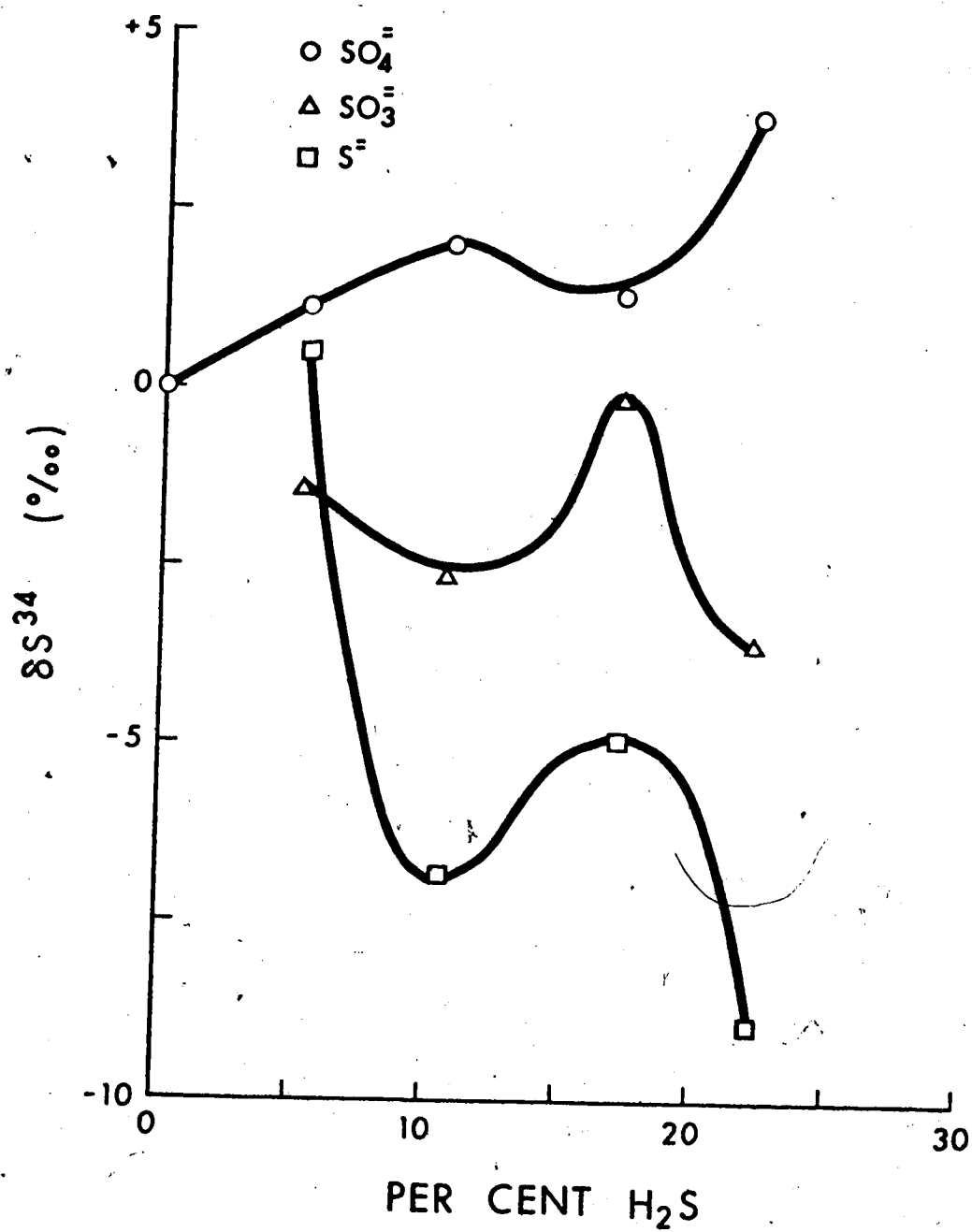
Figure 4-11   Isotope effects during bacterial reduction No. 3 .
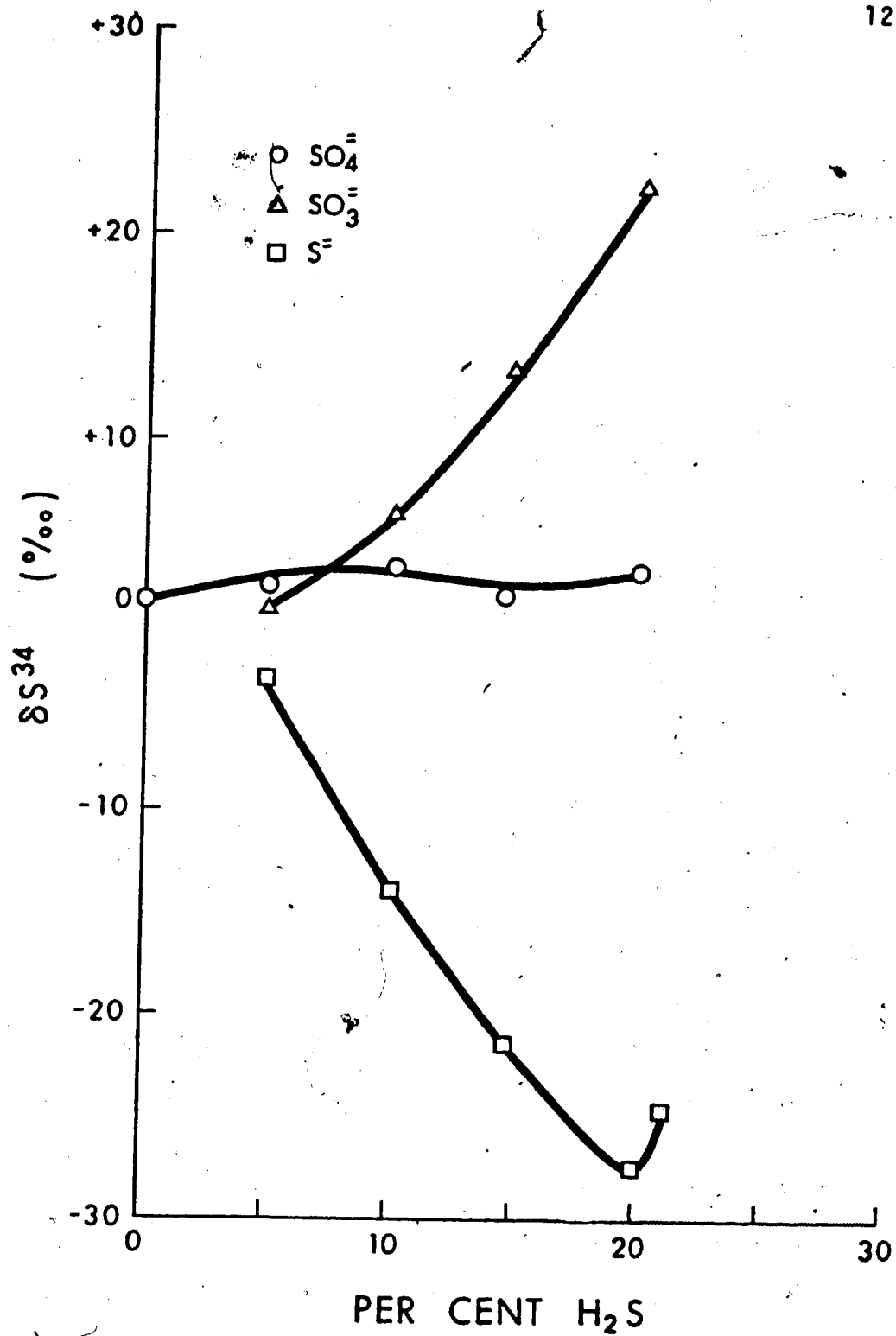
Figure 4-12   Isotope effects during bacterial

reduction No. 4 .

that the sulphite reservoir later stabilized as both the SO_4-SO_3 and SO_3-H_2S reductions proceeded at about the same rate. The final H_2S fraction, however, implied that the SO_4-SO_3 reduction rate rapidly increased. This resulted in a large SO_3 reservoir where the sulphur-32 species were preferentially reduced. Thus a sudden increase in SO_4-SO_3 reduction would have accounted for the low δ S^{34} value of the H_2S. However, it probably would also have produced sufficient quantities of sulphite to cause SO_4-SO_3 reduction cessation, since the SO_4 reducing bacteria are known to be sensitive to high SO_3 concentrations. Sulphite toxicity would have prevented any further reduction of sulphate, but not sulphite, unless there was either a deficiency in nutritional requirements or such a high concentration of sulphite, that even the sulphite reducers found it toxic.

Further verification was obtained from the percentage intermediate present at all stages of the reduction of sulphate. During this reduction experiment, the intermediate consisted of at least 27 per cent of the total sulphur atoms employed for sulphate reduction to hydrogen sulphide. Normally this was greater than 32 per cent, reaching as high as 39 per cent when the reduction ceased. Thus sulphite was not reduced as rapidly as it was formed. The accumulation of a large intermediate reservoir testified to predominant

SO_3 reduction rate control during the latter stages of the
reduction. (Figure 4-7 had previously implied such rate
control.) The general trends of run 3 (Figure 4-7) and
MIZUTANI and RAFTER's (1969) run 1 at $14^{o}C$, seem to be
similar. Their reduction only proceeded to 54 per cent
reaction, in spite of the multiplicity of reducing organisms
present. This could have resulted from an accumulation of
intermediates, which were never reduced to H_2S. During run 3
of the present study, intermediates were always greater than
27 per cent of the total sulphur utilized. Such an accumula-
tion during both reductions was believed to have caused rate
control by the $SO_3 \cdot H_2S$ reducers. They were the only agents
present that could remove the toxic sulphate from the reac-
tion vessel. The general lightening of the H_2S product fur-
ther suggested a modest sulphite reservoir, however the
variations of both run 1 and 4 are difficult to explain.
Table 4- 3 shows that both these reductions had reasonably
large intermediate reservoirs, however MIZUTANI and RAFTER's
(1969) run 3 at $35^{o}C$ had rather confusing variations (Figure
4-8) as well. Their 92 per cent reduction indicated some
unreduced intermediate, but as with the present study, no
meaningful conclusions were forthcoming. No rate controlling
reduction was clearly obvious, but there existed a sufficient-
ly large enough reservoir to have permitted SO_3-H_2S isotopic
selection.

A small sulphite reservoir seems to have been present during the earlier stages of run 2, however, Figure 4-10 shows that this reservoir was soon depleted, since the H_2S fractions became heavier. This would have been due to sulphite reduction occurring as fast as it became available, once the initial reservoir had been exhausted. Thus sulphite was reduced as rapidly as it was formed.

Further proof of a steadily decreasing sulphite reservoir can be seen in Table 4-3, where the per cent sulphite never exceeded 11 per cent of the total amount of sulphur atoms employed. Hence this reservoir was most precarious, threatening the survival of the sulphite reducing bacterial strain. These most adverse conditions suggested sulphate reduction rate control during the reduction of sulphate to the final H_2S product. MIZUTANI and RAFTER (1969) also observed such a rapid reduction of sulphite (run 2 at 25 ^{o}C, Figure 4-8). Their 98 per cent reaction may have only been achieved because of the many reducing organisms employed in their study. Thus other pathways for reduction of sulphate to eventually sulphide may have occurred, but speculation on this point is most impractical.

Clearly, neither sulphate nor sulphite reduction could be solely responsible for rate control during the $SO_4 \to SO_3 \to H_2S$ reduction, rather a competion for control of the rate existed with some other factor determining which step would have controlled the $SO_4 \to H_2S$ bacterial reduction. This variable did certainly influence both the reduction rates, and in addition may have been responsible for the low yields observed with pure culture reduction experiments. Only two conclusions were possible, for such poor yields. Either the sulphate available for SO_4-SO_3 reduction had been depleted, or the available sulphate had not been entirely reduced to some intermediate.

If the sulphate available for reduction had been depleted, then such poor yields were a result of non-reduction of intermediates. If no sulphate was left, then intermediates such as SO_3^- and S_2O_3 were unreacted and present in the reaction vessel. On the other hand, if there was sulphate remaining in the reaction vessel, then the poor yields resulted from a lack of completness of the SO_4-SO_3 reduction.

Now if the sulphate available for reduction had been depleted, then the addition of $BaCl_2$ to the remanent media would have yielded no $BaSO_4$ precipitate. A few drops of HCl were added to the residual media then $BaCl_2$ in excess. The result was as expected. Precipitation of $BaSO_4$ was ob-

served, and the quantities were not negligible. The amount
of unreacted sulphate was then determined to see if the
cause of such poor yields could be explained. Exactly 100
ml of the remanent media and sulphate was used to evaluate
quantitatively the amount of unreacted sulphate. The per-
centages of unreacted sulphur (in sulphate) in the remaining
media were as follows.

Run 1 $BaSO_4$ = .73 mg/ml (S=100 mg/1)= 44.4% of initial
Run 2 $BaSO_4$ = 1.15 mg/ml (S=158 mg/1)= 69.9% of initial
Run 3 $BaSO_4$ = .98 mg/ml (S=135 mg/1)= 59.6% of initial
Run 4 $BaSO_4$ = n. d. , accidentally discarded.

Thus there were large quantities of unreacted sul-
phate present throughout the reduction experiments. If this
unreacted sulphate was ignored, then Figure 4-13 indicates
the relative amounts of intermediate produced during each
sulphate reduction experiment. The unreacted sulphate gave a
further check on the amount of intermediate remaining once
the reduction process ceased. (Previously the sulphate frac-
tions were used to calculate the amount of intermediate pre-
sent during the biological reduction of sulphate.) The
amount of sulphate not reduced when metabolic processes ceased,
indicated that the final amounts of intermediate were as fol-
lows.
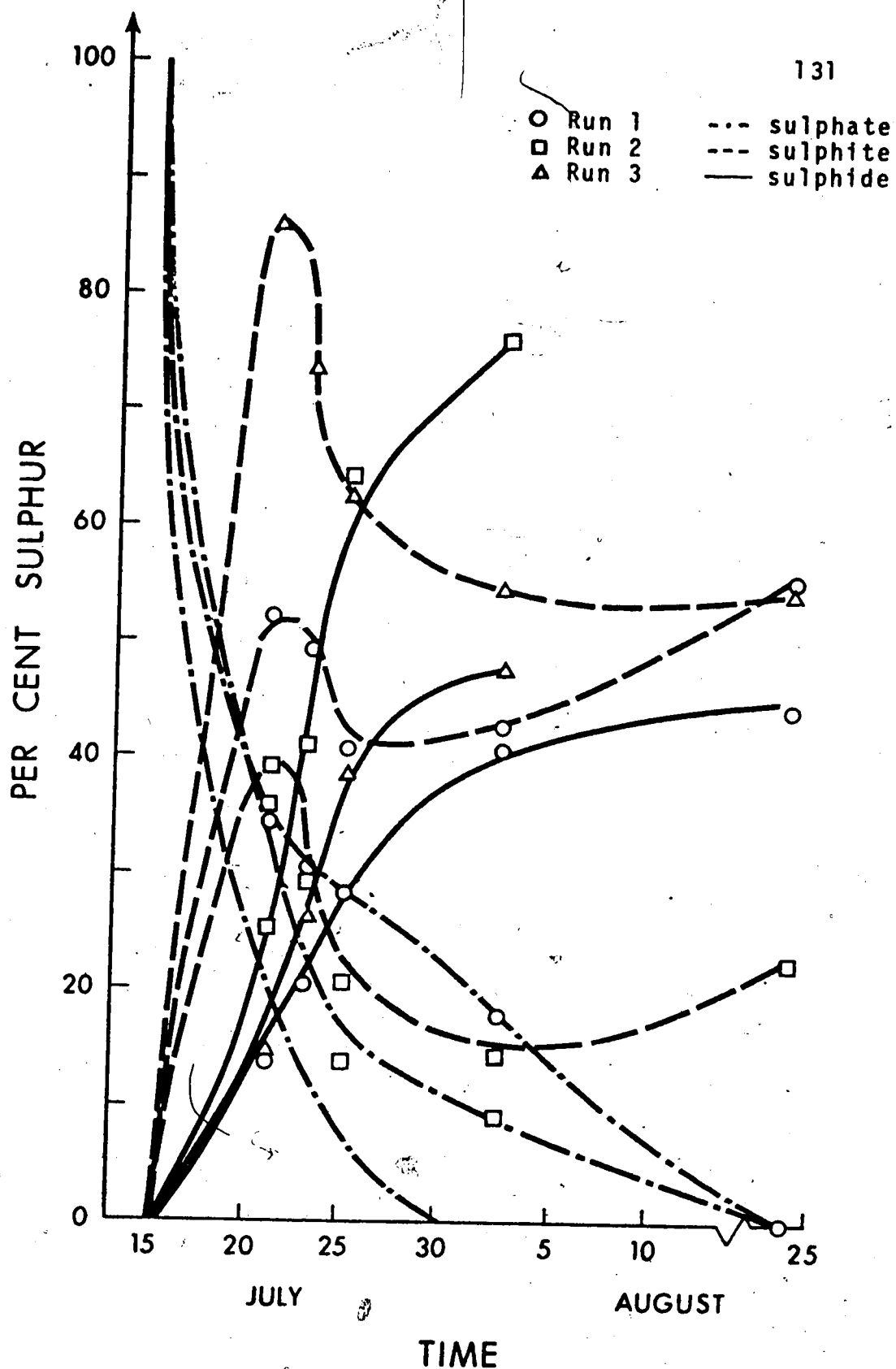
Figure 4-13   Sulphur distribution during the bacterial reduction of sulphate, ignoring unreacted sulphate remaining at the end of the conversion.

	total H_2S	final un SO_4	un SO_4 removed	int. removed	final int.
Run 1	24.4%	28.5%	21.8%	8.5%	16.8%
Run 2	22.7	45.0	27.9	2.0	2.4
Run 3	19.0	38.1	19.8	11.1	12.0
Run 4	19.1	n. d.	25.0	6.4	n. d.

Therefore, there was sulphate available for reduction, but it was not reduced even to sulphite. Within the limits of the chemical preparation and weighing techniques employed, this data confirms the rate control conclusions previously discussed. (Reduction 4 was not available for such an analysis, due to carelessness which resulted in the loss of the final sulphate and media sample.)

Although the per cent reduction was slightly modified by the temperature of the media and inoculum, the poor yields were caused entirely by some other factor. If sulphate was remaining when the reduction had ceased, then the reason would have been sulphite toxicity or simply some metabolic inadequacy. KAPLAN (1962) has stated that sulphite is toxic to most organisms by virtue of its ability to bind carboxyl groups of metabolic intermediates, thus preventing their further metabolism. Such a phenomenon could possibly have accounted for the cessation of microbiological activity in runs 1 and 4. If the reductions did not cease because of

sulphite toxicity, then the question arose as to why such a cessation occurred.

To keep the bacterial strains viable involved removing cultures to new media long before the media was believed to be exhausted of sufficient nutrients for good bacterial growth. Nearly 100 per cent reduction was observed by MIZUTANI and RAFTER (1969), but only when a conglomerate of unknown microorganisms were employed as found in stream mud. KEMP and THODE (1968), in an attempt to obtain greater isotopic fractionation, tried various bacterial strains and physiological states. However, their reductions with pure cultures did not exceed 26 per cent reaction. Complete reductions may be possible with a multitude of reducing bacteria and naturally occurring nutrients, but pure cultures have never escaped this fastidious nature of most microorganisms, when employed in laboratory experiments.

A probable reason for this microbial phenomena, and a possible explanation of why the sulphite reservoir was permitted to develop to such toxic levels, lay hidden in the mechanism involving rate control of the bacterial reduction. HARRISON and THODE (1958), KAPLAN and RITTENBERG (1964), and KEMP and THODE (1968), found the rate of reduction to be inversely proportional to the rate of isotopic fractionation, when lactate was used as the electron donor. However, two

such sulphate reduction experiments of KEMP and THODE (1968) were anomalous. These two reductions occurred at 11° and 20° C, and involved very slow sulphide production coupled with small isotope fractionation. This was attributed to a low temperature effect. What was most startling was that in one series of experiments, the rates of reduction and the isotopic enrichments were not reproducible. This was because there was a direct dependence on the reduction rate, rather than the inverse expected. MIZUTANI and RAFTER (1969) and the present study have also observed such phenomena. This was not a temperature effect, but rather some other factor was responsible for the variations observed.

HARRISON and THODE (1958) stated that S-O bond rupture was rate controlling if the fractionation factor was about 1.025. However, many investigations have since obtained larger values. Thus S-O bond rupture was not rate controlling. KAPLAN and RITTENBERG (1964) concluded that the rate controlling step involved the availability of electrons at the reduction site, hence rate control was dependent upon the electron donor. In the present study the same electron donor was used for all reductions, with the reduction rate and fractionation rate totally independent of the electron donor employed. The reduction rate was observed to be both directly and inversely proportional to the rate of

fractionation at different times during a single bacterial reduction of sulphate experiment. Such was the case for the investigations of MIZUTANI and RAFTER (1969) as well. Hence the rate controlling step during the reduction of sulphate was much more complicated than simple S-0 bond rupture, electron donor, or temperature.

4.6 Conclusions

1. The rate of sulphite reduction was not always proportional to the rate of sulphate reduction. During a single microbiological reduction, both reduction processes were competing for control of the rate, with each rate controlling at various stages of a single $SO_4 \rightarrow SO_3 \rightarrow H_2S$ reduction.

2. Any accumulation of sulphite, during the bacterial reduction of sulphate, is toxic. Sulphite accumulation prevents complete sulphate reduction, because sulphite is toxic to most organisms by virtue of its ability to bind carboxyl groups of intermediates, thus reducing the rate of metabolism.

3. Meaningful microbiological conclusions were only believed possible when a pure culture and known media were employed, in spite of the fact that this is not characteristic of naturally occuring phenomena.

4. Pure cultures of bacteria cannot complete reduction of sulphate because of the simplicity of the media

employed. Only in nature, with its complex forms, nutrients, and pathways, might reduction possibly be complete. Strain acclimatization and poor yields pose the greatest problems in laboratory studies of pure cultures.

5.   An important temperature independent relationship was observed between the O^{18}/O^{16} and the S^{34}/S^{32} ratios of the residual reactant sulphate. The relative enrichment of sulphur-34 to oxygen-18 was found to be approximately 4:1. (This will be discussed further in Sections 5.4 and 5.5.)

6.   Large isotope fractionations were observed between the intermediate and the H_2S final product.   In Run 1 the fractionation reaches as high as 70 per mil (unusually high, since MCCREADY (1974) recently obtained a large fractionation with Yeast of 50 per mil, which to date is considered very high).

Chapter V.   Isotope Effects During Microbiological Sulphate
             Reduction

5.1 Microbiological Sulphate Reduction Mechanisms

In order to explain the observed isotope effects during
microbiological sulphate reduction, it is necessary to ex-
amine mechanisms involved. The basis of these mechanisms is
a redox reaction which occurs when an energy source becomes
oxidized (loss of electrons) and another material becomes
reduced (gain of electrons). Lactate was employed as the
energy and carbon source, donating electrons, while the
sulphate or sulphite ion accepted electrons during the
oxidation - reduction experiments of the present investigation.
KAPLAN and RITTENBERG (1964) assumed that this electron
transfer was the rate controlling step during the microbio-
logical reduction of sulphate.

Once the electron donor, the lactate, has been oxidized,
it is no longer an energy source and may then serve as an
electron acceptor. In such a situation, competition could
occur between sulphate, sulphide, and the oxidized lactate,
to serve as the electron acceptor.

The biological reduction of sulphate mechanisms are
presented in Figure 5-1. These mechanisms are employed
by most sulphate reducers. Although not all sulphate re-
ducers belong to the genus *Desulphovibrio* or *Desulphato-
matulm*, isolotes from nature are most frequently species
of these genuses. These bacterial forms are obligate

SO$_4^=$ (ext.)

cell wall enzyme

SO$_4^=$ (int.)

ATP

ATP sulphurylase

PP

AP

+2e

APS reductase

AMP

SO$_3^=$

+6e

sulphite reductase

S$^=$
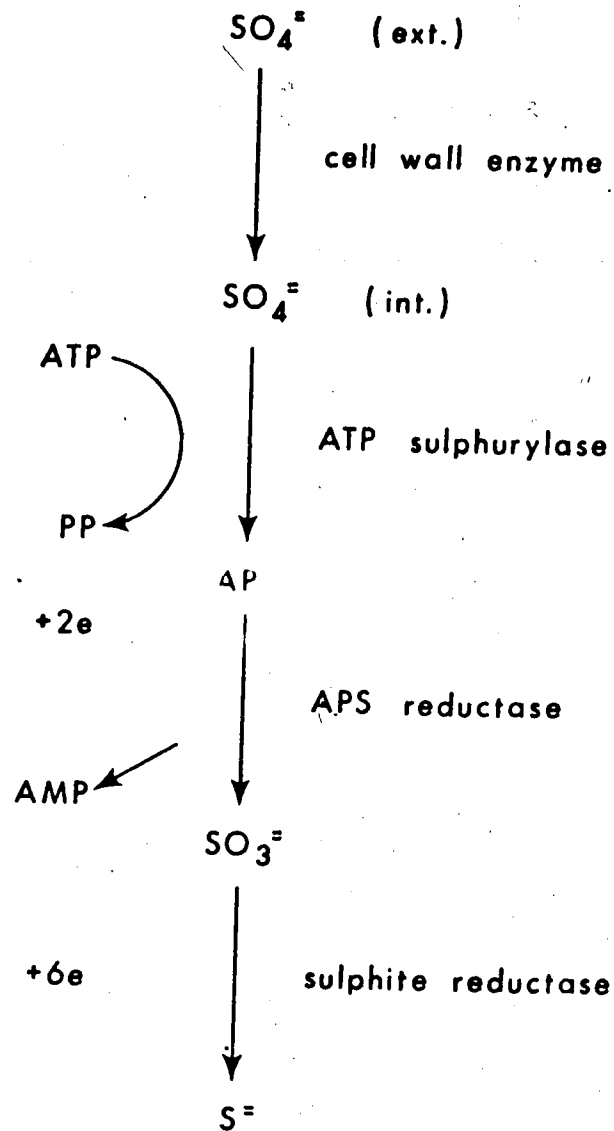
Figure 5-1   Mechanisms of bacterial sulphate reduction (dissimilatory).

anaerobes which can use either H_2 or organic compounds (such as lactate and ethanol) as the energy source.

.During biological sulphate reduction, the energy source transfers electrons through a cytochrome system to the sulphate. This cytochrome is a natural electron carrier, which undergoes oxidation and reduction through a loss or gain of electrons from the iron atoms. These iron atoms were part of the culture media (Fe Cl_2) employed for the reduction experiments. In nature such a process plays a considerable role in the corrosion of iron and steel pipes. This cytochrome electron transfer is the first step of sulphate reduction. That is, with molecular hydrogen as the electron donor, this electron transfer is

$$H_2 + cytochrome^{+3} \rightleftharpoons cytochrome^{+2} + 2H^+.$$
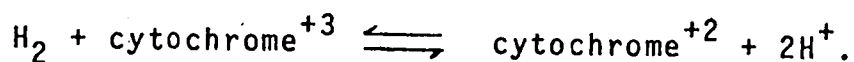
(The cytochrome^{+3} is sometimes written as cytochrome 2 Fe^{+3}, and the cytochrome^{+2} as cytochrome 2 Fe^{+2}.)

This transfer of electrons through a cytochrome system to the sulphate can only occur if the sulphate is in a form which can accept electrons. This is achieved by the high energy phosphorous compound. ATP (adenosine triphosphate), which plays an important role in energy metabolism. The energy derived from oxidation-reduction

is conserved in the high energy bonds of the ATP. This
energy is later released by the enzyme kinares for cell
functions such as mobility, biosynthesis, and growth.
The production of ATP occurs during substrate phospory-
lation. This will occur only if a suitable electron
acceptor is present. Such an electron acceptor is the
sulphate ion.

In order to accept electrons, the sulphate ion must
first be enzymatically converted to APS (adenosine phos-
phosulphate) in the presence of ATP. This is the second
step of the reduction mechanism,

$$ATP + SO_4^{-2} \; \rightleftarrows \; APS + PP \; .$$
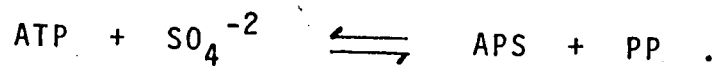
The product PP (pyrophosphate) forms when the sulphate
radical is included in the APS. The sulphate ion is sub-
sequently reduced to sulphite by the enzyme APS reductase.
This third step can be represented as

$$APS \longrightarrow PAP + SO_3^{-2} \; ,$$

NADPH   NADP
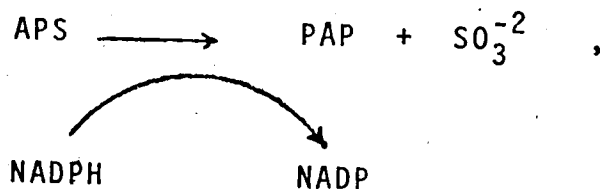
where the APS reductase requires NADPH as an electron
donor-coenzyme.

The fourth step of the SO_4-SO_3-H_2S reduction also
involves an enzyme using NADPH as an electron donor. The
NADPH coenzyme is not permanently attached to enzyme pro-
teins, but rather combines with them only during
the enzymatic reaction. This coenzyme NADPH (nicotinamide
adenine dinucleotide phosphate) is an electron carrier which
donates an electron, becoming the oxidized NADP. (NADPH is
more accurately written as NADP + H^+). Thus the coenzyme
NADPH is necessary in order that the sulphite reductase
enzyme can reduce sulphite to sulphide. That is,

$$SO_3^{-2} \longrightarrow S^{-2}$$

NADPH   NADP
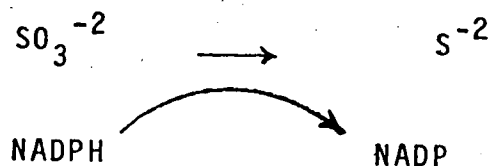
These four steps (see also Figure 5-1) are the
known processes involved in anaerobic respiration. In
such a reduction, the difference in mass of the isotopic
species will be an important factor. Since energy, entropy,
and the free energy of isotopic substances depend on the
vibrational frequencies of the molecules, which in turn
depend on the masses of the atoms in the molecules, it can
be seen that there is a basis for the differences in pro-
perties of isotopic materials. Hence there will be different
rates for each step of the reduction, favoring either the
heavier or lighter isotopic species. Thus isotopic abundance
ratios were believed able to elucidate which mechanisms were

rate controlling during the microbiological reduction of sulphate.

5.2 Reduction Mechanisms and the Isotopic Inversions

The mechanisms of biological sulphate reduction have been outlined (Section 5.1) as presented in BROCK (1970). KEMP and THODE (1968) discussed a similar pathway for the assimilation of sulphate, however, they noted two additional phases of sulphate metabolism which could give rise to isotope effects. They were, the assimilation of sulphate by the cell, and the passage of H_2S out of the cell. Thus the rate controlling step during $SO_4-SO_3-H_2S$ reduction should be one or more of the following:

I. the assimilation of sulphate by the cell,

II. the transfer of electrons at the reduction site,

III. the assimilation of sulphate by ATP,

IV. the reduction of APS to sulphite,

V. analogous complicated enzymatic processes for the reduction of sulphite to sulphide, and

VI. the passage of H_2S out of the cell.

To elucidate the rate controlling mechanisms of microbiological sulphate reduction, it became necessary to examine the isotopic data more closely. An explanation of the observed isotopic inversions (Figure 4.1) was thought to help in determining the rate controlling steps, since

both the sulphur and oxygen data indicated an isotopic inversion phenomena. However, interpretations are restricted since a chemostat was not employed for the bacterial reduction experiments. Thus the cell population and reduction pH were never known.

In such a closed system reduction experiment, the lactate electron donor was believed to have oxidized to acetic acid (CH_3COOH). With no pH regulation available, this sulphate reduction by-product would have lowered the pH of the culture media and inoculum. Since the enzymes associated with bacterial sulphate reduction are known to be very pH sensitive, a lowering of the inoculum pH would have caused cell death (lysis). The resulting intracellular sulphur accumulation would have permitted the remaining viable bacteria to metabolize these organic nutrients rather than the inorganic sources present in the culture media.

The intracellular sulphur present in dead cells has a lower δS^{34} value than the available sulphate (MCCREADY and DIN (1973)). Thus during culture regrowth on metabolites released from lysis, the sulphate isotopic composition would temporarily lighten. (Such an inversion was observed in Figure 4-4.) This regrowth metabolism, however, releases NH_4, which would raise the inoculum pH. Once this regrowth had been established, the pH would increase preventing further lysis. Once the metabolites released from lysis were exhausted, the inorganic sources

in the culture media would have been re-employed. The
isotopic abundance data would then indicate a progressive
enrichment of δS^{34} and δO^{18} in the sulphate. This was
observed experimentally. Soon after this regrowth all
microbiological activity ceased. This was thought to be
a result of the increasing pH, preventing a viable enzy-
matic environment. This conclusion would support the
known pH sensitivity of enzymes. Such an explanation for
low yields is believed valid for similar sulphate reduction
experiments where strict pH and population monitoring are
not employed. Thus complete (100%) sulphate reduction is
not believed possible unless these variables are controlled,
such as can be done in a chemostat.

It should be noted that the intracellular sulphur
released during lysis-metabolism would not have produced
a large isotopic enrichment, because of the limited intra-
cellular reservoir. The present study data did possess
isotopic enrichments of the same order of magnitude as
expected (MCCREADY and DIN (1973) from intracellular accumu-
lations (i.e. less than 0.8 ^{o}/oo for sulphur and 0.2 ^{o}/oo
for oxygen).

The isotopic inversions were believed caused by
critical pH variations. No information concerning SO_4-SO_3-
H_2S reduction rate-control was thought possible from an
examination of the isotopic inversions. Hence pH contributed

to rate control only in so far as it regulated cell populations and isotopic enrichments. The pH variations were also believed to be responsible for some of the very large fractionation factors observed during bacterial reduction of SO_4 experiments. This will be discussed later in Section 5.4.

The isotopic data of Section 5.4 indicate that the bacterial reduction of sulphate was strictly a first order kinetics reaction. The fractionation factors (α) were consistent with first order kinetics, but the fraction fractionation factors (β) certainly were not. REES (1973) has recently discussed isotopic fractionation effects during the reduction of sulphate to hydrogen sulphide in experiments with *Desulphovibrio desulphuricans*. He has presented a steady state model which explains higher fractionation factors in terms of full reversibility of flows between the external sulphate and the internal sulphite. This mo will help to explain the high fractionation factors of the present study, but it involves the addition of back reactions to the forward reactions presented in Section 5.1 as possible rate controlling steps during bacterial sulphate reduction. Thus these high fractionation factors may involve reversibility of the mechanisms of biological sulphate reduction, but they yield no additional possibilities for elucidation of the rate controlling steps. Therefore the forward reaction steps must be modified

to include full reversibility,with the additional steps being:

VII.   the internal sulphate accumulation and a back
       reaction to external sulphate,

VIII. the accumulation of APS and a back reaction to ATP
       liberating the sulphate ion,

IX.   a sulphite accumulation and a back oxidative
       reaction to APS,

X.   an internal H_2S accumulation resulting in the back
       oxidative reaction to sulphite,and

XI.   an accumulation of H_2S in solution which re-enters
       the bacterial cell.

The possible steps involved in biological sulphate
reduction are believed complete. It is now necessary to
discuss each step in terms of known experimental evidence,
to determine which steps are rate controlling.

5.3 The Rate Controlling Steps

An attempt was made by HARRISON and THODE (1958)
to explain the mechanisms controlling fractionation during
sulphate and sulphite reduction. They concluded that the
breaking of the S-O bond during the reduction of sulphate
to sulphite, was the dominant process controlling fraction-
ation at low rates of sulphate reduction. This S-O bond
rupture was believed to possess a kinetic isotope effect

of 22 $^o/oo$, which does not adequately explain more recent data where much greater fractionations have been observed in both laboratory experiments and in nature.

The temperature, sulphate concentration, electron donor, and pH, within the normal physiological range of these parameters, were concluded to influence fractionation only in so far as they influenced the rate of reduction (KAPLAN and RITTENBERG (1964), and KEMP and THODE (1968)). Thus it was concluded that the rate controlling step must be one or more of the forward or backward reaction steps previously outlined.

The initial step (Step I) of the bacterial reduction process is the uptake of sulphate by the bacteria. This process is first order with respect to sulphate at low sulphate concentrations (HARRISON (1957)). At higher concentrations, the rate of sulphate uptake is independent of the sulphate concentration. Here the limiting factor is most probably the supply of an enzyme which complexes with the sulphate ion to transport it across the bacterial cell wall. REES (1973) treated this step as zeroth order with respect to sulphate, and postulated the establishment of steady state conditions at such concentrations. If the rate of entry becomes rate controlling, then no matter what isotope effects are possible in the reduction phase of metabolism, the isotope effects observed will be only those due to entry of the oxidant.

The bacterial uptake of sulphate was concluded by KAPLAN and RITTENBERG (1964), KEMP and THODE (1968), and REES (1973) to have a negligble isotope effect. If the rate of entry of sulphate into the cell was rate controlling, then at low reduction rates the fractionation would have been almost zero. This is not in agreement with experimental evidence, since with lactate or ethanol as the electron donor, the fractionation rate was observed to be inversely proportional to the rate of reduction. REES (1973) noted that this first forward Step I possessed an inverse isotope effect of - 3 ^{o}/oo, so that $S^{34}O_4$ uptake is favored over $S^{32}O_4$ uptake. He also concluded that the isotope effects in either the forward (Step I) or backward (Step VII) steps involving SO_4, uptake, were probably small since they are associated with reactions where the oxidation state of sulphur is not altered.

Hence Step I and Step VII of the reaction sequence were concluded to be not rate controlling, except at low sulphate concentrations. These processes, as concluded by REES (1973), were then zeroth order with respect to sulphate concentration. On the other hand, the passage of H_2S from the cell is presumably fast and hence not rate controlling since H_2S is toxic towards cell constituents. REES (1973) concluded that Step VI, the passage of H_2S from the cell, involved only a small isotope effect and

was not rate controlling. He also concluded that the backward Step Xl possessed a small isotope effect and was similarly not rate controlling. Thus the rate controlling steps are to be found during the reduction of sulphate and/or sulphite in the bacterial cell.

Steps V and X involve analogous but more complicated enzymatic processes than are involved in $SO_4 \rightarrow SO_3$ reduction. The present study (Section 4.5) and the investigation of MIZUTANI and RAFTER (1969) have shown that the rate of controlling step occurred in both SO_4-SO_3 and SO_3-H_2S reduction, hence it must have involved some biochemical process common (analogous) to each.

The bacterial reduction of Sulphate solely by a sulphate reducer as *Desulphovibrio desulphuricans* did not permit other investigators to realize that rate control was common to both SO_4-SO_3 and SO_3-H_2S reductions.

Although detailed biochemical mechanisms of sulphite reduction are not known, it is assumed that a similar enzymatic pathway should have been the reduction mechanism for $SO_3 - H_2S$ reduction. This would have been somewhat more complicated in that three oxygen atoms are removed as compared to only one in the $SO_4 - SO_3$ reduction. Regardless, both reduction processes were observed to be rate controlling when all other parameters apparently were constant. Hence the rate controlling step was not sulphate

nor sulphite reduction, but rather some bacterial process common to both reductions. Thus Steps V and X can be eliminated from the list of possible rate controlling steps.

It should be noted that the poor yields of other investigators and the present study were most probably a result of the experimental media employed. MIZUTANI and RAFTER (1969) and NAKAI and JENSEN (1964) obtained good yields employing media found in nature, since these materials are much more complex than simple laboratory preparations. Also the electron donors used in previous investigations were not materials found commonly in nature (lactate itself is not so abundant in nature). Acclimatization of the bacteria to these artificial nutrients was then a possible explanation for the poor yields. KEMP and THODE (1968) found strains that would never acclimatize to laboratory media. Hence the apparent fastidious nature of microorganisms in laboratory experiments may have been solely a result of poor medium acclimatization, poor choice of electron donor, enzymatic and cell deficiencies in the media, and difficulty in microbiological utilization of non-natural energy sources.

Actual rate control may be linked to a poor acclimatization of simplified media constituents and inorganic energy sources. Furthermore, the detailed biochemical

mechanisms of energy source reduction by sulphate and sulphite reducers is still not fully understood. The SO_4 - SO_3 reduction process has been investigated as outlined previously from BROCK (1970). KEMP and THODE (1968) mentioned specific details of this reduction that were noted in BROCK (1970), but were the results of investigations by POSTGATE (1956), ISHIMOTO, KOYAMA, and NAGAI (1954), ISHIMOTO (1959), and PECK (1959, 1960, 1961, 1962). At present the enzymatic requirements involved are not understood well enough to permit good laboratory experiments to be carried out (i.e. 100 % reduction of available sulphate).

If enzymatic requirements are the basis for the poor yields reported, then only further microbiological investigations will clarify this lack of understanding concerning the microbiological reduction of sulphate and sulphite. If poor acclimatization by the bacterial strains is not rate controlling, then the possible steps that could have controlled the reduction rate should be:

II. the transfer of electrons at the reduction site,
III. the assimilation of sulphate by ATP,
IV. the reduction of APS to sulphite, and
IX. a sulphite accumulation involving a back oxidative reaction to APS.

Extensive study is still needed to completely under-
stand rate control during microbiological sulphate reduc-
tion, and isotope studies may not be solely capable of
providing all the solutions. However, isotope data can be
analyzed to yield information about some of the physical
processes involved.

5.4 Isotope Effects

When a substance continuously loses material that
had a fixed isotopic ratio relative to that of the remain-
ing substance, the Rayleigh distillation equation is app-
licable. To express the change in isotopic ratio in terms
of the "del" notation, the Rayleigh equation

$$\delta - \delta_0 = 1000 (\alpha - 1) \ln F$$

is employed. Here δ_0 is the isotopic composition of the
original   sulphate, δ is the isotopic abundance ratio of
the sulphate when the fraction of sulphate remaining is F,
and α is the average kinetic fractionation factor for this
process. The enrichment factor $1000(\alpha-1)$ was calculated for
both sulphur and oxygen isotopes in the sulphate ion. The
results appear in Table 5-1. The ratio of the sulphur-34
enrichment to the oxygen-18 enrichment in the sulphates was
found to be approximately 4:1. That is, the enrichment re-

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Table 5-1 Calculation of the kinetic fractionation factors (α) for oxygen and sulphur isotopes in sulphate during the bacterial reduction of sulphate

Specimen Number	Sulphate $(\delta S^{34}_t - \delta S^{34}_o)$	$(\delta O^{18}_t - \delta O^{18}_o)$ (°/oo)	Fraction F	-ln F	$1000(\alpha-1)$ S	$1000(\alpha-1)$ O	$\dfrac{1000(\alpha_s-1)}{1000(\alpha_o-1)}$
1 - 0	0.00	0.00	1.000	0.000	---	---	
1 - 1	2.16	0.58	.642	.443	4.9	1.3	
1 - 2	2.02	0.47	.619	.480	4.2	1.0	
1 - 3	1.98	0.44	.606	.501	4.0	0.9	
1 - 4	2.65	0.66	.549	.600	4.4	1.1	
1 - 5	3.73	0.90	.442	.816	4.6	1.1	
					22.0	5.4	$\dfrac{22.03}{5.37} = 4.10$
2 - 0	0.00	0.00	1.000	0.000	---	---	
2 - 1	1.93	0.46	.819	.200	9.7	2.3	
2 - 2	1.33	0.32	.797	.227	5.9	1.4	
2 - 3	2.45	0.69	.752	.285	8.6	2.4	
2 - 4	3.84	1.02	.735	.308	12.5	3.3	
					36.6	9.4	$\dfrac{36.58}{9.44} = 3.88$
3 - 0	0.00	0.00	1.000	0.000	---	---	
3 - 1	1.11	0.26	.553	.592	1.9	0.4	
3 - 2	2.16	n.d.	.563	.574	3.8	n.d.	
3 - 3	1.37	0.33	.540	.616	2.2	0.5	
3 - 4	3.34	0.86	.549	.600	5.6	1.4	
					9.6	2.4	$\dfrac{9.62}{2.41} = 3.99$
4 - 0	0.00	0.00	1.000	0.000	---	---	
4 - 1	1.35	0.33	.717	.333	4.1	1.0	
4 - 2	1.94	0.49	.690	.371	5.2	1.3	
4 - 3	1.15	0.29	.708	.345	3.3	0.8	
4 - 4	1.58	0.45	.664	.409	3.9	1.1	
					16.5	4.3	$\dfrac{16.47}{4.25} = 3.87$
							avg. = 3.96

lationship between the two kinetic fractionation factors was calculated to be

$$\frac{\alpha_s - 1}{\alpha_0 - 1} = 3.96 \quad .$$

The kinetic fractionation factors (α) calculated using the Rayleigh distillation equation, were average fractionation factors for the bacterial reduc on process. The relative enrichment ratio of the two iso pic species was in agreement with that observed by MIZUTANI and RAFTER (1969) during their independent but concurrent investigation. In spite of the differences in media, organisms, and techniques of both studies, this 4:1 ratio remained relatively independent of all factors save the presence of sulphate reducing bacteria. An extensive analysis of their data appeared somewhat out of order because of their employment of several flasks. However, since with pure cultures (present investigation) a similar phenomena was observed, it seemed reasonable to analyze their observations further.

The kinetic (average) fractionation factors (α) of both studies yielded enrichment ratios of about 4:1. The fraction fractionation factors (β) for each sulphate fraction removed did not appear to follow the trend of the kinetic fractionation factors (α). The fraction fractionation ctors (β) were calculated for both investigations as follows.

Sample 1-1   removed at 35.8% reaction, $\alpha_s = 1.0049$

$$\alpha_o = 1.0013$$

Sample 1-2   removed at 38.1 reaction, $\alpha_s = 1.0042$

$$\alpha_o = 1.0010$$

For this period, the sulphur β_{1-2} was evaluated using the relationship

$$(\beta_{1-2})(\% \text{ reacted}) = [(\% \text{ reaction})(\alpha_{1-2})] - [(\% \text{ reaction})(\alpha_{1-1})] \,,$$

where numerically for the interval under examination

$$(\beta_{1-2})_s(38.1 - 35.8) = (38.1)(1.0042) - (35.8)(1.0049)$$

$$2.3 \, (\beta_{1-2})_s = 38.260 - 35.975 = 2.285$$

$$(\beta_{1-2})_s = \frac{2.285}{2.3} = 0.9935 \quad .$$

Similarily for the oxygen isotope fractionation,

$$(\beta_{1-2})_o = \frac{38.137 - 35.847}{2.3} = 0.9957 \quad .$$

For the designated interval, the resultant enrichment factor then became

$$\frac{(\beta_{1-2} - 1)_s}{(\beta_{1-2} - 1)_o} = 1.50$$

The fraction fractionation factors (β) for the bacterial reduction of sulphate experiments are recorded in Table 5-2. The ratio of the sulphur to oxygen isotopic enrichment yielded values much removed from the consistent 4:1 observed with the kinetic fractionation factors (α). The data of MIZUTANI and RAFTER (1969) also was investigated and similar results were noted (Table 5-2). Both studies yielded varying enrichment ratios, but the mean was approximately 4:1. These fraction fractionation factors are expected to give wider fluctuations than the kinetic fractionation factors because of the accumulation of random errors involved in their calculation. Of interest, is that even the wildest fraction fractionation factors are consistent with the 4:1 ratio.(Apparently first order kinetics are not applicable unless just the kinetic fractionation factors are considered. Recently REES (1973) concluded that such bacterial reductions were not first order. This will be discussed more fully in Section 5.2.) The fractionations observed in the present investigation will also be discussed later (Section 5.2), and explained in terms of non-microbiological fractionation arising from enzyme mediated chemical equilibrium.)

The relative isotopic enrichment observed during the bacterial reduction of sulphate was calculated to be

Table 5-2   The fraction fractionation factors (β) for oxygen and sulphur isotopes in sulphate during the bacterial reduction of sulphate

	Present Study			Mizutani and Rafter (1969)		
Specimen Number	β_s	β_o	$\dfrac{\beta_s-1}{\beta_o-1}$	β_s	β_o	$\dfrac{\beta_s-1}{\beta_o-1}$
1 - 1	1.0049	1.0013	3.73			
1 - 2	0.9935	0.9957	1.50			
1 - 3	0.9966	0.9980	1.65			
1 - 4	1.0078	1.0026	2.98	1.0115	1.0015	7.66
1 - 5	1.0052	1.0011	4.68	1.0320	1.0123	2.60
2 - 1	1.0097	1.0023	4.20	1.0108	1.0023	4.70
2 - 2	0.9747	0.9941	4.28	1.0123	1.0050	2.46
2 - 3	1.0210	1.0070	3.01	1.0018	1.0001	-
2 - 4	1.0689	1.0151	4.56	0.9937	1.0034	-1.85
3 - 1	1.0019	1.0004	4.23	1.0140	1.0045	3.11
3 - 2	0.9094	n. d.	n. d.	1.0361	1.0189	1.91
3 - 3	0.9716	n. d.	n. d.	0.9933	0.9875	0.54
3 - 4	0.8343	0.9560	3.76	0.9644	0.9971	12.27
4 - 1	1.0041	1.0010	4.09			
4 - 2	1.0176	1.0048	3.68			
4 - 3	1.0361	1.0091	3.96			
4 - 4	1.0074	1.0239	0.31			

mean = 3.38

mean = 3.71

about 4.0. The isotopic data as presented in Figure 4-5 confirmed this ratio. The relative enrichment observed was not unique. The oxygen and sulphur isotopic composition of sulphate from water in Lake Vanda, Antartica, was reported by RAFTER and MIZUTANI (1967b) to possess such an enrichment of the isotopes. A plot of their δS^{34} values for the sulphate against the δO^{18} values yielded an approximate straight line of slope 4.0. At depth, this lake consisted of strongly density-stratified non-convective saline water. RAFTER and MIZUTANI (1967b) assumed that the enrichment was due to biological fractionation. (Biological activity was known to exist in this lake.)

MIZUTANI and RAFTER (1969), as previously mentioned, conducted three biological reduction of sulphate experiments employing sulphate free wet stream mud as the inoculum. In each case, they found the sulphur-34 enrichment of the sulphate to be about four times the oxygen-18 enrichment. Also, this ratio was observed to be independent of the temperature of the reduction. No explanation was given as to why this relative enrichment ratio was approximately four, nor was any attempt made to determine the microbiological activity in the stream mud inoculum.

The results of the present study confirmed the relative enrichments observed by MIZUTANI and RAFTER (1969).

However, this confirmation came from four most unusual sul-
-phate reduction experiments. The inversions were decidedly
independent of the kinetic or fraction fractionation factors,
and the isotopic compositions of the sulphate in Figure 4-5
showed that the inversions only produced changes of direction
along the "iso-enrichment" slope of 4:1. The relative enrich-
ment of the unreacted sulphate remained constant, whether
enriched or depleted in sulphur-34 or oxygen-18. Thus the
biological reduction of sulphate was assumed to always follow
such an "iso-enrichment" process, with the reduction demanding
sulphur and oxygen isotopic correlation with respect to the
relative enrichment of both isotopic species.

5.5 The 4:1 Relative δS^{34} to δO^{18} Enrichment

The 4:1 relative enrichment ratio of the sulphur and
oxygen isotopic compositions was most intriguing. A simple
explanation involves the initial S - O bond rupture.

The enrichment in sulphur - 34 of the unreacted
sulphate was observed to be four times that of the oxygen -
18 . For the initial S - O bond rupture, there is a choice
of four oxygen atoms in a given sulphate ion; however only
one sulphur atom is involved. Thus simple statistics sug-
gests a per atom enrichment in sulphur to be four times
as great as that for the oxygen. This simple argument does
not consider bond energies which are identified on a theo-

retical basis with kinetic isotope effects.

This relative enrichment ratio should also be able to be predicted theoretically, since reaction rate constants are different for competitive reactions of isotopic molecules. This can be done from a statistical point of view, using the theory of "absolute rates". The ratio of the rate constants of two isotopic molecules can be expressed as a simple function of the vibrational energy levels of the two molecules. BIGELEISEN and MAYER (1947) have approximated this ratio of the rate constants (κ) to be;

$$\frac{\kappa_1}{\kappa_2} = \frac{s_1}{s_2} \cdot \frac{s_2\dagger}{s_1\dagger} \cdot \frac{k_1}{k_2} \cdot \left(\frac{m_2{}^*}{m_1{}^*} \right)^{1/2} \cdot \left(1 + \right.$$

$$\left. \sum_{i}^{3n-6} G(u_i) \, \Delta u_i - \sum_{i}^{3n'-6} G(u_i{}^\dagger) \, \Delta u_i{}^\dagger \right) ,$$

where , s = the symmetry number of the reactants,

s = the symmetry number of the activated complex,

k = the transmission coefficient,

m^* = the effective mass,

$u = h\nu/KT$,

ν = the vibrational frequency of the molecule, and

$G(u) = 1/2 - 1/u + 1/(e^u - 1)$.

The difficulty in utilizing the Bigeleisen - Mayer relation rests in the lack of information concerning the activated complex. One also has the problem of deciding a value for the effective mass. In practice, various approximations are made (REES and THODE (1965)) to see which theoretical model best fits the experimental data.

The simplest model to consider is that where the reaction is approximated by a simple S - O rupture. Two cases can be considered. The activated complex is the completely dissociated molecule, in which case

$$\sum_{i}^{3n'-6} G(u_i^\dagger) \, \Delta u_i^\dagger = 0 \quad (\text{ Case I })$$

The second possibility is that the activated complex is similar to the reactant S - O bond, in which case the kinetic isotope effect is simply given by the effective mass term (Case II). In both situations, different effective mass terms may be tried, such as S - O (atoms) and SO_3 - O (fragments).

Since G(u) has been tabulated by BIGELEISEN and MAYER (1947) as a function of u in a convenient form for rapid calculation, the ratios of the rate constants for isotopic substitution in these two cases can be evaluated. The calculations are summarized for each case as follows.

RATIO	CASE I		CASE II	
	Atoms	Fragments	Atoms	Fragments
$\dfrac{K_{32}}{K_{34}}$	1.02695	1.01891	1.00994	1.00204
$\dfrac{K_{16}}{K_{18}}$	1.10645	1.11772	1.03931	1.04990
$\dfrac{\dfrac{K_{32}}{K_{34}} - 1}{\dfrac{K_{16}}{K_{18}} - 1}$	0.253	0.161	0.253	0.041

(All data (vibrational frequency, etc.) from HERTZBERG.)

Thus it can be seen that the 4:1 ratio of $\delta S^{34}:\delta O^{18}$ is not predicted by a simple S - O rupture model. Indeed, the relative enrichments, as shown in the last line of the above data, of sulphur - 34 and oxygen - 18 are reversed.

This whole problem is further complicated by the recent findings of MIZUTANI and RAFTER (1973) , where the δO^{18} value of the sulphate remaining in the bacterial reduction was observed to depend upon the δO^{18} value of the water in which the sulphate was reduced by the bacteria. They inter-preted this as an oxygen isotope exchange between the sul-

phate oxygen and the water through intermediates in the bacterial reduction of sulphate.

The laboratory experiments of MIZUTANI and RAFTER (1973) involved both mixed and purified cultures. Extrapolation of their findings yields δH_2O^{18} values which would give 4:1 relative enrichments, however, the corresponding δH_2O^{18} values differ for each experiment and range widely (+12 ^{o}/oo to -10 ^{o}/oo). The implications of their work seem to suggest that the 4:1 ratio does not have particular significance. However, at this time the 4:1 observation should not be so readily dismissed. The work of MIZUTANI and RAFTER (1973) cannot adequately explain why so many workers have found the 4:1 ratio in laboratory experiments (LLOYD (1968).; MIZUTANI and RAFTER (1969), the present study) and in terrestrial situations (MIZUTANI and RAFTER (1967), MIZUTANI and RAFTER (1969)).

No doubt, the fact that their reductions were carried out for up to 89 days was a factor in promoting oxygen isotope exchange, and can account to some extent for the difference between their work and that of others. However, this argument is not the limitation since the 4:1 ratio has been observed terrestrially.

The study of MIZUTANI and RAFTER (1973) did not follow first order kinetics. Their data indicated that back oxidative reactions do occur, and are an integral part

of biological sulphate reduction. This was also reported by TRUDINGER and CHAMBERS (1973) using S^{35} label. Thus the pathways available for $SO_4 \rightarrow SO_3 \rightarrow H_2S$ reduction are many, and the role played by the intermediates and back oxidative reactions has yet to be fully understood. The relative enrichment of $\delta S^{34} : \delta O^{18}$ has to depend upon the physical and chemical effects involved in both the forward and backward reactions.

It is hard to believe that the 4:1 relative enrichment ratio does not have special significance when in this thesis, it was encountered persistently during both normal and inverse kinetic isotope effects.

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              / COMMONLY USED VARIABLES AND SUBROUTINES
              *30
0030   0000   INTTME,   0000
0031   0000   GMTIME,   0000
0032   0000   XORSOR,   0000
0033   0520   WRITE,    NWRITE
0034   0200   CHAR01,   LIST01
0035   0213   CHAR02,   LIST02
0036   0226   CHAR03,   LIST03
0037   0243   CHAR04,   LIST04
0040   0271   CHAR05,   LIST05
0041   0305   CHAR06,   LIST06
0042   0321   CHAR07,   LIST07
0043   0357   CHAR10,   LIST10
0044   7775   M3,       -3
0045   0003   K3,       3
0046   0722   CRLF,     NCRLF
0047   0730   TYPE,     NTYPE
0050   0550   LISN,     NLISN
0051   0534   BELL,     NBELL
0052   0560   EXPON,    NEXPON
0053   0740   TAB,      NTAB
0054   0000   GMCT,     0000
0055   0400   TRIAPT,   TRIPAD
0056   1200   DOUBPT,   DOUBLE
0057   0441   TRIDIV,   TRIPDV
0060   0061   OLXIPT,   OLXI
0061   0000   OLXI,     0000
0062   0000   OLHI,     0000
0063   0000   OLLO,     0000
0064   5200   STORE,    5200
0065   0600   DECPRT,   SDPRNT
0066   0067   GMPT,     GMHI
0067   0000   GMHI,     0000
0070   0000   GMMED,    0000
0071   0000   GMLOW,    0000
0072   0000   TEMPTM,   0000
0073   0000   TEMM,     0000
0074   0000   TEMN,     0000
0075   0000   TEMO,     0000
0076   0000   TEMP,     0000
0077   0000   TEMQ,     0000
0100   0000   TEMR,     0000
0101   0000   TEMS,     0000
0102   0000   TEMT,     0000
0103   4200   LOCTON,   4200
0104   4600   LOCTEN,   4600
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0105   0000   NUMT,      0000
0106   0000   CNTR,      0000
0107   0000   QUOT1,     0000
0110   0000   QUOT2,     0000
0111   0000   DVSOR1,    0000
0112   0000   DVSOR2,    0000
0113   0000   DV1,       0000
0114   0000   DV2,       0000
0115   0000   DV3,       0000
0116   0000   DV4,       0000
0117   0113   DVDPT,     DV1
0120   0111   DVSPT,     DVSOR1
0121   2000   DMULPT,    DMUL
0122   0200   DBDVPT,    DUBDIV
0123   0124   TENSPT,    TENS
       0030   TENS,      30
0125   3240              3240
0126   0000   ADD,       0000
0127   0107   QUOTPT,    QUOT1
0130   1000   RDVMPT,    READVM
0131   1611   RATAGN,    AGNRAT
0132   2141   DV2PT,     2141
0133   1130   SETIS,     TSETIS
0134   1600   RATIOS,    RATIO
0135   0756   DEPOST,    DEPXOS
0136   4040   MEANNF,    NMEANS
0137   1400   DVDRTO,    DVDRAT
0140   1676   STDDEV,    STDDEV
0141   0752   DPCHAG,    DEPCHG
0142   1707   STDTW,     STDTWO
0143   2400   INCASE,    TWCASE
0144   1461   SQRT,      SQRTNO
0145   1441   HAPPY,     HAPPYS
0146   2511   AGAIN,     AGAIN3
0147   2537   STXORS,    STXOS
0150   2624   ZERDEC,    DECZER
0151   2633   ZTYPE,     ZRTYPE
0152   3016   GOON,      ONGO
0153   2600   CNTRO,     CNTROS
0154   2711   LINE,      LINES
0155   0000   TABCNT,    0000
0156   2671   DVONE,     ONEDV
0157   3051   NEGT,      NEGIT
0160   2142   R1CNT,     2142
0161   2143   R2CNT,     2143
0162   2731   XOSMB,     MBXOS
0163   0266   SAYLCN,    266
0164   0000   NEWXOS,    0000
0165   3000   DEVSET,    SETDEV
0166   3014   BLAH,      3014
0167   0000   EXTRA,     0000
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0170   2747   KORECT,    CORECT
0171   1131   BACKST,    1131
____   1334   STAR70,    STARS
       1673   CNTXOS,    XOSCT
       3200   DELETE,    DELEIT
0175   3400   MUMCRT,    CRTMUM
0176   3500   TAB50,     TABA50
0177   3550   NUMTCR,    CRNUMT
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                      *200
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0201    0311                 311            / INTTME=2**
0202    0316                 316
0203    0324                 324
0204    0324                 324
0205    0315                 315
0206    0305                 305
0207    0275                 275
0210    0262                 262
0211    0252                 252
0212    0252                 252
0213    0213    LIST02,      .            / -12 (-10 DEC)
0214    0307                 307            / GMTIME=2**
0215    0315                 315
0216    0324                 324
0217    0311                 311
0220    0315                 315
0221    0305                 305
0222    0275                 275
0223    0262                 262
0224    0252                 252
0225    0252                 252
0226    0226    LIST03,      .            -14 (-12 DEC)
0227    0324                 324          / THIS SET IS
0230    0310                 310
0231    0311                 311
0232    0323                 323
0233    0240                 240
0234    0323                 323
0235    0305                 305
0236    0324                 324
0237    0240                 240
0240    0311                 311
0241    0323                 323
0242    0240                 240
0243    0243    LIST04,      .            25 (-21 DEC)
0244    0306                 306          / FIRST SET MUST BE S !
0245    0311                 311
0246    0322                 322
0247    0323                 323
0250    0324                 324
0251    0240                 240
0252    0323                 323
0253    0305                 305
0254    0324                 324
0255    0240                 240
0256    0315                 315
0257    0325                 325
0260    0323                 323
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0261   0324              324
0262   0240              240
0263   0302              302
0264   0305              305
0265   0240              240
0266   0323              323
0267   0240              240
0270   0241              241
0271   0271   LIST05,    .
0272   0315              315
0273   0305              305
0274   0301              301
0275   0316              316
0276   0240              240
0277   0322              322
0300   0301              301
0301   0324              324
0302   0311              311
0303   0317              317
0304   0240              240
0305   0305   LIST06,    .
0306   0323              323
0307   0324              324
0310   0304              304
0311   0240              240
0312   0304              304
0313   0305              305
0314   0326              326
0315   0240              240
0316   0311              311
0317   0323              323
0320   0240              240
0321   0321   LIST07,    .
0322   0324              324
0323   0331              311
0324   0320              320
0325   0305              305
0326   0240              240
0327   0331              311
0330   0240              240
0331   0311              311
0332   0306              306
0333   0240              240
0334   0304              304
0335   0305              305
0336   0326              326
0337   0240              240
0340   0323              323
0341   0301              301
0342   0324              324
```

/ -16 (-14 DEC)
/ MEAN RATIO IS

/ -13 (-11 DEC)
/ STD DEV IS

/ -33 (-27 DEC)
/ TYPE Y IF DEV SATSFACTORY

0343	0311	311
0344	0323	323
0345	0306	306
0346	0301	301
0347	0303	303
0350	0324	324
0351	0317	317
0352	0322	322
0353	0331	331
0354	0277	277
0355	0240	240
0356	0240	240
0357	0357	LIST10, •
0360	0255	255
0361	0240	240
0362	0317	317
0363	0316	316
0364	0314	314
0365	0331	311
0366	0240	240
0367	0263	263
0370	0240	240
0371	0323	323
0372	0305	305
0373	0324	324
0374	0323	323

/ -15 (-13 DEC)
/ - ONLY 3 SETS

```
                    / TRIPLE PRECISION ADD OF DOUBLE PRECISION
                    / NUMBERS . CALLING SEQUENCE:
                    /      1) JMS TRIPAD WITH FIRST ADDRESS IN ACC
                    /      2) RETURN SUPERHI ADDRESS IN ACC
                    / REQUIRES ' INTEGRATION TIME ' ON PAGE 0 .
                    *400
   0400  0000   TRIPAD,    0000
   0401  3237              DCA HIPTL       / FIRST HIGH POINTER
   0402  7300              CLL CLA
   0403  3063              DCA OLLO        / TIDY UP REGISTERS
   0404  3062              DCA OLHI
   0405  3061              DCA OLXI
   0406  1030              TAD INTTME      / HOW MANY ?
   0407  7041              CIA
   0410  3236              DCA LOCTME
   0411  1237              TAD HIPTL
   0412  7001              IAC
   0413  3240              DCA LOPTL
   0414  1640   ADDE,      TAD I LOPTL     / ADD LOW ORDER
   0415  1063              TAD OLLO
   0416  3063              DCA OLLO
   0417  7004              RAL
   0420  1637              TAD I HIPTL     / ADD HIGH ORDER
   0421  1062              TAD OLHI
   0422  3062              DCA OLHI
   0423  7004              RAL             / CARRY
   0424  1061              TAD OLXI
   0425  3061              DCA OLXI
   0426  2237              ISZ HIPTL
   0427  2237              ISZ HIPTL       / SHIFT ADDRESS BY 2 .
   0430  2240              ISZ LOPTL
   0431  2240              ISZ LOPTL
   0432  2236              ISZ LOCTME
   0433  5214              JMP ADDE        / FINISHED ?
   0434  1060              TAD OLXIP       / NO
   0435  5600              JMP I TRIPAD    / YES
   0436  0000   LOCTME,    0000
   0437  0000   HIPTL,     0000
   0440  0000   LOPTL,     0000

                    / DIVIDE TRIPLE PRECISION NUMBER BY A POWER
                    / OF TWO . CALLING SEQUENCE:
                    /      1) JMS TRIPDV WITH HIGH ADDRESS IN ACC
                    /      2) RETURN ACC CLEAR AND RESULT IN THE
                    /      MEDIUM AND LOW ORDER PART OF T. P. .
                    / DIVISOR IN ' INTTME ' ON PAGE 0 .
   0441  0000   TRIPDV,    0000
   0442  3313              DCA QUOTHI      / POINTER
```

```
0443    1313              TAD QUOTHI
0444    7001              IAC
0445    3314              DCA QUOTMD
0446    1314              TAD QUOTMD
0447    7001              IAC
0450    3315              DCA QUOTLO
0451    3316              DCA POWER
0452    7100              CLL
0453    1030              TAD INTTMD        / MUST BE POWER OF 2 !!
0454    7010              RAR
0455    7010              RAR
0456    2316              ISZ POWER         / TWOS EXPONENT
0457    7420              SNL
0460    5255              JMP .-3
0461    7200              CLA
0462    1316              TAD POWER         / IF NOT 2**N
0463    7041              CIA
0464    3316              DCA POWER
0465    7100    TWOSDV,   CLL
0466    1713              TAD I QUOTHI      / FETCH PARTS NOW
0467    7010              RAR
0470    3713              DCA I QUOTHI
0471    1714              TAD I QUOTMD
0472    7010              RAR
0473    3714              DCA I QUOTMD
0474    1715              TAD I QUOTLO
0475    7010              RAR
0476    3715              DCA I QUOTLO
0477    7004              RAL
0500    3317              DCA ROUND         / SAVE ROUNDOFF BIT
0501    2316              ISZ POWER         / FINISHED ?
0502    5265              JMP TWOSDV        / NO
0503    7300              CLA CLL           / YES
0504    1715              TAD I QUOTLO
0505    1317              TAD ROUND
0506    3715              DCA I QUOTLO
0507    7004              RAL
0510    1714              TAD I QUOTMD
0511    3714              DCA I QUOTMD      / CARRY
0512    5641              JMP I TRIPDV
0513    0000    QUOTHI,   0000
0514    0000    QUOTMD,   0000
0515    0000    QUOTLO,   0000
0516    0000    POWER,    0000
0517    0000    ROUND,    0000

0520    0000    NWRITE,   0000              / WRITE OUT MESSAGES .
0521    7100              CLL                / IN AFTER JMS FROM A
0522    3010              DCA 10             / TAD CHARXX AS IN *200
0523    1720              TAD I NWRITE       / AFTER JMS IS OCTAL OF
0524    3317              DCA ROUND          / CHARACTERS AS *200 .
```

```
0525    2320            ISZ NWRITE
0526    6046            TLS
0527    1410            TAD I 10
0530    4447            JMS I TYPE
0531    2317            ISZ ROUND
0532    5327            JMP .-3
0533    5720            JMP I NWRITE

0534    0000    NBELL,  0000                / RING BELL FOUR TIMES
0535    7300            CLA CLL
0536    4446            JMS I CRL
0537    1346            TAD M4
0540    3317            DCA ROUND
0541    1347            TAD K207
0542    4447            JMS I TYPE
0543    2317            ISZ ROUND
0544    5341            JMP .-3
0545    5734            JMP I NBELL
0546    7774    M4,     -4
0547    0207    K207,   207

0550    0000    NLISN,  0000                / READ X OR S FROM THE
0551    6031            KSF                 / KEYBOARD
0552    5351            JMP .-1
0553    6036            KRB
0554    6046            TLS
0555    7100            CLL
0556    3032            DCA XORSOR
0557    5750            JMP I NLISN

0560    0000    NEXPON,  0000               / RAISE ACC TO A
0561    3317            DCA ROUND           / POWER OF TWO
0562    1717            TAD I ROUND
0563    7041            CIA
0564    3316            DCA POWER
0565    7001            IAC
0566    7004            RAL
0567    2316            ISZ POWER
0570    5366            JMP .-2
0571    3717            DCA I ROUND
0572    5760            JMP I NEXPON
```

```
/ SIGNED DECIMAL PRINT , DOUBLE PRECISION
/ CALLING SEQUENCE:
/        1)   JMS SDPRNT    / CALLED
/        2)   HIADDR        / ADDRESS OF HIGH ORDER
       *600
0600  0000   SDPRNT,    0000
0601  4550              JMS I ZERDEC    / ZERO CHECK
0602  1600              TAD I SDPRNT    / GET ADDRESS
0603  3302              DCA SDGET
0604  1702              TAD I SDGET     / HIGH ORDER WORD
0605  7700              SMA CLA         / NEGATIVE ?
0606  1271              TAD SDPLUS      / NO
0607  1272              TAD SDMNS       / YES
0610  1276              TAD SDTWO
0611  4330              JMS NTYPE
0612  1702              TAD I SDGET
0613  7510              SPA             / POSITIVE ?
0614  7061              CMA CML
0615  3274              DCA SDHIGH
0616  2302              ISZ SDGET
0617  1702              TAD I SDGET     / LOW ORDER WORD
0620  7430              SZL             / LINK SET ?
0621  7141              CMA CLL IAC
0622  7430              SZL             / OVERFLOW ?
0623  2274              ISZ SDHIGH
0624  3275              DCA SDLOW
0625  1266              TAD SDLOOP      / SET DIGIT COUNTER
0626  3273              DCA SDCNT
0627  1267              TAD SDADDR      / SET POINTER
0630  3303              DCA SDPTR
0631  2200              ISZ SDPRNT      / SET LINKAGE
0632  1703   SDARND,    TAD I SDPTR     / POWER OF TEN
0633  2303              ISZ SDPTR
0634  3276              DCA SDHSUB
0635  1703              TAD I SDPTR
0636  2303              ISZ SDPTR
0637  3277              DCA SDLSUB
0640  7100   SDDO,      CLL             / DOUBLE PRECISION
0641  1277              TAD SDLSUB      / SUBTRACTION
0642  1275              TAD SDLOW
0643  3301              DCA SDTEML
0644  7004              RAL
0645  1276              TAD SDHSUB
0646  1274              TAD SDHIGH
0647  7510              SPA             / UNDERFLOW ?
0650  5256              JMP SDOUT       / NO
0651  2300              ISZ SDBOX       / YES
0652  3274              DCA SDHIGH
```

```
0653   1301                   TAD  SDTEML
0654   3275                   DCA  SDLOW
0655   5240            SDOUT, JMP  SDDO
0656   7200                   CLA              / PICK UP DIGIT
0657   1300                   TAD  SDBOX
0660   1270                   TAD  SDTWO
0661   4551                   JMS  I ZTYPE
0662   3300                   DCA  SDBOX
0663   2273                   ISZ  SDCNT        / TYPED 7 DIGITS ?
0664   5232                   JMP  SDARND       / NO
0665   5600                   JMP  I SDPRNT     / YES
0666   7771            SDLOOP, -7               / COUNT 7
0667   0704            SDADDR, SDCONL           / BASE FOR DIGITS
0670   0260            SDTWO,  260
0671   7763            SDPLUS, -15
0672   7775            SDMNS,  -3
0673   0000            SDCNT,  0                / STORAGE LOCATIONS
0674   0000            SDHIGH, 0
0675   0000            SDLOW,  0
0676   0000            SDHSUB, 0
0677   0000            SDLSUB, 0
0700   0000            SDBOX,  0
0701   0000            SDTEML, 0
0702   0000            SDGET,  0
0703   0000            SDPTR,  0
0704   7413            SDCONL, 7413             / POWERS OF TEM
0705   6700                    6700             / -1,000,000
0706   7747                    7747             / -100,000
0707   4540                    4540
0710   7775                    7775             / -10,000
0711   4360                    4360
0712   7777                    7777             / -1,000
0713   6030                    6030
0714   7777                    7777             / -100
0715   7634                    7634
0716   7777                    7777             / -10
0717   7766                    7766
0720   8777                    7777             / -1
0721   7777                    7777


0722   0000            NCRLF,  0000             / CARRIAGE RETURN
0723   1336                    TAD  K215        / AND LINE FEED
0724   4330                    JMS  NTYPE
0725   1337                    TAD  K212
0726   4330                    JMS  NTYPE
0727   5722                    JMP  I NCRLF

0730   0000            NTYPE,  0000             / TYPE CHARACTER
0731   6041                    TSF              / FROM KEYBOARD
```

```
0732    5331              JMP  .-1
0733    6046              TLS
0734    7300              CLA  CLL
0735    5730              JMP  I  NTYPE
0736    0215     K215,    215
0737    0212     K212,    212


0740    0000     NTAB,    0000          / TAB 10 SPACES
0741    1044              TAD  M3
0742    3350              DCA  TABCT
0743    1351              TAD  K240
0744    4330              JMS  NTYPE
0745    2350              ISZ  TABCT
0746    5343              JMP  .-3
0747    5740              JMP  I  NTAB
0750    0000     TABCT,   0000
0751    0240     K240,    240
0752    0000     DEPCHG,  0000
0753    1103              TAD  LOCTON
0754    3374              DCA  XORSLC
0755    5752              JMP  I  DEPCHG


0756    0000     DEPXOS,  0000          / DEPOSIT X OR S
0757    7300              CLA  CLL        / STARTING 3600
0760    4340              JMS  NTAB
0761    1032              TAD  XORSOR
0762    4330              JMS  NTYPE
0763    4322              JMS  NCRLF
0764    4322              JMS  NCRLF
0765    1070              TAD  GMMED
0766    3774              DCA  I  XORSLC
0767    2374              ISZ  XORSLC
0770    1071              TAD  GMLOW
0771    3774              DCA  I  XORSLC
0772    2374              ISZ  XORSLC
0773    5756              JMP  I  DEPXOS
0774    0000     XORSLC,  0000
```

```
                        / READ VOLTMETER VALUES AND FIND THE MEAN
                        *1000
    1000   0000    READVM,     0000                / CLEAR LOCATIONS
    1001   7300            CLA CLL
    1002   1327            TAD MMM6
    1003   3155            DCA TABCNT
    1004   7300            CLA CLL
    1005   3067            DCA GMHI
    1006   3070            DCA GMMED
    1007   3071            DCA GMLOW
    1010   1031            TAD GMTIME
    1011   7041            CIA
    1012   3054            DCA GMCT
    1013   7300    MORE,       CLA CLL             / MORE TO DO !
    1014   1064            TAD STORE
    1015   3244            DCA POINTR
    1016   1030            TAD INTTME
    1017   7041            CIA
    1020   3326            DCA MINTT               / NEGATE
    1021   6322            6322                    / SET FLAG FOR A
    1022   6301    READVT,     6301                / VOLTMETER VALUE
    1023   5222            JMP .-1
    1024   6302            6302                    / GET HIGH PART
    1025   3644            DCA I POINTR
    1026   2244            ISZ POINTR
    1027   6304            6304                    / GET LOW PART
    1030   3644            DCA I POINTR
    1031   2244            ISZ POINTR
    1032   6322            6322                    / SET FLAG
    1033   2326            ISZ MINTT               / FINISHED ?
    1034   5222            JMP READVT              / NO
    1035   7300            CLA CLL                 / YES
    1036   1030            TAD INTTME
    1037   7041            CIA                     / BCD TO BINARY
    1040   3326            DCA MINTT               / RESET COUNTER
    1041   1064            TAD STORE
    1042   3244            DCA POINTR
    1043   4456    CONVRT,     JMS I DOUBPT
    1044   0000    POINTR,     0000                / HIGH BCD NUMBER
    1045   3324            DCA TEMPAD
    1046   1724            TAD I TEMPAD
    1047   3644            DCA I POINTR            / IN OLD BCD
    1050   2244            ISZ POINTR
    1051   2324            ISZ TEMPAD
    1052   1724            TAD I TEMPAD
    1053   3644            DCA I POINTR
    1054   2244            ISZ POINTR
    1055   2326            ISZ MINTT               / FINISHED ?
```

```
1056    5243                        JMP  CONVRT          / NO
1057    7300                        CLA  CLL             / YES
1060    1064                        TAD  STORE           / ADDRESS IN ACC
1061    4455                        JMS I TRIAPT         / ADDRESS OF OLXI IN
1062    4457                        JMS I TRIDIV         / ACC ON RETURN
1063    1060                        TAD  OLXIPT
1064    7001                        IAC
1065    4554                        JMS I LINE
1066    7300                        CLA  CLL
1067    1071                        TAD  GMLOW
1070    1063                        TAD  OLLO
1071    3071                        DCA  GMLOW
1072    7004                        RAL
1073    1062                        TAD  OLHI
1074    1070                        TAD  GMMED
1075    3070                        DCA  GMMED
1076    7004                        RAL
1077    1067                        TAD  GMHI
1100    3067                        DCA  GMHI
1101    2054                        ISZ  GMCT            / COUNTER FOR MEAN
1102    5213                        JMP  MORE

                    / GET MORE AVERAGES FOR GRAND MEAN

1103    1030                        TAD  INTTME          / SET UP FOR MEAN
1104    3325                        DCA  TEMP
1105    1031                        TAD  GMTIME
1106    3030                        DCA  INTTME
1107    1066                        TAD  GMPT
1110    4457                        JMS I TRIDIV
1111    1325                        TAD  TEMP            / RESTORE INTTME
1112    3030                        DCA  INTTME
1113    1066                        TAD  GMPT
1114    7001                        IAC
1115    3322                        DCA  GMPRPT
1116    4446                        JMS I CRLF
1117    4446                        JMS I CRLF
1120    4453                        JMS I TAB
1121    4465                        JMS I DECPRT
1122    0000    GMPRPT,     0000
1123    5600                        JMP I READVM
1124    0000    TEMPAD,     0000
1125    0000    TEMP,       0000
1126    0000    MINTT,      0000
1127    7772    MMM6,       -6


1130    0000    TSETIS,     0000                    / WHAT IS THIS
1131    7300    STBACK,     CLA CLL                 / SET - X OR S ?
1132    1036                        TAD  CHAR03
```

```
1133    4433                JMS I WRITE
1134    7764                -14
1135    6032                KCC
1136    4450                JMS I LISN
1137    1032                TAD XORSOR
1140    3373                DCA SAMPLE
1141    7300                CLA CLL
1142    1373                TAD SAMPLE
1143    1372                TAD M330
1144    7440                SZA
1145    7410                SKP
1146    5365                JMP ONWARD          / X
1147    7300                CLA CLL
1150    1373                TAD SAMPLE
1151    1371                TAD M323
1152    7440                SZA
1153    7410                SKP
1154    5365                JMP ONWARD          / S
1155    7300                CLA CLL
1156    1373                TAD SAMPLE
1157    1370                TAD M310
1160    7440                SZA
1161    7410                SKP
1162    5774                JMP I PARMPT        / H ( HALT )
1163    4451                JMS I BELL          / ERROR MADE !!
1164    5331                JMP STBACK
1165    4570    ONWARD,     JMS I KORECT        / CORRECT SYMBOL
1166    4200                JMS READVM
1167    5730                JMP I TSETIS
1170    7470    M310,       -310
1171    7455    M323,       -323
1172    7450    M330,       -330
1173    0000    SAMPLE,     0000
1174    4000    PARMPT,     PARM
```

```
/ DOUBLE PRECISION BCD TO BINARY CONVERSION
/ CALLING SEQUENCE:
/      1) JMS DOUBLE
/      2) ADDRESS OF HIGH
/ RETURN WITH ADDRESS OF HIGH IN ACC

/ ALSO CONTAINS SINGLE PRECISION BCD TO BINARY
/ CALLING SEQUENCE:
/      1) C(AC) = 3 BCD CHARACTERS
/      2) JMS BCDBIN
/ RETURN WITH ANSWER IN C(AC)

                    /*1200
1200    0000    DOUBLE,    0000
1201    7300               CLA CLL
1202    1600               TAD I DOUBLE        / FETCH HIGH ADDRESS
1203    3273               DCA LOW1
1204    2200               ISZ DOUBLE
1205    1673               TAD I LOW1          / FETCH HIGH ORDER
1206    4300               JMS BCDBIN          / CONVERT IT
1207    3274               DCA HIGH1           / AND STORE
1210    2273               ISZ LOW1
1211    1673               TAD I LOW1          / FETCH LOW ORDER
1212    4300               JMS BCDBIN          / CONVERT IT
1213    3273               DCA LOW1            / AND STORE
1214    1274               TAD HIGH1
1215    7112               CLL RTR
1216    7012               RTR
1217    7010               RAR                 / MULTIPLY HIGH ORDER
1220    3300               DCA BCDBIN          / PART BY 128
1221    1300               TAD BCDBIN
1222    0332               AND K177
1223    3275               DCA HIGH
1224    1300               TAD BCDBIN
1225    7010               RAR
1226    0330               AND K7600
1227    3276               DCA LOW
1230    1274               TAD HIGH1           / MULTIPLY HIGH ORDER
1231    7104               CLL RAL             / BY THREE
1232    1274               TAD HIGH1           / FORM 128*HIGH-3*HIGH
1233    7141               CIA CLL
1234    1276               TAD LOW
1235    3276               DCA LOW
1236    7420               SNL
1237    7040               CMA
1240    1275               TAD HIGH
1241    3275               DCA HIGH            /  125*HIGH
1242    1275               TAD HIGH            / NOW MULTIPLY BY 8
```

```
1243    7106              CLL RTL
1244    7004              RAL
1245    0331              AND K7770          / MASK 9 BITS
1246    3275              DCA HIGH
1247    1276              TAD LOW
1250    7106              CLL RTL
1251    7004              RAL
1252    3276              DCA LOW
1253    1276              TAD LOW
1254    7004              RAL
1255    0327              AND K7              / 3 BITS
1256    1275              TAD HIGH
1257    3275              DCA HIGH
1260    1276              TAD LOW
1261    0331              AND K770            / 9 BITS
1262    7100              CLL
1263    1273              TAD LOW1            / ADD LOW PART
1264    3276              DCA LOW             / AND STORE
1265    1275              TAD HIGH
1266    7430              SZL
1267    7001              IAC
1270    3275              DCA HIGH
1271    1277              TAD HIPTR           / RETURN WITH HIGH
1272    5600              JMP I DOUBLE        / ADRESS IN ACC
1273    0000    LOW1,     0
1274    0000    HIGH1,    0
1275    0000    HIGH,     0
1276    0000    LOW,      0
1277    1275    HIPTR,    HIGH
```

/ SINGLE PRECISION CONVERSION

```
1300    0000    BCDBIN,   0000
1301    3275              DCA HIGH
1302    1275              TAD HIGH
1303    0333              AND K7400          / LEFT DIGIT
1304    7112              CLL RTR
1305    3276              DCA LOW
1306    1276              TAD LOW
1307    7010              RAR
1310    1276              TAD LOW
1311    7041              CIA
1312    1275              TAD HIGH
1313    3275              DCA HIGH
1314    1275              TAD HIGH
1315    0326              AND K7760
1316    7112              CLL RTR
1317    3276              DCA LOW
1320    1276              TAD LOW
1321    7010              RAR
```

```
1322   1276,              TAD LOW
1323   7041              CIA
1324   1275              TAD HIGH
1325   5700              JMP I BCDBIN

1326   7760   K7760,     7760
1327   0007   K7,        7
1330   7600   K7600,     7600
1331   7770   K7770,     7770
1332   0177   K177,      177
1333   7400   K7400,     7400


1334   0000   STARS,     0000            / LINE OF STARS AND
1335   4446              JMS I CRLF       / CRLF'S TO END THE
1336   1347              TAD M70          / PROGRAM
1337   3350              DCA STARLN
1340   1351              TAD KK252
1341   4447              JMS I TYPE
1342   2350              ISZ STARLN
1343   5340              JMP .-3
1344   4446              JMS I CRLF
1345   5353              JMP .+6
1346   5752              JMP I PARMPT
1347   7672   M70,       -70
1350   0000   STARLN,    0000
1351   0252   KK252,     252
1352   3532   PARMPT,    PARM

1353   4446              JMS I CRLF
1354   4446              JMS I CRLF
1355   4446              JMS I CRLF
1356   4446              JMS I CRLF
1357   4446              JMS I CRLF
1360   4446              JMS I CRLF
1361   4446              JMS I CRLF
1362   4446              JMS I CRLF
1363   4446              JMS I CRLF
1364   4446              JMS I CRLF
1365   4446              JMS I CRLF
1366   3077              DCA TEMTQ
1367   1376              TAD MN260
1370   3777              DCA I LCN1
1371   1375              TAD MIN3
1372   3774              DCA I LCN1
1373   5346              JMP PAROUT
1374   3372   LCN1,      3372
1375   7775   MIN3,      -3
1376   7520   MN260,     -260
1377   3346   LCN1,      3346
```

```
                    / DOUBLE PRECISION DIVIDE
                    *1400
1400    0000    DVDRAT,     0000                    / EXPECT STORED
1401    1117                TAD DVDPT               / DV1, DV2, DVSOR1,
1402    3206                DCA MULT+1              / AND DVSOR2
1403    1123                TAD TENSPT
1404    3207                DCA MULT+2
1405    4521    MULT,       JMS I DMULPT
1406    0000                0000
1407    0000                0000
1410    3113                DCA DV1
1411    1132                TAD DV2PT
1412    3126                DCA ADD
1413    1526                TAD I ADD
1414    3114                DCA DV2
1415    2126                ISZ ADD
1416    1526                TAD I ADD
1417    3115                DCA DV3
1420    2126                ISZ ADD
1421    1526                TAD I ADD
1422    3116                DCA DV4
1423    1120                TAD DVSPT
1424    3227                DCA ADDRS1
1425    1117                TAD DVDPT
1426    4522                JMS I DBDVPT
1427    0000    ADDRS1,     0000
1430    3126                DCA ADD
1431    1526                TAD I ADD
1432    3107                DCA QUQT1
1433    2126                ISZ ADD
1434    1526                TAD I ADD
1435    3110                DCA QUOT2
1436    1127                TAD QUOTPT
1437    4554                JMS I LINE
1440    5600                JMP I DVDRAT


1441    0000    HAPPYS,     0000                    / 'Y' TYPED IF THE
1442    1042                TAD CHAR07              / RESULTS ARE SAT-
1443    4433                JMS I WRITE             / ISFACTORY .THREE
1444    7745                -33                     / MORE SETS OTHER-
1445    4453                JMS I TAB               / WISE .
1446    4453                JMS I TAB
1447    6032                KCC                     / 'S' STARTS !!
1450    4450                JMS I LISN
1451    7000                NOP
1452    4575                JMS I MUMCRT
1453    4574                JMS I DELETE
```

```
1454  7000              NOP
1455  4446              JMS I CRLF
1456  4446              JMS I CRLF
1457  4446              JMS I CRLF
1460  4536              JMS I MEANNF

1461  0000   SQRTNO,    0000              / DOUBLE PRECISION
1462  7300              CLA CLL           / SQUARE ROOT OF A
1463  3350              DCA ROOT          / DOUBLE PRECISION
1464  1352              TAD M1            / NUMBER , EXPECTED
1465  3351              DCA SQXT          / IN QUOT1 AND QUOT2
1466  1352              TAD M1            / WITH RESULT IN
1467  3354              DCA MM            / QUOT2 .
1470  1110   SQX,       TAD QUOT2         / SINGLE PRECISION .
1471  1361              TAD SQXT
1472  3110              DCA QUOT2
1473  7004              RAL
1474  1107              TAD QUOT1
1475  1354              TAD MM
1476  3107              DCA QUOT1
1477  7420              SNL
1500  5331              JMP SQRF
1501  7100              CLL
1502  2350              ISZ ROOT
1503  1351              TAD SQXT
1504  1356              TAD K4000
1505  7450              SNA
1506  5315              JMP COMPLX
1507  7300              CLA CLL
1510  1353              TAD M2
1511  1351              TAD SQXT
1512  3351              DCA SQXT
1513  7100              CLL
1514  5270              JMP SQX
1515  7300   COMPLX,    CLA CLL           / DOUBLE PRECISION
1516  1353              TAD M2
1517  1354              TAD MM
1520  3354              DCA MM
1521  1354              TAD MM
1522  1355              TAD K3776
1523  7450              SNA
1524  5331              JMP SQRF
1525  7300              CLA CLL
1526  1352              TAD M1
1527  3351              DCA SQXT
1530  5270              JMP SQX
1531  7300   SQRF,      CLA CLL           / ROUND OFF
1532  1351              TAD SQXT
1533  7041              CIA
1534  1110              TAD QUOT2
```

```
1535   3110                   DCA  QUOT2
1536   1110                   TAD  QUOT2
1537   1110                   TAD  QUOT2
1540   1351                   TÁD  SQXT
1541   7500                   SMA
1542   2350                   ISZ  ROOT
1543   7300                   CLA  CLL
1544   1350                   TAD  ROOT
1545   3110                   DCA  QUOT2
1546   3107                   DCA  QUOT1
1547   5661                   JMP  I  SQRTNO
1550   0000    ROOT,          0000
1551   0000    SQXT,          0000
1552   7777    M1,            -1
1553   7776    M2,            -2
1554   0000    MM,            0000
1555   3776    K3776,         3776
1556   4000    K4000,         4000
```

```
                    *1600
1600    0000    RATIO,      0000                / EVALUATION OF AN
1601    3164                DCA NEWXOS          / X/S RATIO
1602    1274                TAD M6
1603    3106                DCA CNTR
1604    1274                TAD M6
1605    3155                DCA TABCNT
1606    1103                TAD LOCTON
1607    3275                DCA XORSL
1610    5217                JMP RATCON
1611    0000    AGNRAT,     0000                / SET COUNTERS
1612    7300                CLA CLL
1613    1044                TAD M3
1614    3106                DCA CNTR
1615    1274                TAD M6
1616    3155                DCA TABCNT
1617    3272    RATCON,     DCA SOXCNT
1620    7300                CLA CLL
1621    1272    RATIOT,     TAD SOXCNT
1622    1106                TAD CNTR
1623    7650                SNA CLA
1624    5264                JMP ISWHAT
1625    1275                TAD XORSL           / X/S OR S/X ?
1626    4547                JMS I STXORS
1627    7300                CLA CLL
1630    1273                TAD XOSCNT
1631    7440                SZA
1632    5235                JMP .+3
1633    2273                ISZ XOSCNT
1634    5253                JMP FINELY
1635    7300                CLA CLL
1636    1112    SETX,       TAD DVSOR2          / AN X/S !
1637    3072                DCA TEMPTM
1640    1114                TAD DV2
1641    3112                DCA DVSOR2
1642    1072                TAD TEMPTM
1643    3114                DCA DV2
1644    1111                TAD DVSOR1
1645    3072                DCA TEMPTM
1646    1113                TAD DV1
1647    3111                DCA DVSOR1
1650    1072                TAD TEMPTM
1651    3113                DCA DV1
1652    3273                DCA XOSCNT
1653    4537    FINELY,     JMS I DVDRTO        / DIVIDE IT
1654    1107                TAD QUOY1
1655    3675                DCA I XORSL
1656    2275                ISZ XORSL
1657    1110                TAD QUOT2
```

```
1660   3675            DCA I XORSL
1661   2272            ISZ SOXCNT
1662   2275            ISZ XORSL
1663   5221            JMP RATIOT
1664   1106   ISWHAT,  TAD CNTR         / RETURN WHERE ?
1665   7041            CIA
1666   1274            TAD M6
1667   7440            SZA
1670   5611            JMP I AGNRAT      / 3 MORE SETS
1671   5600            JMP I RATIO       / MAIN SIX
1672   0000   SOXCNT,  0000
1673   0000   XOSCNT,  0000
1674   7772   M6,      -6
1675   0000   XORSL,   0000


1676   0000   STDDVN,  0000             / EVALUATE STANDARD
1677   7300            CLA CLL           / DEVIATION FOR
1700   1103            TAD LOCTON        / FIRST SIX VALUES
1701   3375            DCA XORSCB
1702   3105            DCA NUMT
1703   3376            DCA STDCNT
1704   1274            TAD M6
1705   3106            DCA CNTR
1706   5312            JMP STDON
1707   0000   STDTWO,  0000             / AFTER 3 MORE SETS
1710   7300            CLA CLL
1711   3376            DCA STDCNT
1712   7300   STDON,   CLA CLL          / SUM OF SQUARES
1713   1376            TAD STDCNT
1714   1106            TAD CNTR
1715   7700            SMA CLA
1716   5366            JMP STDFOR
1717   1375            TAD XORSCB
1720   3377            DCA XORS
1721   2377            ISZ XORS
1722   1775            TAD I XORSCB
1723   7040            CMA
1724   3113            DCA DV1
1725   1777            TAD I XORS
1726   7450            SNA
1727   5333            JMP .+4
1730   7041            CIA
1731   3114            DCA DV2
1732   5335            JMP .+3
1733   2113            ISZ DV1
1734   3114            DCA DV2
1735   1114            TAD DV2
1736   1110            TAD QUOT2
1737   3114            DCA DV2
1740   7004            RAL
```

```
                    / SIGNED DOUBLE PRECISION MULTIPLY
                    / CALLING SEQUENCE:
                    /    1) JMS DMUL
                    /    2) ADDRESS OF MULTIPLICAND ( HIGH )
                    /       ADDRESS OF MULTIPLIER  ( HIGH )
                    / RETURN , HIGH PRODUCT IN AC , NEXT IN
                    / B , C , AND D , ETC .
                    *2000
  2000   0000   DMUL,      0000
  2001   7300        CLA CLL
  2002   4306        JMS TSIGN            / FETCH AND SET SIGN
  2003   1337        TAD MLTH
  2006   3334        DCA MULTH            / HIGH MULTIPLICAND
  2007   1336        TAD MLTL
  2010   3335        DCA MULTL            / LOW MULTIPLICAND
  2011   4306        JMS TSIGN            / FETCH AND SET SIGN
  2012   1335        TAD MULTL
  2013   3301        DCA MP2
  2014   1336        TAD MLTL
  2015   4344        JMS MP4              / MULTIPLY LOWS
  2016   3343        DCA D
  2017   1373        TAD MP5
  2020   3342        DCA C
  2021   1334        TAD MULTH
  2022   3301        DCA MP2
  2023   1336        TAD MLTL
  2024   4344        JMS MP4              / MULTIPLY HIGHS
  2025   1342        TAD C
  2026   3342        DCA C
  2027   7004        RAL                  / GET CARRY
  2030   1373        TAD MP5
  2031   3341        DCA B
  2032   7004        RAL
  2033   3340        DCA A
  2034   1335        TAD MULTL
  2035   3301        DCA MP2
  2036   1337        TAD MLTH
  2037   4344        JMS MP4
  2040   1342        TAD C
  2041   3342        DCA C
  2042   7004        RAL
  2043   1373        TAD MP5
  2044   1341        TAD B
  2045   3341        DCA B
  2046   7004        RAL
  2047   1340        TAD A
  2050   3340        DCA A
  2051   1334        TAD MULTH
```

```
2052    3301    Y       DCA MP2
2053    1337            TAD MLTH
2054    4344            JMS MP4
2055    1341            TAD B
2056    3341            DCA B
2057    7004            RAL
2060    1373            TAD MP5
2061    1340            TAD A
2062    5600            JMP I DMUL        / EXIT WITH HIGH
2063    7402            HLT


                MP1,
2106    0000    TSIGN,      0000
2107    1600            TAD I DMUL        / FETCH ADDRESS
2110    3340            DCA ADDRSS
2111    1740            TAD I ADDRSS      / HIGH ORDER
2112    7100            CLL
2113    7510            SPA               / IS IT < 0 ?
2114    7060            CMA CML           / YES
2115    3337            DCA MLTH
2116    2340            ISZ ADDRSS
2117    1740            TAD I ADDRSS      / LOW ORDER
2120    7430            SZL
2121    7141            CMA CLL IAC
2122    3336            DCA MLTL
2126    7430            SZL
2127    2337            ISZ MLYH
2130    2200            ISZ DMUL
2131    5706            JMP I TSIGN

2134    0000    MULTH,      0000
2135    0000    MULTL,      0000
2136    0000    MLTL,       0000
2137    0000    MLTH,       0000
                ADDRSS,
2140    0000    A,          0
2141    0000    B,          0
2142    0000    C,          0
2143    0000    D,          0
2144    0000    MP4,        0000          / UNSIGNED MULTIPLY
2145    3306            DCA MP1
2146    3373            DCA MP5
2147    1374            TAD M12           / 12 BITS
2150    3372            DCA MP3
2151    7100            CLL
2152    1306            TAD MP1
2153    7010            RAR
2154    3306            DCA MP1
2155    1373            TAD MP5
```

```
2156    7420          SNL              / A 1 ?
2157    5362          JMP  .+3         / NO
2160    7100          CLL              / YES
2161    1301          TAD  MP2
2162    7010          RAR
2163    3373          DCA  MP5
2164    2372          ISZ  MP3         / 12 BITS ?
2165    5352          JMP  MP4+6       / NO
2166    1306          TAD  MP1         / YES
2167    7010          RAR
2170    7100          CLL
2171    5744          JMP  I  MP4
2172    0000    MP3,  0
2173    0000    MP5,  0
2174    7764    M12,  -14
```

```
/ DOUBLE PRECISION DIVIDE
/ CALLING SEQUENCE:
/    1) C(AC)=ADDRESS HIGH ORDER DIVIDEND-
/    2) JMS DUBDIV
/    3) ADDRESS OF HIGH ORDER DIVISOR
/ RETURN : C(AC)  =HIGH QUOTIENT
/               C(DIVND4)=LOW QUOTIENT
/            _  C(DIVND1)=HIGH REMAINDER
/               C(DIVND2)=LOW REMAINDER
/ EXIT WITH ADDRESS


                    *2200
2200    0000    DUBDIV,   0000               / DIVIDEND ADDRESS
2201    3334              DCA ADDRS
2202    1734              TAD I ADDRS        / HIGH DIVIDEND
2205    3335              DCA DIVND1
2206    2334              ISZ ADDRS
2207    1734              TAD I ADDRS        / DIVIDEND
2210    3336              DCA DIVND2
2211    2334              ISZ ADDRS
2212    1734              TAD I ADDRS        / DIVIDEND
2213    3337              DCA DIVND3
2214    2334              ISZ ADDRS
2215    1734              TAD I ADDRS        / DIVIDEND/
2216    3340              DCA DIVND4
2217    5237              JMP DIVGO1         / GET DIVISOR
2220    1342    RDCONT,   TAD LDIVSR
2221    1336              TAD DIVND2
2222    3336              DCA DIVND2
2223    7004              RAL
2224    1341              TAD HDIVSR
2225    1335              TAD DIVND1
2226    3335              DCA DIVND1
2227    7100              CLL
2230    1335              TAD DIVND1         / HIGH=0 ?
2231    7440              SZA
2232    5234              JMP .+2            / NO
2233    1336              TAD DIVND2         / YES
2234    7510              SPA
2235    5347              JMP RDCON+1
2236    5346              JMP RDCON

                / FETCH DIVISOR
2237    1600    DIVGO1,   TAD I DUBDIV
2240    2200              ISZ DUBDIV
2241    3334              DCA ADDRS
2242    1734              TAD I ADDRS        / HIGH DIVISOR
```

```
2243   7100            CLL
2244   7500            SMA                      / DIVISOR>0 ?
2245   7060            CMA CML                  / YES
2246   3341            DCA HDIVSR
2247   2334            ISZ ADDRS
2250   1734            TAD I ADDRS              / LOW DIVISOR
2251   7430            SZL
2255   7141            CMA IAC CLL
2256   3342            DCA LDIVSR
2257   7430            SZL                      / CARRY ?
2260   2341            ISZ HDIVSR               / YES
2261   1345            TAD M25
2262   3344            DCA DIVCNT
2263   7100            CLL
2264   5307            JMP DIV2
2265   1336    DIV3,   TAD DIVND2               / SHIFT HIGH
2266   7004            RAL                      / DIVIDEND LEFT
2267   3336            DCA DIVND2
2270   1335            TAD DIVND1
2271   7004            RAL
2272   3335            DCA DIVND1
2273   1336            TAD DIVND2               / COMPARE DIVISOR
2274   1342            TAD LDIVSR               / WITH DIVISOR
2275   3334            DCA ADDRS
2276   7004            RAL                      / CARRY
2277   1335            TAD DIVND1
2300   1341            TAD HDIVSR
2301   7420            SNL
2302   5306            JMP DIV2-1
2303   3335            DCA DIVND1
2304   1334            TAD SDDRS
2305   3336            DCA DIVND2
2306   7200            CLA
2307   1340    DIV2,   TAD DIVND4               / ROTATE LOW
2310   7004            RAL
2311   3340            DCA DIVND4
2312   1337            TAD DIVND3               / QUOTIENT BITS
2313   7004            RAL
2314   3337            DCA DIVND3
2315   2344            ISZ DIVCNT               / DONE 24. ?
2316   5265            JMP DIV3                 / NO
2317   7300            CLA CLL                  / YES
2320   1336            TAD DIVND2
2321   1336            TAD DIVND2
2322   3336            DCA DIVND2
2323   7004            RAL
2324   1335            TAD DIVND1
2325   1335            TAD DIVND1
2326   3335            DCA DIVND1
2327   5220            JMP RDCONT
```

```
2331    1333    OUT,            TAD DVNDPT          / EXIT
2332    5600                    JMP I DUBDIV
2333    2337    DVNDPT,         DIVND3
2334    0000    ADDRS,          0
2335    0000    DIVND1,         0
2336    0000    DIVND2,         0
2337    0000    DIVND3,         0
2340    0000    DIVND4,         0
2341    0000    HDIVSR,         0
2342    0000    LDIVSR,         0
2344    0000    DIVCNT,         0
2345    7747    M25,            -31                 /       (10)
2346    2340    RDCON,          ISZ DIVND4
2347    7300                    CLA CLL
2350    5331                    JMP OUT

2355    3371                    DCA SET1            / SORT SETS
2356    1771                    TAD I SET1
2357    7650                    SNA CLA
2360    5364                    JMP .+4
2361    1371                    TAD SET1
2362    3772                    DCA I SET2
2363    5773                    JMP I SET3
2364    2371                    ISZ SET1
2365    1771                    TAD I SET1
2366    7640                    SZA CLA
2367    5363                    JMP .-4
2370    5375                    JMP .+5
2371    0000    SET1,           0000
2372    3156    SET2,           3156
2373    3076    SET3,           3076
2374    3140    SET4,           3140
2375    3561                    DCA I R2CNT
2376    5774                    JMP I SET4
```

```
                    / EVALUATION OF STANDARD DEVIATION
                    *2400
2400   0000   TWCASE,       0000                    / SET OF SIX
2401   1105           .     TAD NUMT
2402   7640                 SZA CLA
2403   5274                 JMP NFFS
2404   5271                 JMP FIRFS
2405   4577   TWCASS,       JMS I NUMTCR            / SET OF THREE
2406   1104                 TAD LOCTEN
2407   3306                 DCA XOSSTD
2410   3336                 DCA AGNCNT
2411   3115                 DCA DV3
2412   3116                 DCA DV4
2413   1336   CASEON,       TAD AGNCNT              / SET UP ARRAY
2414   1106                 TAD CNTR
2415   7700                 SMA CLA
2416   5237                 JMP DVCASE
2417   1306                 TAD XOSSTD
2420   3305                 DCA STDXOS
2421   2305                 ISZ STDXOS
2422   1116                 TAD DV4
2423   1705                 TAD I STDXOS
2424   3116                 DCA DV4
2425   7004                 RAL
2426   1115                 TAD DV3
2427   1706                 TAD I XOSSTD
2430   3115                 DCA DV3
2431   7430                 SZL
2432   4553                 JMS I CNTRO
2433   2306                 ISZ XOSSTD
2434   2306.                ISZ XOSSTD
2435   2336                 ISZ AGNCNT
2436   5213                 JMP CASEON              / ALL DONE ?
2437   1105   DVCASE,       TAD NUMT                / NO
2440   3112                 DCA DVSOR2              / SO ADD UP
2441   3111                 DCA DVSOR1
2442   3113                 DCA DV1
2443   3114                 DCA DV2
2444   1120                 TAD DVSPT
2445   3250                 DCA ADDRS5
2446   1117                 TAD DVDPT
2447   4522                 JMS I DBDVPT
2450   0000   ADDRS5,       0000                    / FIND DEVIATION
2451   3307                 DCA RESULT
2452   1707                 TAD I RESULT
2453   3107                 DCA QUOTI
2454   2307                 ISZ RESULT
2455   1707                 TAD I RESULT
```

```
2456    3110            DCA  QUOT2
2457    4544            JMS  I SQRT          / TAKE ROOT
2460    1041            TAD  CHAR06
2461    4433            JMS  I WRITE
2462    7765            -13
2463    1127            TAD  QUOTPT
2464    3266            DCA  ADDRS4
2465    4465            JMS  I DECPRT        / PRINT IT
2466    0000    ADDRS4, 0000
2467    4446            JMS  I CRLF
2470    5600            JMP  I TWCASE
2471    1304    FIRF5,  TAD  K5              / SET OF SIX
2472    3105            DCA  NUMT
2473    5205            JMP  TWCASS
2474    1105    NFF5,   TAD  NUMT            / SET OF THREE
2475    1045            TAD  K3
2476    3105            DCA  NUMT
2477    5205            JMP  TWCASS
2504    0005    K5,     5
2505    0000    STDXOS, 0
2506    0000    XOSSTD, 0
2507    0000    RESULT, 0
2510    0000            0

2511    0000    AGAIN3, 0000                 / DEPOSIT AND DO
2512    7300            CLA  CLL              / MORE RATIOS
2513    3336            DCA  AGNCNT
2514    4562            JMS  I XOSMB
2515    1037            TAD  CHAR04
2516    4433            JMS  I WRITE          / DO MESSAGE
2517    7753            -25
2520    1043            TAD  CHAR10
2521    4433            JMS  I WRITE
2522    7763            -15
2523    4446            JMS  I CRLF
2524    1336            TAD  AGNCNT
2525    1044            TAD  M3
2526    7700            SMA  CLA
2527    5334            JMP  .+5
2530    2336            ISZ  AGNCNT
2531    4533            JMS  I SETIS
2532    4535            JMS  I DEPOST
2533    5324            JMP  .-7
2534    4531            JMS  I RATAGN
2535    5711            JMP  I AGAIN3
2536    0000    AGNCNT, 0000

2537    0000    STXOS,  0000                 / STORE RATIO
2540    3375            DCA  XOS
2541    1775            TAD  I XOS
```

```
2542   3111          DCA  DVSOR1
2543   2375          ISZ  XOS
2544   1775          TAD  I  XOS
2545   3112          DCA  DVSOR2
2546   2375          ISZ  XOS
2547   1775          TAD  I  XOS
2550   1775          TAD  I  XOS
2551   3113          DCA  DV1
2552   2375          ISZ  XOS
2553   1775          TAD  I  XOS
2554   1775          TAD  I  XOS
2555   3114          DCA  DV2
2556   7004          RAL
2557   1113          TAD  DV1
2560   3113          DCA  DV1
2561   2375          ISZ  XOS
2562   1775          TAD  I  XOS
2563   1111          TAD  DVSOR1
2564   3111          DCA  DVSOR1
2565   2375          ISZ  XOS
2566   1775          TAD  I  XOS
2567   1112          TAD  DVSOR2
2570   3112          DCA  DVSOR2
2571   7004          RAL
2572   1111          TAD  DVSOR1
2573   3111          DCA  DVSOR1
2574   5737          JMP  I  STXOS        / READY FOR MEAN
2575   0000    XOS,  0000
```

```
                    / OVERFLOW PROTECTION
                    *2600
2600    0000    CNTROS,     0000                    / SEND MESSAGE
2601    7300                CLA CLL
2602    1206                TAD ERRMES
2603    4433                JMS I WRITE
2604    7763                -15
2605    5600                JMP I CNTROS
2606    2606    ERRMES,     .                       / -15 (-13 DEC)
2607    0215                215                     / OVERFLOW
2610    0212                212
2611    0240                240
2612    0317                317
2613    0326                326
2614    0305                305
2615    0322                322
2616    0306                306
2617    0314                314
2620    0317                317
2621    0327                327
2622    0212                212
2623    0215                215

2624    0000    DECZER,     0000                    / DECIMAL LOCATION
2625    7300                CLA CLL
2626    3231                DCA ZRCNT
2627    3232                DCA ZERCNT
2630    5624                JMP I DECZER
2631    0000    ZRCNT,      0
2632    0000    ZERCNT,     0

2633    0000    ZRTYPE,     0000
2634    3264                DCA ZLCN
2635    1232                TAD ZERCNT
2636    7640                SZA CLA
2637    5244                JMP NOZER
2640    1264                TAD ZLCN
2641    1266                TAD MZ269
2642    7650                SNA CLA
2643    5247                JMP ZERO
2644    2232    NOZER,      ISZ ZERCNT              / SUPRESS ZEROS
2645    1264                TAD ZLCN
2646    5251                JMP .+3
2647    6046    ZERO,       TLS                     / TYPE A BLANK
2650    1267                TAD B240
2651    4447                JMS I TYPE
```

```
2652    2201            ISZ  ZRCNT
2653    1231            TAD  ZRCNT
2654    1265            TAD  MMM2
2655    7650            SNA  CLA
2656    5260            JMP  DECZR
2657    5633            JMP  I  ZRTYPE

2660    1270    DECZR,  TAD  D256
2661    4447            JMS  I  TYPE
2662    2232            ISZ  ZERCNT
2663    5633            JMP  I  ZRTYPE
2664    0000    ZLCN,   0
2665    7776    MMM2,   -2
2666    0260    MZ260,  260
2667    0240    B240,   240
2670    0256    D256,   256


2671    0000    ONEDV,  0000            / DIVIDE
2672    1120            TAD  DVSPT
2673    3276            DCA  ADDRS7
2674    1117            TAD  DVDPT
2675    4522            JMS  I  DBDVPT
2676    0000    ADDRS7, 0000
2677    3310            DCA  RSULT
2700    1710            TAD  I  RSULT
2701    3107            DCA  QUOT1
2702    2310            ISZ  RSULT
2703    1710            TAD  I  RSULT
2704    3110            DCA  QUOT2
2705    4446            JMS  I  CRLF
2706    4446            JMS  I  CRLF
2707    5671            JMP  I  ONEDV
2710    0000    RSULT,  0000


2711    0000    LINES,  0000            / PRINT OUTPUT ON
2712    3325            DCA  LINLCN      / A LINE OF SIX
2713    1155            TAD  TABCNT
2714    7640            SZA  CLA
2715    5322            JMP  .+5
2716    4446            JMS  I  CRLF
2717    1330            TAD  LCN
2720    3155            DCA  TABCNT
2721    5324            JMP  .+3
2722    2155            ISZ  TABCNT
2723    7000            NOP
2724    4465            JMS  I  DECPRT   / PRINT ONE
2725    0000    LINLCN, 0000
2726    4453            JMS  I  TAB
2727    5711            JMP  I  LINES
```

```
2731    0000    MBXOS,      0000                    / X OR S FOR
2732    1164                TAD NEWXOS              / NEW SET ?
2733    7640                SZA CLA
2734    5341                JMP .+5
2735    1345                TAD PL323
2736    3563                DCA I SAYLCN
2737    2164                ISZ NEWXOS
2740    5344                JMP .+4
2741    1346                TAD PL330
2742    3563                DCA I SAYLCN
2743    3164                DCA NEWXOS
2744    5731                JMP I MBXOS
2745    0323    PL323,      323
2746    0330    PL330,      330


2747    0000    CORECT,     0000                    / OPERATOR CORRECT ?
2750    7300                CLA CLL
2751    1102                TAD TEMTT
2752    7640                SZA CLA
2753    5363                JMP .+8
2754    1032                TAD XORSOR
2755    1374                TAD MC323
2756    7640                SZA CLA
2757    5372                JMP .+11
2760    2102                ISZ TEMTT
2761    4446                JMS I CRLF
2762    5747                JMP I CORECT           / YES
2763    1032                TAD XORSOR
2764    1375                TAD MC330
2765    7640                SZA CLA
2766    5372                JMP .+4
2767    3102                DCA TEMTT
2770    4446                JMS I CRLF
2771    5747                JMP I CORECT           / YES
2772    4446                JMS I CRLF
2773    5571                JMP I BACKST           / NO
2774    7455    MC323,      -323
2775    7450    MC330,      -330
```

```
                        *3000
3000   0000   SETDEV,   0000            / DEPOSIT DEVIATIONS
3001   7300             CLA CLL         / SEPARATE FROM THE
3002   1214             TAD DEVT1       / RATIOS ON *3600
3003   3215             DCA DEVT2
3004   2215             ISZ DEVT2
3005   1560             TAD I R1CNT
3006   3614             DCA I DEVT1
3007   1561             TAD I R2CNT
3010   3615             DCA I DEVT2
3011   2214             ISZ DEVT1
3012   2214             ISZ DEVT1
3013   5600             JMP I SETDEV
3014   0000   DEVT1,    0
3015   0000   DEVT2,    0


3016   0000   ONGO,     0000            / INTIME AND GMTIME
3017   1034             TAD CHAR01      / SETS . HOW MANY ?
3020   4433             JMS I WRITE
3021   7766             -12
3022   6032             KCC
3023   4450             JMS I LISN
3024   1032             TAD XORSOR
3025   4360             JMS TEST        / CORRECT NUMBER ?
3026   3030             DCA INTTME
3027   4446             JMS I CRLF
3030   1247             TAD BASE1
3031   4452             JMS I EXPON     / EXPODENTIATE
3032   1035             TAD CHAR02      / INTIME FIRST
3033   4433             JMS I WRITE
3034   7766             -12
3035   6032             KCC
3036   4450             JMS I LISN
3037   1032             TAD XORSOR
3040   4360             JMS TEST        / CORRECT NUMBER ?
3041   3031             DCA GMTIME
3042   4446             JMS I CRLF
3043   1250             TAD BASE2
3044   4452             JMS I EXPON     / EXPODENTIATE
3045   5616             JMP I ONGO,     / GMTIME NOW
3046   7520   M260,     -260
3047   0030   BASE1,    INTTME
3050   0031   BASE2,    GMTIME


3051   0000   NEGIT,    0000            / EVALUATE THE NEW
3052   3354             DCA EXTCNT      / STANDARD DEVIATION
```

```
3053   1103              TAD LOCTON        / SINCE THREE MORE
3054   3355              DCA XOSEXT        / SETS
3055   1104              TAD LOCTEN
3056   3357              DCA EXTSOX
3057   1167              TAD EXTRA
3060   7440              SZA               / MORE ?
3061   5265              JMP .+4           / NO
3062   1353              TAD MD9
3063   3167              DCA EXTRA
3064   5267              JMP .+3
3065   1044              TAD M3
3066   3167              DCA EXTRA
3067   7300     EXTON,   CLA CLL           / SO ADD THEM
3070   1354              TAD EXTCNT
3071   1167              TAD EXTRA
3072   7700              SMA CLA
3073   5350              JMP EXFINI
3074   1355              TAD XOSEXT
3075   5776              JMP I SETS
3076   2356              ISZ XOSEXT
3077   1755              TAD I SOXEXT
3100   7040              CMA
3101   3113              DCA DV1
3102   1756              TAD I SOXEXT
3103   7450              SNA
3104   5310              JMP .+4
3105   7041              CIA
3106   3114              DCA DV2
3107   5312              JMP .+3
3110   2113              ISZ DV1
3111   3114              DCA DV2
3112   1114              TAD DV2
3113   1110              TAD QUOT2
3114   3114              DCA DV2
3115   7004              RAL
3116   1113              TAD DV1
3117   1107              TAD QUOT1
3120   3113              DCA DV1
3121   7100              CLL
3122   1117              TAD DVDPT
3123   3327              DCA EXTML+1
3124   1117              TAD DVDPT
3125   3330              DCA EXTML+2
3126   4521     EXTML,   JMS I DMULPT      / SQUARE DOUBLE
3127   0000              0                 / PRECISION
3130   0000              0
3131   0000              SZA CLA
3132   4553              JMS I CNTRO
3133   1532              TAD I DV2PT
3134   7640              SZA CLA
3135   4553              JMS I CNTRO
3136   1560              TAD I RICNT
```

```
3137   3757                 DCA I EXTSOX        / AND DEPOSIT THEM
3140   2357                 ISZ EXTSOX
3141   1561                 TAD I R2CNT
3142   3757                 DCA I EXTSOX
 143   2357                 ISZ EXTSOX
.3144  2354                 ISZ EXTCNT
3145   2355                 ISZ XOSEXT
3146   2355                 ISZ XOSEXT
3147   5267                 JMP EXTON
-3150  1167    EXFINI,      TAD EXTRA
3151   3106                 DCA CNTR
3152   5651                 JMP I NEGIT
3153   7767    MD9,         -9
3154   0000    EXTCNT,      0
3155   0000    XOSEXT,      0
3156   0000    SOXEXT,      0
3157   0000    EXTSOX,      0

3160   0000    TEST,        0000               / VALID VALUES ?
3161   1246                 TAD M260
3162   7550                 SPA SNA
3163   5371                 JMP QUES           / NO
3164   1374                 TAD MN7
3165   7540                 SMA SZA
3166   5371                 JMP QUES           / NO
3167   1375                 TAD PL7
3170   5760                 JMP I TEST         / YES
3171   7300    QUES,        CLA CLL
3172   4446                 JMS I CRLF
3173   5217                 JMP ONGO+1
3174   7771    MN7,         -7
3175   0007    PL7,         7
3176   2355    SETS,        2355
```

```
                    *3200
3200   0000   DELEIT,   0000            / DELETE A RATIO
3201   7300             CLA CLL
3202   1032             TAD XORSOR
3203   1327             TAD MM331
3204   7650             SNA CLA
3205   4572             JMS I STAR70
3206   1032             TAD XORSOR
3207   1325             TAD MM304
3210   7640             SZA CLA
3211   5600             JMP I DELEIT
3212   5731   OKAY,     JMP I CHECK     / VALIDITY CHECK
3213   7041             CIA
3214   3333             DCA NCNTR
3215   3334             DCA MUMCNT
3216   4453             JMS I TAB
3217   4453             JMS I TAB
3220   7300   DELAGN,   CLA CLL         / WHICH RATIO TO
3221   1336             TAD DELCH        / DELETE ?
3222   4433             JMS I WRITE
3223   7771             -7
3224   6032             KCC
3225   4450             JMS I LISN
3226   1032             TAD XORSOR
3227   3101             DCA TEMTS
3230   1106             TAD CNTR
3231   1346             TAD CNTCTR
3232   1032             TAD XORSOR
3233   7540             SNA SZA         / WAS IT CORRECT ?
3234   5263             JMP DELWR
3235   1333             TAD NCNTR
3236   7550             SPA SNA
3237   5263             JMP DELWR
3240   5732   FINE,     JMP I CHCIT     / YES , SO DELETE IT
3241   1335             TAD DELLCN
3242   1335             TAD DELLCN
3243   1103             TAD LOCTON
3244   1324             TAD MN2
3245   3335             DCA DELLCN
3246   3735             DCA I DELLCN
3247   1335             TAD DELLCN
3250   1330             TAD P400
3251   3347             DCA LCNDEL
3252   3747             DCA I LCNDEL
3253   2335             DCA DELLCN
3254   2347             ISZ LCNDEL
3255   3735             DCA I DELLCN
```

```
3256    3747                    DCA  I LCNDEL
3257    2334                    ISZ  MUMCNT
3260    4446                    JMS  I CRLF
3261    4576                    JMS  I TAB50
3262    5220                    JMP  DELAGN
3263    7300    DELWR,          CLA  CLL           / NO, SO ASK AGAIN
3264    4446                    JMS  I CRLF
3265    1032                    TAD  XORSOR
3266    1326                    TAD  MM316
3267    7650                    SNA  CLA
3270    5274                    JMP  .+4
3271    4451                    JMS  I BELL
3272    4576                    JMS  I TAB50
3273    5220                    JMP  DELAGN
3274    4446    DELOUT,         JMS  I CRLF        / SET UP COUNTERS.
3275    4446                    JMS  I CRLF        / FOR EVALUATION
3276    1077                    TAD  TEMTQ
3277    1334                    TAD  MUMCNT
3300    3077                    DCA  TEMTQ
3301    2350                    ISZ  MUMOK
3302    1103                    TAD  LOCTON
3303    3756                    DCA  I NXOSLL
3304    3757                    DCA  I NSTRGE
3305    3760                    DCA  I NSTAGE
3306    1334                    TAD  MUMCNT
3307    7041                    CIA
3310    1752                    TAD  I CONT1
3311    3752                    DCA  I CONT1
3312    1334                    TAD  MUMCNT
3313    7041                    CIA
3314    7000                    NOP
3315    3753                    DCA  I CONT2
3316    1354                    TAD  CONT3
3317    3755                    DCA  I CONT4
3320    2361                    ISZ  NUMCTR
3321    1076                    TAD  TEMTP
3322    3106                    DCA  CNTR
3323    5362                    JMP  NEG
3324    7776    MN2,            -2
3325    7474    MM304,          -304
3326    7462    MM316,          -316
3227    7447    MM331,          -331
3230    0400    P400,           400
3231    3420    CHECK,          3420
3232    3436    CHCIT,          3436
3233    0000    NCNTR,          0
3234    0000    MUMCNT,         0
3235    0000    DELLCN,         0
3236    3336    DELCH,          .             / -7 (-7 DEC)
3237    0304                    304           / DELETE
```

```
3240    0305                    ⋈ 305
3241    0314                      314
3242    0305                      305
3243    0324                      324
3244    0305                      305
3245    0240                      240
3246    7520    CNTCTR,          -260
3247    0000    LCNDEL,          0000
3250    0000    MUMOK,           000✓
3251    4047    CONT,            4047
3252    4142    CONT1,           4142
3253    0045    CONT2,           0045
3254    5776    CONT3,           5776
3255    4130    CONT4,           4130
3256    4145    NXOSLL,          4145
3257    4146    NSTRGE,          4146
3260    4147    NSTAGE,          4147
3261    0000    NUMCTR,          0000

3262    1372    NEG,            TAD MX3              / NEGATE
3263    3767                    DCA I NEXTRA
3264    1370                    TAD NLCT
3265    3771                    DCA I NGLCT
3266    5751                    JMP I CONT
3267    0167    NEXTRA,         0167
3270    2405    NLCT,           2405
3271    3051    NGLCT,          3051
3272    0000    MX3,            0000
3273    3520    SET,            3520
```

```
                        *3400
3400    0000    CRTMUM,     0000                / DCA 0 IN RATIC TO BE
3401    1614                TAD I NMUMOK        / DELETED
3402    7650                SNA CLA
3403    5600                JMP I CRTMUM
3404    3614                DCA I NMUMOK
3405    1215                TAD PL6
3406    3616                DCA I NK6
3407    1314                TAD PL3
3410    3617                DCA I NK3
3411    1315                TAD K1106
3412    3716                DCA I N4130
3413    5600                JMP I CRTMUM
3414    3350    NMUMOK,     3350
3415    0006    PL6,        6
3416    4142    NK6,        4142
3417    0045    NK3,        0045
3420    7300    CHECK,      CLA CLL             / SET COUNTERS
3421    1106                TAD CNTR
3422    1235                TAD DP6
3423    7650                SNA CLA
3424    5231                JMP .+5
3425    1106                TAD CNTR
3426    3076                DCA TEMTP
3427    1044                TAD M3
3430    5234                JMP .+4
3431    1106                TAD CNTR
3432    3076                DCA TEMTP
3433    1106                TAD CNTR
3434    5665                JMP I OKAY+1
3435    0006    DP6,        6
3436    3666    CHCIT,      DCA I DELCN         / DEPOSIT ZEROS
3437    1106                TAD CNTR
3440    1235                TAD DP6
3441    7650                SNA CLA
3442    5244                JMP .+2
3443    5256                JMP .+11
3444    1667                TAD I NCNTRN
3445    3100                DCA TEMTR
3446    5670                JMP I FINE+1
3447    1100                TAD TEMTR
3450    1666                TAD I DELCN
3451    3666                DCA I DELCN
3452    1100                TAD TEMTR
3453    1045                TAD K3
3454    3100                DCA TEMTR
3455    5670                JMP I FINE+1
```

```
3456    1076                    TAD TEMTR
3457    7000                    NOP
3460    1100                    TAD TEMTR
3461    7650                    SNA CLA
3462    5264                    JMP .+2
3463    5247                    JMP .-12
3464    5272                    JMP DELOTE
3465    3213    OKAY+1,         3213
3466    3335    DELCN,          3335
3467    3333    NCTNRN,         3333
3470    3241    FINE+1,         3241
3471    1442    DELOT,          1442
3472    4446    DELOTE,         JMS I CRLF
3473    4446                    JMS I CRLF
3474    5671                    JMP I DELOT


3500    0000    TABA50,         0000              / TAB 50 SPACES
3501    7300                    CLA CLL
3502    1311                    TAD M50
3503    3312                    DCA LCN50
3504    1313                    TAD KS240
3505    4447                    JMS I TYPE
3506    2312                    ISZ LCN50
3507    5304                    JMP .-3
3510    5700                    JMP I TABA50
3511    7730    M50,            -50
3512    0000    LCN50,          .0
3513    0240    KS240,          240
3514    0003    PL3,            3
3515    1106    K1106,          1106
3516    4130    N4130,          4130
3517    0000
3520    7300                    CLA CLL           / SORT LOCATIONS
3521    1747                    TAD I CNTC
3522    1346                    TAD PL3
3523    3747                    DCA I CNTC
3524    1345                    TAD X
3525    7640                    SZA CLA
3526    5331                    JMP .-3
3527    5366                    JMP MUMCT+1
3530    5744                    JMP I CONTX
3531    5372                    JMP MUMCT+5
3532    7300                    CLA CLL
3533    3345                    DCA X
3534    5743                    JMP I PAROUT


3543    4000    PAROUT,         PARM              / RESET TO START
3544    4136    CONTX,          4136              / OVER COMPLETE
3545    0000    X,              0000              / PROGRAM
```

```
3546    0003    PL3,            3
3547    3346    CNTC,           3346
3550    0000    CRNUMT,         0000            / SET START COUNTERS
3551    7300                    CLA CLL
3552    1764                    TAD I NUMCT
3553    7650                    SNA CLA
3554    5750                    JMP I CRNUMT
3555    1765                    TAD I MUMCT
3556    7041                    CIA
3557    1105                    TAD NUMT
3560    3105                    DCA NUMT
3561    3764                    DCA I NUMCT
3562    7300                    CLA CLL
3563    5750                    JMP I CRNUMT
3564    3361    NUMCT,          3361
3565    3334    MUMCT,          3334
3566    1747                    TAD I CNTC
3567    1346                    TAD PL3
3570    3747                    DCA I CNTC
3571    2345                    ISZ X
3572    1106                    TAD CNTR
3573    1346                    TAD PL3
3574    3776                    DCA I MXM3
3575    5330                    JMP PAROUT ,-13
3576    3372    MXM3,           3372
```

```
                        *4000
        4000    7300    PARM,       CLA CLL          / START OF MAINLINE
        4001    3106                DCA CNTR         / SET COUNTERS
        4002    3167                DCA EXTRA
        4003    3164                DCA NEWXOS
        4004    1104                TAD LOCTEN
        4005    3566                DCA I BLAH
        4006    4353                JMS COMMET       / START OUTPUT FORMAT
        4007    4446                JMS I CRLF
        4010    4552                JMS I GOON
        4011    4541                JMS I DPCHAG
        4012    1103                TAD LOCTON
        4013    3345                DCA LOCTON
        4014    3346                DCA STORGE
        4015    3347                DCA STOAGE
        4016    3351                DCA MEANCT
        4017    5226                JMP MEANS

        4020    2351    MBES,       ISZ MEANCT       / ASK HOW MANY TO DO
        4021    1037                TAD CHAR04
        4022    4433                JMS I WRITE
        4023    7753                -25
        4024    4446                JMS I CRLF
        4025    5226                JMP MEANS
        4026    1351    MEANS,      TAD MEANCT       / AND COLLECT DATA
        4027    7450                SNA
        4030    5220                JMP MBES
        4031    1343                TAD M9
        4032    7700                SMA CLA
        4033    4240                JMS NMEANS
        4034    2351                ISZ MEANCT
        4035    4533                JMS I SETIS      / AND SORT IT
        4036    4535                JMS I DEPOST     / AND DEPOSIT IT
        4037    5226                JMP MEANS

        4040    0000    NMEANS,     0000             / DO MEAN RATIO
        4041    1106                TAD CNTR
        4042    7640                SZA CLA
        4043    5246                JMP .+3
        4044    5372                JMP CLEAR
        4045    5247                JMP MEANON
        4046    4546                JMS I AGAIN
        4047    7300    MEANON,     CLA CLL
        4050    3350                DCA STOCNT
        4051    1350    .SUMTOT,    TAD STOCNT       / FROM STORAGE
        4052    1106                TAD CNTR
        4053    7700                SMA CLA
        4054    5273                JMP SUMMON
```

```
4055   1345              TAD XOSLL
4056   3352              DCA FIXS
4057   2352              ISZ FIXS
4060   1346              TAD STORGE
4061   1752              TAD I FIXS
4062   3346              DCA STORGE
4063   7004              RAL
4064   1347              TAD STOAGE
4065   1745              TAD I XOSLL
4066   3347              DCA STOAGE
4067   2345              ISZ XOSLL
4070   2345              ISZ XOSLL
4071   2350              ISZ STOCNT        / ALL DONE ?
4072   5251              JMP SUMTOT        / NO
4073   1346  SUMMON,     TAD STORGE        / YES , SO DO
4074   3116              DCA DV4           / THE MEAN OF THEM
4075   1347              TAD STOAGE
4076   3115              DCA DV3
4077   3113              DCA DV1
4100   3114              DCA DV2
4101   3111              DCA DVSOR1
4102   1106              TAD CNTR
4103   1342              TAD K6
4104   7640              SZA CLA           / 9 OR 6 VALUES ?
4105   5312              JMP .+5
4106   1342              TAD K6            / 6 TO AVERAGE
4107   3344              DCA MUM
4110   3106              DCA CNTR
4111   5315              JMP READI
4112   1344              TAD MUM
4113   1045              TAD K3            / 9 TO AVERAGE
4114   3344              DCA MUM
4115   1344  READI,      TAD MUM           / SO PRINT THEM
4116   3112              DCA DVSOR2        / AND DO RATIO
4117   4556              JMS I DVONE       / MEAN
4120   1040              CHAR05
4121   4433              JMS I WRITE
4122   7765              -13
4123   1127              TAD QUOTPT
4124   3326              DCA ADDRS3
4125   4465              JMS I DECPRT      / PRINT MEAN
4126   0000  ADDRS3,     0000
4127   4446              JMS I CRLF
4130   1106              TAD CNTR
4131   7640              SZA CLA
4132   5777              JMP I FIX
4133   4550              JMS I STDDEV      / DO STANDARD
4134   4543              JMS I INCASE      / DEVIATION NOW
4135   5340              JMP .+3
4136   4542              JMS I STDTW       / REPEAT FOR THREE
4137   4543              JMS I INCASE      / MORE SETS
```

```
4140    4545                JMS  I  HAPPY          /  FINISHED THIS
4141    4572                JMS  I  STAR70         /  SAMPLE ?
4142    0006    K6,         6
4143    7767    M9,         -11
4144    0000    MUM,        0
4145    0000    XOSLL,      0
4146    0000    STORGE,     0
4147    0000    STOAGE,     0
4150    0000    STOCNT,     0
4151    0000    MEANCT,     0
4152    0000    FIXS,       0


4153    0000    COMMET,     0000                   /  ONE LINE FOR
4154    6046                TLS                    /  COMMENTS
4155    4562                JMS  I  XOSMB
4156    4446                JMS  I  CRLF
4157    7300                CLA  CLL
4160    4450                JMS  I  LISN
4161    1032                TAD  XORSOR
4162    1371                TAD  MN215
4163    7640                SZA  CLA
4164    5357                JMP  .-7               /  ENDS WITH CRLF
4165    4446                JMS  I  CRLF
4166    4446                JMS  I  CRLF
4167    3102                DCA  TEMTT
4170    5753                JMP  I  COMMET
4171    7563    MN215,      -215
4172    7300    CLEAR,      CLA  CLL
4173    3573                DCA  I  CNTXOS
4174    4534                JMS  I  RATIOS
4175    5247                JMP  MEANON
4176    3052    NEGIT+1,    3052
4177    3520    FIX,        3520
```

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