FINAL REPORT

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PART I - LIFE DETECTION

A. INTRODUCTION.

In this final report, we present the work that has been accomplished in the past year on contract NASw-1054 for the study and development of a new and promising technique for the detection of extraterrestrial life. This part consists of the following sections:

A. Introduction.

B. Summary of the Features of the Method.

C. Theoretical Basis of the Method.

D. Discussion of Program.

Summary of:

1. Work accomplished in each area of investigation.

2. Summary of information still needed.

E. Literature Survey on Oxyanions and Enzymes.

F. Experimental Details.

G. Bibliography.

B, SUMMARY OF THE FEATURES OF THE METHOD.

The basis of this proposed life detection system is that of detecting the presence of enzymes (and thereby life) by means of their ability to catalyze the exchange of O^{18} between a labeled oxyanion and water (for a full discussion see part C). A summary of the features inherent in this system are:

FOREWORD

Under Contract NASw-1054, research has been carried out on:

I. a study of the feasibility of detecting extraterrestrial life based on the exchange between water and oxyanions;

II. a study of energy exchange in autotrophic life.

This Final Report is organized into two parts, following the topics stated above.

In Part I, extensive data from the research work is presented herein.

In Part II, the research work is summarized, and reference is made to a number of publications which present the research findings.

1) There are many terrestrial enzymes and enzyme systems known which catalyze oxygen exchange between oxyanions and water. However, it is not necessary to know the specific chemical reactions involved nor their mechanisms in order to recognize the presence of enzymes that catalyze the exchanges. Thus the method has great generality.

2) The background rates of exchange for the oxyanions in which we are interested are very slow, and so far we have discovered no example of significant non-enzymic catalysis of exchange. Catalysis of exchange is a property which appears to be unique to living systems; thus the method is unambiguous.

3) The oxygen-18 labeled inorganic oxyanions which are of most interest are stable to temperatures of at least 200°C. Some, (phosphate, sulfate) can be heated to red heat. Thus sterilization is no problem.

4) The only chemical compounds required are the oxygen-18 labeled anions and water. The appearance of oxygen-18 in the water is followed by electrolyzing the water and measuring the relative abundances of mass 32 ($O^{16}O^{16}$) and mass 34 ($O^{16}O^{18}$). The mass spectrometer could be pretuned on these masses and need not scan. Thus the method has simplicity.

5) Our anticipation that the rate of the exchange reactions might be much greater than the net reactions catalyzed by the enzymes is borne out in part by the accompanying data. Thus, in terms of μmoles of reaction occurring, the method has great inherent sensitivity.

6) The equipment is versatile, so that in addition to providing a yes or no answer to the presence of life, it can provide more sophisticated measurements of the nature of this life as well as collect basic geochemical data.

7) Finally, it has been shown by experiment that with various preparations such as whole cells, cell-free extracts, whole insects, plant extract and with various random soil samples, that the rate of exchange of O^{18} from PO_4 and NO_3 is appreciable and measurable, and that the system as a detector of life, even at this early stage of development, is one which shows <u>great promise</u>.

C. THEORETICAL BASIS OF THE METHOD.

The idea that life might exist on other planets, notably Mars, has raised considerable interest among many scientists for a number of years. As yet, there is no compelling evidence either for or against the presence of life on Mars, primarily due to lack of sufficient clearly established information. The literature on this subject has been reviewed recently (59)* and from this report one would think that conditions on Mars are within reasonable limits for the survival of a life system.

So far we have no assurance that extraterrestrial life will have a functional chemistry similar to terrestrial life. Tests for life based solely or too closely upon analogy to earth life, therefore, are perhaps risky. A limited number of probes for extraterrestrial life, therefore,

*See page 54 for references.

should include one or more experiments which will recognize the presence of some fundamental property of life in an unambiguous fashion. The property we have chosen is enzymic (non-inorganic) catalysis involving oxyanions.

The experiments depends upon the detection of enzymic catalysis of oxygen exchange between oxyanions (AO) and water, or net metabolic transfer of oxygen from the oxyanion to the water.

These reactions can be monitored by using an isotope of oxygen (O^{18}) in the anion.

$$\text{Exchange:}\quad AO^{18} + H_2O^{16} \rightleftharpoons AO^{16} + H_2O^{18} \qquad (1)$$

$$\text{Net Transfer:}\quad AO^{18} + (H) \longrightarrow A(H) + H_2O^{18}. \qquad (2)$$

If exchange or transfer occurs, that is if O^{18}, originally only present in the anion is now found in the water, the presence of an enzymic (living) system is indicated.

The following assumptions underlying this proposed investigation are applicable to all earth forms of life, and because of their generality appear to be inescapable features upon which another life system will depend, even though it may have evolved along different paths.

1) THE LIVING SYSTEM IS A CHEMICAL ONE AND IS BASED UPON AN AQUEOUS MEDIUM. In view of the unique solvent, ionic, and thermal properties of water, this seems to be a reasonable assumption. In fact, it is hard to visualize living systems which would not require an aqueous solution as their reaction medium.

2) THE CHEMICAL PROCESSES INVOLVED IN THE LIFE SYSTEM WILL BE CATALYZED BY SOME SPECIAL MEANS. It seems reasonable to assume that maintenance and growth of living organisms involves a constant flux of energy which is provided by the synthesis and utilization of certain high-energy (metastable) compounds. For this system to function efficiently, various reactions must be catalyzed enzymatically so that they can proceed at rates higher than would occur spontaneously.

3) THE HIGH-ENERGY COMPOUNDS USE OR CONSIST OF SOME FORM OF OXYGEN-CONTAINING INORGANIC ANIONS. The ubiquity of these low atomic number elements, their different levels of oxidation, the change in energy involved in transitions between these states and the high solubility of the oxyanions in water make this assumption reasonable.

4) A NET TRANSFER OR EXCHANGE BETWEEN THE OXYGEN PRESENT IN THE ANION AND THE OXYGEN IN WATER WILL ACCOMPANY THE REACTIONS WHICH THE OXYANIONS UNDERGO. Any reduction of the anion necessarily implies a release of its oxygen. In terrestrial life, this oxygen generally is found in water.

On earth a very important role is played by high energy phosphor-oxygen linkages (such as in ADP, ATP). The formation and breakage of these bonds is accompanied by the exchange of oxygen from the anion into water. It is reasonable to assume that these or similar transfer or exchange reactions occur in other life systems.

Reduction processes, such as Eq. 2, will yield a transfer of oxygen from the anions to water. On earth, for example, plants and microbes use cellular reducing power (H) to reduce nitrate to the ammonium level so that it can be incorporated into amino acids. As can be seen in Eq. 3, the three labeled oxygens from the nitrate will appear in water. Similarly, the reduction of sulfate leads to a transfer of four labeled oxygens into water.

$$HNO_3^{18} + 8\ (H) \longrightarrow NH_3 + 3H_2O^{18}. \qquad (3)$$

There is also the possibility that Eqs. 2 and 3 describe only the overall balance of more complex mechanisms in which more oxygen is transferred from the anion to water than would correspond to the net rate of the overall process. In case of phosphate, it was shown by M. Cohn (19) that the enzyme inorganic pyrophosphatase catalyzed the exchange of oxygen at a rate 500 times faster than the rate of pyrophosphate hydrolysis proper. This implies that the measurement of exchange could be about 500x more sensitive than a direct measurement of the hydrolysis of pyrophosphate. It is not yet known to what extent such rapid exchange is a general property of cell-free preparations systems.

D. DISCUSSION OF THE PROGRAM.

1. Summary of work accomplished in each area of investigation.

(a) Anions and uncatalyzed exchange.

From our knowledge of life here on earth, there are several oxyanions that we know are involved in metabolic pathways. Phosphate is of

course the most common, along with nitrate and sulphate. Other anions of interest, as possibly being involved in Martian life systems are bromate, chlorate, nitrite, borate, silicate, sulfite, phosphite, arsenate, and organic acids.

In order for the method to be unambiguous, it is essential that the spontaneous rate of isotope exchange (non-enzymic) between the oxyanion and water be sufficiently low so that no appreciable uncatalyzed exchange will occur during the time interval of the measurement. It is also important to know to what extent non-enzymic catalysis will affect the rate of exchange.

To investigate these problems we have conducted an extensive survey of the literature (see section E). From this survey it was found that there were sufficient data available to calculate rates of exchange for nitrate, chlorate, bromate, arsenate, and several organic acids with a fair degree of reliability (see Table I). With the other anions, there was insufficient information, and what was available is detailed in section E.

It appears that for silicate carbonate, arsenate and borate, exchange is too rapid to be at all useful in our program.

Although there is good evidence that the rate of PO_4 exchange is low, exact values under the conditions we chose are not available, and it would be very worthwhile to have this information.

The data available for sulfate exchange appears to be quite inadequate, in that although exchange seems slow in concentrated solution, some conflicting reports leave the values at the conditions we have chosen unknown, and with some doubts about whether some slow exchange may or may not occur in dilute solution.

Conflicting evidence also suggests an investigation of sulfite, before this anion's exchange properties are known.

Should phosphite come under consideration, the data available indicate that it will be useful, but more complete information is required for the chosen conditions to make the data complete.

The information available for nitrite is very good for a temperature of 0°, but for higher temperatures (25° and 70°) was uncalculable, and therefore must still be determined.

From all these data, it appears that the exchange rates for nitrate, chlorate, bromate, organic acids, phosphate and phosphite are all of sufficiently low magnitude to allow their use in our scheme. It also appears that silicate, carbonate borate, and arsenate are not at all suitable. Nitrite may be useful or not, depending on the situation and conditions finally selected, but at present it would seem to be excluded from this program.

Sulfate, chlorate and bromate are important anions where insufficient, or conflicting information is available. Although the exchange rates would seem to be very low, and most likely well within the range needed for this program, we feel that for completeness these anions exchange properties should be more fully investigated.

Finally, it appears that inorganic catalysis effects the exchange rate but very little, and it remains only to determine any possible organic chemical catalysis.

Summary of Survey on Oxyanions

Suitable	Probably suitable	Uncertain	Unsuitable
PO_4	SO_4	SO_3	SiO_4
NO_3	PO_3		BO_4
NO_2	ClO_3		CO_3
some organic acids	BrO_3		As_O4
			Amino acids

(b) Enzymatic catalysis.

In order to uncover what is already known in this area we have conducted a literature survey (see sect. E and Table II). This study showed that in earth life there are a number of enzymes that will catalyze exchange between water and phosphate. There is also an indication that this exchange may occur at a faster rate than that of the reaction normally mediated by the enzyme. Since it is also known that many organisms utilize nitrate and sulfate, it would not be unexpected that there might be enzymes available to exchange between water and these anions as well.

To further investigate the generality and feasibility of our method we have conducted a series of experiments which show that intact cells, cell-free extracts, and soil samples will actually exchange O^{18} out of labeled PO_4 and NO_3 into water at an easily measurable rate (see section F 2,3). Other ancillary experiments included detecting exchange from cut up whole insects and plants.

In all cases recently tried (excluding some February soil samples) detectable exchange was observed with one or another anion after reasonable incubation times.

Thus we feel that even in its currently crude form, this method provides a sensitive, reliable, general life detection system.

(c) Possible procedure for the experiment.

At this early stage in the development of our program we have not made specific decisions as to the actual procedure that would be followed aboard the Martian probe.

The general procedure we plan to follow is to introduce a sample of Martian surface material into a small vessel containing water (H_2O^{16}) in which is dissolved the salt(s) of one or more O^{18} labeled oxyanions (e.g. $KH_2PO_4^{18}$). After a suitable interval of time, the water is analyzed by mass spectrometry for its H_2O^{18} content. If exchange has occurred, that is, if O^{18} has been transferred from the phosphate to the water, this would indicate the presence of a catalytic (living) system.

During incubation, a number of parameters should be selected. Such are temperature, pH and gas atmosphere (gases can be either preselected on earth or collected from Martian atmosphere). Since it will not be possible to test many temperatures and pH's, we have decided to pick three realistic values of each upon which to focus our attention. Since the ranges of surface temperatures on Mars are not known accurately, we are considering 0°, 25°, and $70^\circ C$ with the intent of covering the most reasonable band of temperatures to be expected. Many organisms here on earth

are known to be hardy enough to survive, and sometimes even require quite acid or alkaline conditions, and accordingly we have chosen pH values of 2, 7, and 10. With terrestrial materials, one would hesitate to use high concentrations of oxyanions; therefore, one might set an arbitrary limit at 0.1 molar.

The rapid uncatalyzed exchange between silicate, carbonate, or amino acids and water may introduce a complication, but it affects our method only in a quantitative fashion. For instance, in a hypothetical mixture containing equal amounts of silicate-O^{16} and H_2O^{18}, the O^{18} will distribute itself equally between the water and the silicate, leaving one-half of the original label to be detected by our method. Since the method essentially yields a yes or no answer, this means a decrease in sensitivity only. In this connection we should mention the necessity of control measurements because the natural abundance of O^{18} in the Martian atmosphere and soil is not known.

(d) Mass spectrometer and sample handling.

A mass spectrometer is used to measure the content of the stable oxygen isotope O^{18} in water. In fact, the idea of this life detection method grew from the development at RIAS of a very simple mass spectrometer inlet system which has been used successfully in the last few years in various studies of biological gas exchange. Up to this point in the development of the program a conventional instrument of relatively low accuracy has been used. We have used both CO_2 equilibration and electrolysis to analyze the samples.

Details of sample analysis, the inlet, and the mass spectrometer are given in section F1.

2. Summary of information still needed.

(a) The various oxyanions that are known from earth life systems should be examined, as well as others that might reasonably be expected to be found in extraterrestrial life. Their rate of spontaneous exchange should be accurately determined under all the experimental conditions that will be used in the actual experiments on Mars. The possibility of non-enzymic catalysis both by inorganic and organic (but not enzyme) molecules should be investigated.

(b) The oxyanions that are shown through the above studies to have suitable inorganic properties to be useful in our method should be tested in a wide variety of terrestrial organisms (animals, plants, microbes, soils) in various states (whole organisms, cells, cell-free preparations, with and without energy source). From this work it can be determined for each of the anions just how general a phenomenon such exchange is in terrestrial life, and what the optimal conditions are for promoting exchange.

(c) The actual experiment that will be performed on Mars should be designed. On the basis of the above study the techniques for sample handling and the conditions for incubation and assay could be chosen with the purpose of combining the most general and sensitive features available.

(d) The equipment needed to handle the sample and monitor exchange (mass spectrometer) plus associated instrumentation should be designed and built to fit the specifications of the experiment.

E. LITERATURE SURVEY.

1. Non-Enzymatic Exchange between Oxyanions and Water.

In order accurately to evaluate the ability of an enzyme to catalyze O^{18} exchange between water and an oxyanion, it is first necessary to know the amount of exchange that will occur uncatalyzed, or catalyzed by various non-enzymatic systems. This particular area has been the subject of considerable investigation, and various reviews (28, 29, 30, 51, 60, 66) up to 1964 are available (numbers refere to the bibliography).

The oxyanions that are most seriously considered as being involved in possible Martian life systems are bromate, chlorate, nitrate, nitrite, sulfate, phosphate, borate, silicate, and organic acids. Also included as anions of possible future interest are sulfite, phosphite, and arsenate. From the literature it has been possible to calculate for some of these species the rate of exchange and corresponding time of half-exchange (that time necessary for 1/2 of the oxygen in the anion to be exchanged). These values are presented in Table I for the pH's of 2, 7, and 10, and the temperatures of 0°, 25° and $70^{\circ}C$*. In subsequent tables, the specific rate constants, k, bear notes as to whether they were observed or calculated, and the Ea values used for this calculation are included along with the temperature range over which they were obtained. One obvious difficulty with all these results, except for acetate, phosphite, and phosphate is that the assumption is made that the only exchange reaction possible is

*These figures are chosen because they seem reasonable conditions to expect on Mars.

TABLE I

T 1/2 in Minutes

Temp (°C)	pH	NO_3	ClO_3	BrO_3 **	NO_2	NO_2 * (buffer)	Acetic Acid	Benzoic Acid
0	2	10^{14}	2.79×10^{11}	4.33×10^{5}	1.03×10^{-3}	4.76×10^{-2}	2.29×10^{4}	4.3×10^{4}
	7	10^{24}	2.79×10^{21}	4.33×10^{15}	3.20×10^{6}	1.48×10^{7}	3.74×10^{9}	4.3×10^{9}
	10	10^{30}	2.79×10^{27}	4.33×10^{21}	3.20×10^{12}	1.48×10^{13}	3.75×10^{9}	4.3×10^{12}
25	2	2.2×10^{12}	4.23×10^{9}	4.47×10^{4}		5.33×10^{-5}	2.31×10^{3}	3.6×10^{3}
	7	2.2×10^{22}	4.23×10^{19}	4.47×10^{14}		5.33×10^{5}	1.91×10^{8}	3.6×10^{8}
	10	2.2×10^{28}	4.23×10^{25}	4.47×10^{20}		5.33×10^{11}	1.91×10^{8}	3.6×10^{11}
70	2	8.66×10^{8}	1.05×10^{7}	1.98×10^{3}			7.35×10	9.2×10
	7	8.66×10^{18}	1.05×10^{17}	1.98×10^{13}			1.43×10^{6}	9.2×10^{6}
	10	8.66×10^{24}	1.05×10^{23}	1.98×10^{19}			1.42×10^{6}	9.2×10^{9}

* At 0° the buffer was acetate (.01M).

At 25° the buffer was phosphate.

For complete details, see Appendix.

** The alternate data in the literature yield 1.63 x larger values (see text).

that which is acid catalyzed, and that it follows the observed kinetic dependencies (found in acid solution) even in alkaline solutions. That this is probably not too far from the truth is borne out by the few observations that have been made in alkaline solution, though these are only indicative and far from illustrating any kinetic behavior. There is a possibility that in alkaline solution another mechanism of exchange may occur, and in some cases it will be necessary to furthur explore this possibility for our purposes.

The careful work of Anbar (1) and Hoering (38) for bromate, and Hoering (39) for chlorate in acid solution is certainly to be considered reliable, and the only question that can be raised is whether some other mechanism operates in exchange reactions at other than the low pH's studied. Although it seems that this is probably not to be expected, we feel at least a check of the exchange rate at pH's 7 and 10 should be done at all three temperatures, in order to clarify the results reported by Hall (35), which showed what appear to be appreciable exchange, and are far from what the kinetic data would predict.

The values for nitrate (1) seem to be fairly accurate, but with nitrous acid the effects of catalysis and competing reactions have not been completely clarified.

Silicate, it appears, is probably not useful because of its very rapid exchange with H_2O (100% in 1 min. at 100°C (71)), although a careful study at lower temperatures and various pH's is indicated adequately to define its exchange parameters. Borate (71), however, appears to be much

too fast, exchanging completely in less than 10 seconds (31), and in the range of pH 3-10 the rate is too fast to be measured at present.

Sulfate has been studied in strong acid solution (40) and the exchange has been found to be slow and acid dependent. Unfortunately, the relationship is not a simple one because of the high concentrations involved and the results are not readily or accurately convertible to the conditions in which we are interested. The best indication that exchange will be slow under the conditions we have chosen is that the T 1/2 of 1.04 molar solution of H_2SO_4 in H_2O was observed to have a value of 8.4×10^2 min. at 100°. With the observed first order dependence on $[H^+]$ and $[HSO_4^-]$, a solution of .01 M $SO_4^=$ at pH 2 would have a T 1/2 about 10^4 greater, or $\sim 8.4 \times 10^6$ min. At a lower temperature this would be even higher, and of course, at higher pH, very much higher again. Calculations are <u>not</u> included, since as mentioned, the data will not be accurate enough to be anywhere near reliable. In terms of actual observation, the following information <u>is</u> useful:

<u>Sulfate</u>

Ref: 71. Na_2SO_4 in 0.988 N - NaOH for 29 hrs. at 100° gave 0% exchange

Na_2SO_4 in 1.028 N + H_2SO_4 for 37 hrs. at 100° gave 0% exchange

35. Na_2SO_4 in H_2O for 14 hrs. at 95-100° gave 4% exchange

Na_2SO_4 in H_2O for 46 hrs. at 95-100° gave 4% exchange

Na_2SO_4 in NaOH for 15 hrs. at 95-100° gave 0% exchange

Na_2SO_4 in NaOH for 18 hrs. at 95-100° gave 4% exchange

Na_2SO_4 in HCl for 12 hrs. at 95-100° gave 23% exchange

71. $KHSO_4$ in H_2O for 23 hrs. at 100° gave 0% exchange

55. $KHSO_4$ in H_2O for 26 hrs. at 100° gave partial exchange

$KHSO_4$ in H_2O for 24 hrs. at 100° gave partial exchange

$KHSO_4$ in H_2O for 1/60 hrs. at 100° gave 0% exchange

70. $KHSO_4$ at 100° exchanges completely within several hundred hours T 1/2 from 20-200 hrs., depending upon concentrations.

Some earlier work with Na_2SO_4 in alkaline solution (22), however, showed 100% exchange in 26 hrs., being thus in oppossition (and recognized by the authors) with the above work and leaving a question about behavior in basic solution.

Because of the discrepancies found, and the lack of information that allows us to extrapolate values to our conditions, we believe that an exploration of sulfate, at least under our chosen conditions, is definitely warranted.

Some work with sulfite ion exchange, although brief, is also included here:

Sulfite

Ref: 35. K_2SO_3 in H_2O for 9 hrs. at 95-100° gave 112% exchange

K_2SO_3 in H_2O for 8 hrs. at 95-100° gave 102% exchange

K_2SO_3 in NaOH for 5 hrs. at 95-100° gave 0% exchange

55. K_2SO_3 in H_2O for 1/2 hrs. at 19.3° gave 0% exchange

K_2SO_3 in H_2O for 24 hrs. at 19.3° gave 0% exchange

K_2SO_3 in H_2O for 24 hrs. at 100° gave 0% exchange.

Unfortunately, there is also some more recent work (36) that appears to contradict both these sets of results, and shows exchange in both acid and basic solutions to be quite rapid, and thus this anion must be examined before being at all usable.

A recent paper on phosphite (61) indicates that under the desired conditions, exchange with water will be slow enough to permit its use as a test oxyanion, but the exact values for our purposes must still be determined.

The following information illustrates the lower limits of T 1/2:

Phosphite

Temp. (°C)	pH	T 1/2 (obs.)
80	1.00	6.08×10^2 min.
100	2.3	1.20×10^4 min.
100	6.00	3.68×10^5 min.
100	8.00-10.00	5.52×10^5 min.

Early work with phosphate yielded the following observations:

Phosphate (1)

Ref: 71. KH_2PO_4 in H_2O for 41 hrs. at 100° gave 10% exchange

70. KH_2PO_4 in H_2O at 100° had a T 1/2 190 hours

71. Na_2HPO_4 in H_2O for 23 hrs. at 100° gave 0% exchange

Na_3PO_4 in H_2O for 46 hrs. at 100° gave 0% exchange

70. $Na_4P_3O_7$ in H_2O for 41 hrs. at 100° gave 0% exchange

$Na_4P_2O_7$ in NaOH for 41 hrs. at 100° gave 2.5% exchange

$Na_4P_2O_7$ in H_2SO_4 for 64 hrs. at 100° gave 15% exchange.

A recent study (11) of orthophosphate at 100°C has yielded some very interesting results. It was found that the rate of exchange varies with change in acidity and actually went through a maximum. This maximum occurred at pH 4.5 - 5.5, a region where the predominant specie was $H_2PO_4^-$. A plot of the rate vs. pH is shown in the accompanying Figure I, and also indicates the contributions made by each specie to the overall exchange. The notation k_E here refers to a first order rate constant calculated from the atom % excess (N_t) as follows:

$$k_E = 4(2.3/t) \log N_\infty - N_t)$$

where t is time in seconds. The values of T 1/2 at 100°, calculated from observed data and listed below, place an upper limit on the rate of exchange to be expected at the lower temperatures of interest to us.

Because of the extreme importance of phosphate in the study of life systems here on earth (both from a basic research viewpoint and as a model for our life detection system) we feel that it is imperative to obtain at least the data for our chosen conditions, or preferably to obtain curves such as shown in Fig. I at each of the temperatures of interest.

Phosphate (2)

Temp.	pH	T 1/2 (obs.)
100°	2.21	1.63×10^4 min.
100°	4.00	1.15×10^4 min.
100°	8.61	2.88×10^5 min.

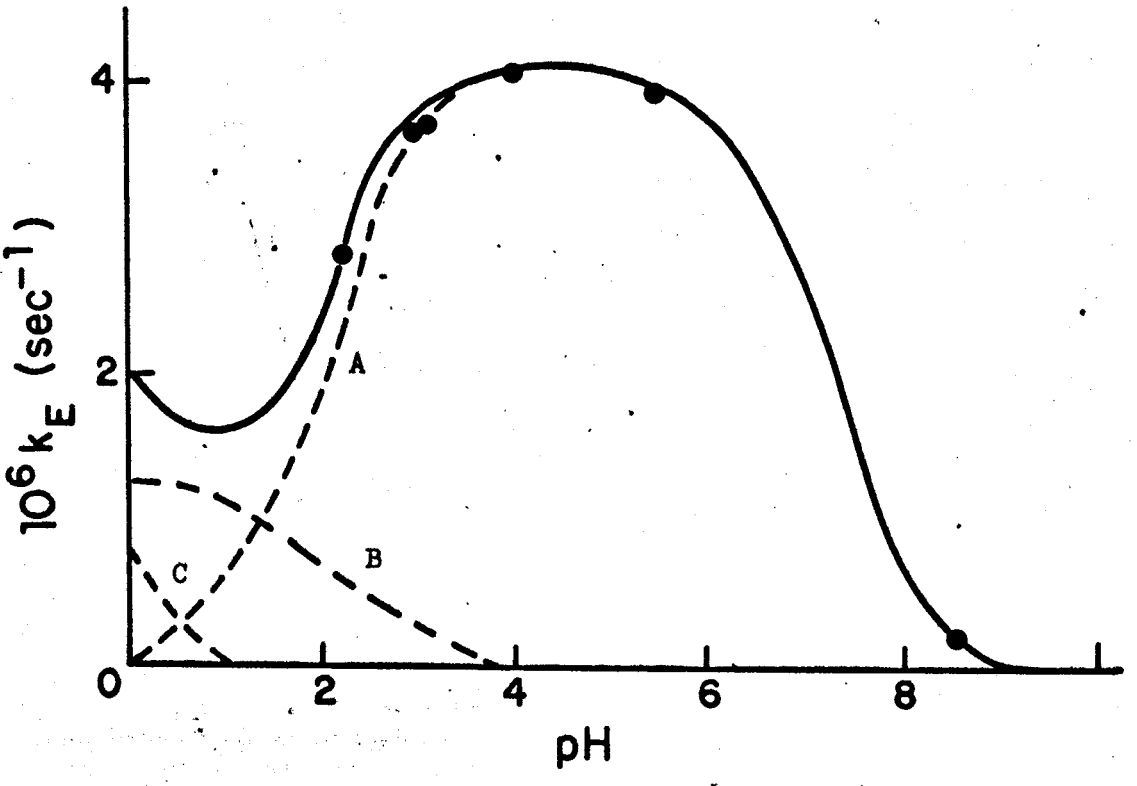


Figure I

Oxygen exchange of orthophosphate at pH 0-9 and 100.1°C. Contribution of (A) exchange of monoanion, (B) neutral exchange, and (C) acid-catalysed exchange. Full line is calculated curve.

Finally, arsenate-water exchange has been studied (43), and although it was too fast to be measured at pH 2, at higher pH the values are given below as follows:

Arsenate

Temp. °C	pH	T 1/2 (obs.)	Ea Temp. range	Ea kcal
32	6	20 min.	4° - 14°	4.044
32	8	44 min.	14° - 32°	3.874
32	10	55 min.	32° - 55°	3.325
4	8	78 min.		
14	8	62 min.		
55	8	30 min.		

In two papers, (8, 56) the rate constants of exchange for oxalic acid were determined and the E and A parameters calculated, for example, at 79°C and pH 2.587, the T 1/2 is about 200 minutes. It was determined also that the rate was proportional to the acidity up to 4 M acid.

The values for acetate with no buffer are good, but the kinetics for acetate in phosphate buffer do not take into account phosphate ion involvement (14). From the results with acetic acid, it is clear that the mechanism of exchange is different at a pH above the pKa than below, and that in alkaline solution the rate is at a plateau minimum. This same behavior is probably to be found in benzoic (or any other carboxylic acid) and as such the figures for pH 7.0 and 10.0 are most likely incorrect.

A recent paper shows the kinetics and rates of exchange of trifluoracetic and trichloracetic acids (50) with rate constants as follows:

Trifluoracetic Acid (ionic strength = 4)

Temp.	k_1 $l^2/mole^2$ sec	k_2 l/mole sec	k_4 l/mole sec
0.0°	3.57×10^{-6}	1.24×10^{-6}	1.12×10^{-2}
25.0	25.3×10^{-6}	14.0×10^{-6}	7.39×10^{-2}
45.3	1.89×10^{-6}	46.0×10^{-6}	23.7×10^{-2}

Trichloracetic Acid (ionic strength = 4)

Temp.	k_1 $l^2/mole^2$ sec	k_2 l/mole sec	k_3 l/mole sec
25.5°	1.92×10^{-6}	1.42×10^{-6}	3.11×10^{-4}
55.0	20.3×10^{-6}	11.0×10^{-6}	27.8×10^{-4}

Where R = rate of exchange, x = stoichiometric concentration of RCO_2H, C = concentration of total carboxylic acid, $R = xC(k_1[H^+]\ [H_2O] + k_2[H_2O] + k_3[OH^-] + (1-x)C\ k_4[O\bar{H}])$. Also calculated were E_a, A and ΔS parameters at 25° and pH_2 about 2, the T 1/2 is about 7.13×10^2 min for tri-fluoracetic acid and 3.8×10^3 min for trichloracetic acid.

From these data, it appears that the organic acids all have exchange rates of about the same order of magnitude, and that the same phenomenon of alkaline plateau as was found for acetic acid probably prevail for all these species.

Several amino acids have also been investigated. Glycine alone was found not to exchange with water at 100°C after 24 hours. When the solution was acidified to pH 2 and treated at this temperature and period of time, exchange was complete (52, 53). In another study of glycine (57) the estimated T 1/2 of exchange at 76°C and pH of 2 is 3×10^2 min. All the parameters k's, E_a and A were calculated.

No exchange was observed at 100° for tyrosine in 40 hours, or glycylglycine in 24 hours (53). The inability of D- and L-Phenylalanine and carbobenzoxy-L-Phenylalanine to exchange should be noted (64).

Of the oxyanions, it appears that bromate, chlorate, nitrate, phosphite, phosphate, sulfate, and the organic acids (represented by acetic and benzoic acids) have an exchange rate with water that is slow, and is not appreciably affected by the presence of other anions (see bromate). It should be noted, however, that more information on most of these anions is still to be desired. Nitrous acid, amino acids and arsenate at low pH's exchange much too rapidly to be useful, while at higher pH's the rates are sufficiently slow to be useful. Borate and silicate appear to be much too rapidly exchanged at all pH values to be useful, but the latter perhaps should be investigated further. Sulfite, if it is seriously to be considered, should be investigated more thoroughly, because the present information is ambiguous.

From the literature the catalytic effects of various anions appear to involve, as a maximum, rate increases of one order of magnitude. To ascertain completely all the possible catalytic effects of various compounds, organic and inorganic, much remains to be done.

Complete details for NO_3, ClO_3, BrO_3, NO_2, HOAc and ϕCO_2H are listed in the Appendix.

2. Enzyme-catalyzed Exchange.

Although our ultimate interest lies in exchanges occurring between oxyanions and water, in the following discussion we shall include information dealing with other systems that are of wide occurrence and of general importance, in order to demonstrate the range of applicability and specificity that enzymes are responsible for in these exchanges.

The area of enzyme catalyzed exchange of various oxygen containing compounds with H_2O has recently been reviewed (60) and much of the information discussed below is mentioned in this review.

It has been observed that both intact mitochondria (5, 7, 18, 20, 27) and soluble enzyme systems isolated from rat liver mitochondria (6, 15, 21, 33) will catalyze an exchange of oxygen-18 between inorganic phosphate and water. The rate of exchange was such that at pH 7.3 and 30° (in the presence of mitochondria equivalent to 2.5 mg protein per ml.), there was a 37% exchange of O^{18} 53 μAo/mg. protein hour from inorganic phosphate (30 μm per ml.) into water in 20 minutes (33). Although only rat liver mitochondria have been studied so far, it is most likely that this exchange is promoted by all mitochondria.

The enzyme alkaline phosphatase has also been found (65) to catalyze exchange between water and phosphate, while with prostatic acid phosphatase (13) no exchange was observed.

In the process of studying O^{18} exchange in muscle and muscle proteins (16, 17, 24, 25, 42, 45-48, 72) it was found that muscle alone (16, 17, 25, 34) as well as the purified proteins myosin, H-meromyosin and actomyosin, under the proper conditions (24, 65, 67, 72) catalyzed an exchange of O^{18} between water and phosphate. In one experiment, (4.5 mg of actomyosin per ml. at 23° and pH 7.4 exchanged 2.1 μm/hr. H_2O^{18} in 0.01 M PO_4) the apparent Km for this reaction was found to be 50 μm.

The acid carboxyl group is a common anion in earth life chemistry and its exchange properties are therefore, also included. A recent paper (32) has reported the catalysis of O^{18} exchange between water and long chain fatty acids by rat liver mitochondria, although the preliminary note has no experimental detail or data. Chymotrypsin has been found to exchange O^{18} into 3,5 dibromo-tyrosine (26) and into carbobenxozy-L-phenylalanine (64) from labeled water. This same enzyme also catalyzed exchange of O^{18} from labeled benzoyl-L-phenylalanine and acetyl-L-tryptophan back into water (3). It should be noted that chymotrypsin did <u>not</u> catalyze exchange of benzoyl-D-phenylalanine, β-phenylpropionic acid, (3), or L-phenylalanine (64) with water, indicating that these materials are not capable of reacting with the active site of the enzyme surface, even though they are held to it (thus they are competitive inhibitors).

Pepsin has also been found (62) to catalyze the exchange of O^{18} between H_2O and carbobenzoxy-L-phenylalanine and N-acetyl-L-tyrosine, (although <u>not</u> with benzoic acid, D-, or L-phenylalanine, again demonstrating

substrate specificity). This exchange was found to be 36% complete in 4.5 hours, and 90-100% complete in 22 hours with the substrate and compound 3 mg. per ml. and the solution at 37° and pH 4.7.

Acetylcholinesterase has been found to catalyze exchange between water and the formate, acetate, propionate, and butyrate anions (4). Although these exchanges proceeded at a good rate, it should be noted that there was exchange under control conditions, and that for formate, this control exchange was three times the catalyzed. It was also found that lipase A caused exchange in the butyrate anion, but not in acetate.

Urease has been reported (63) to catalyze an exchange between H_2O^{18} and phosphate in the presence of urea. With 0.33 mg per ml. of enzyme and .19 M phosphate at pH 6.0 and 30° there was 57% exchange in 1 hour.

It has been reported (41) that 5'-nucleotidase in the presence of adenosine will also catalyze an H_2O^{18}: phosphate exchange.

A study of yeast inorganic pyrophosphatase (19), has shown that extensive exchange of O^{18} occurred between labeled phosphate and water. This reaction is perhaps one of the best that can be used as a model system. With an optimum concentration of magnesium ion in the solution, a pH of 7.2, ionic strength 0.2, phosphate concentration 0.01 M and temperature 30°, the rate of oxygen exchange was 44 μmoles of oxygen per γ enzyme per hour. From the data in the following section it will be seen that O^{18} exchange with phosphate alone under these conditions is negligible. Interesting also is the observation that the enzyme catalyzes an oxygen exchange between phosphate and water that is 500 times faster than the rate of complete reversal of the hydrolysis of pyrophosphate.

TABLE II

Enzyme or Preparation	Rate of Exchange (μA 0/mg. prot./hr.)
1. Rat liver mitochondria	53
2. Muscle actomyosin	0.5
3. Pepsin	0.5
4. Urease	1300
5. Yeast inorganic pyrophosphatase	44,000

A collection of data on the rates of O^{18} exchange between PO_4 and water is shown in Table II for the purposes of illustrating the range of exchange rates that may be encountered in living systems.

F. EXPERIMENTAL DETAILS.

1. Mass Spectrometer, inlet, measurement of O^{18}, and sensitivity.

The mass spectrometer which we are using is the Consolidated Electrodynamics Corporation type 21-613 Residual Gas Analyzer, as shown in Fig. II modified to include a continuous inlet system without gold leak assembly. The only unusual feature of this device is the inlet system (Fig. II) and the reaction vessel. A thin teflon membrane separates the mass spectrometer inlet from a liquid or gaseous sample, the size of which need be only a few microliters. Water and (dissolved) gases diffuse through the membrane into the mass spectrometer at a rate which allows continuous and rapid detection (1-10 seconds). Figure III shows a configuration which allows the measurement of either a liquid sample or gases evolved by electrolysis of water.

Three possible methods for analysis of the O^{18} content of H_2O are:

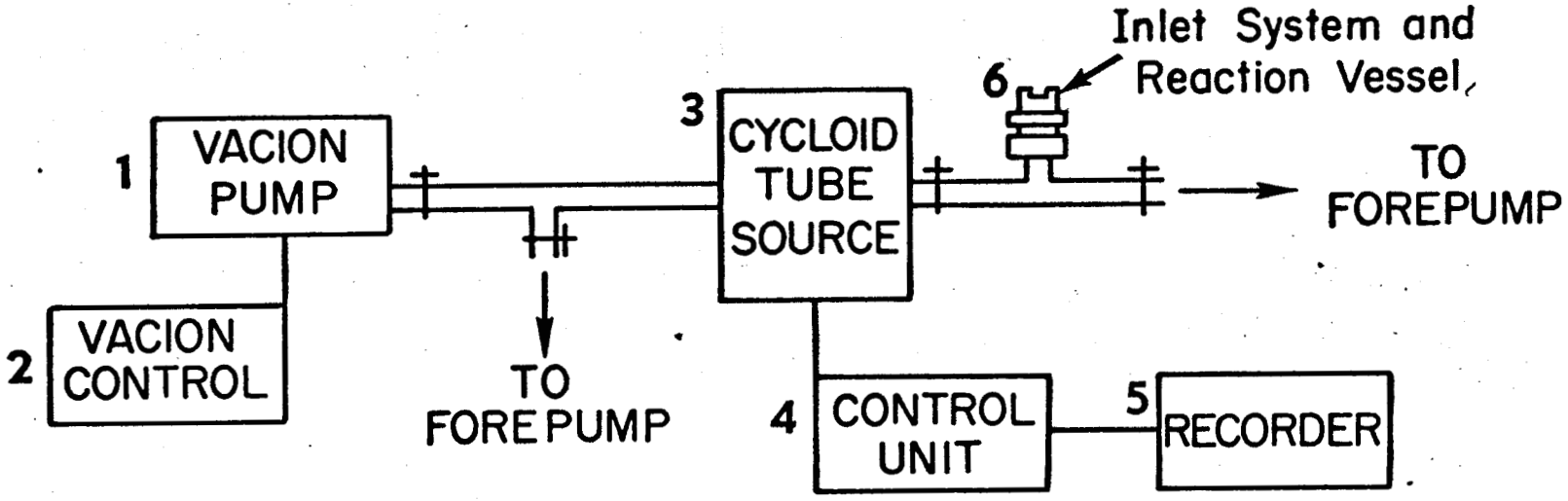
(1) Assay of the H_2O^{18} directly by tuning on mass numbers, $20(H_2O^{18})$ and $18(H_2O^{16})$ or mass numbers $19(HO^{18})$ and $17(HO^{16})$.

(2) Assay of the H_2O^{18} indirectly by equilibrating it with carbon dioxide (adding bicarbonate and carbonic anhydrase) and tuning on mass numbers $46(CO^{16}O^{18})$ and $44(CO^{16}O^{16})$.

(3) Assay of the H_2O^{18} indirectly by electrolyzing the water and tuning on mass numbers $34(O^{16}O^{18})$ and $32(O^{16}O^{16})$.

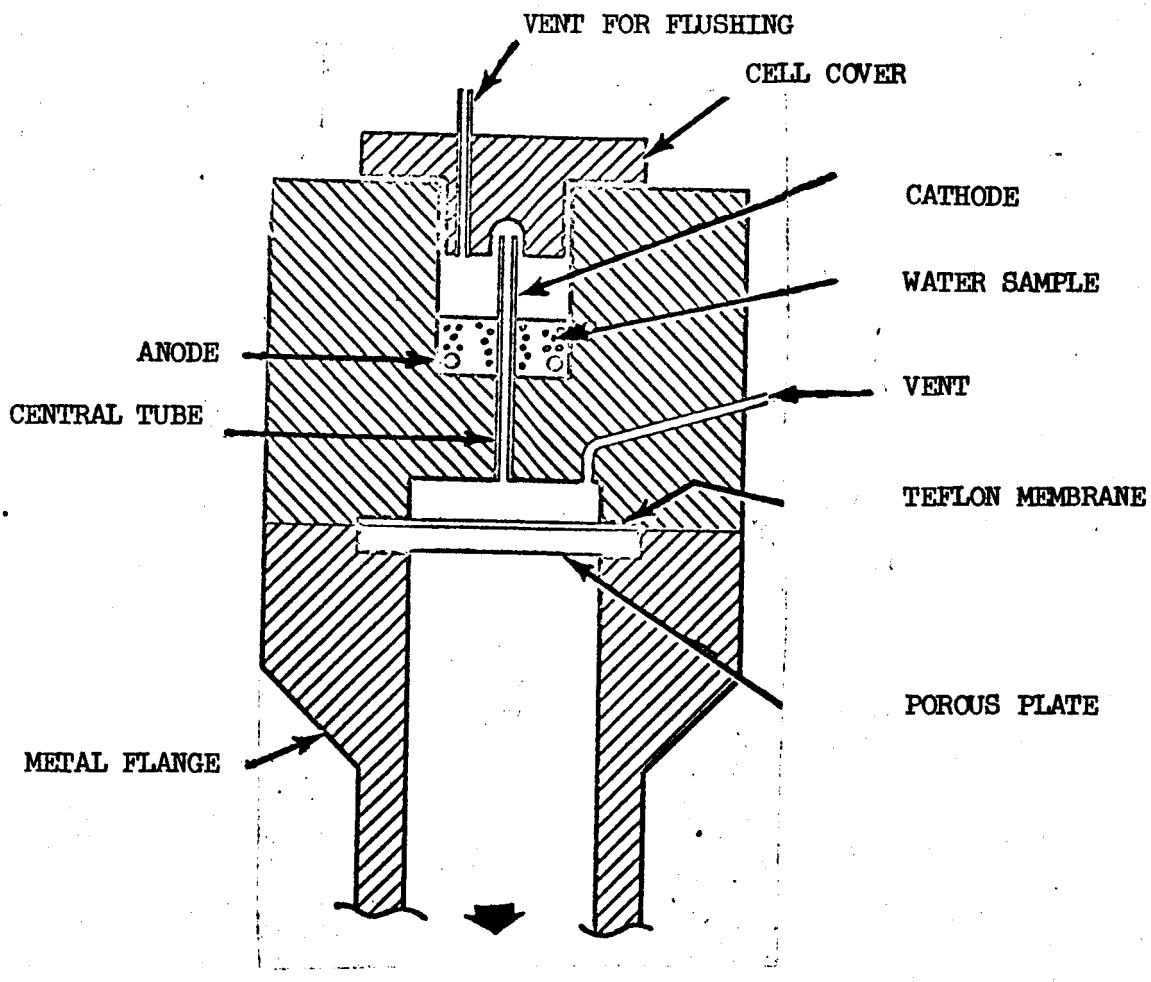
FIGURE II

MASS SPECTROMETER



1. Varian Associates
   Vacion Pump
   Type 912-6001
2. Varian Associates
   Vacion Pump Control Unit
   Model 921-0012
3. Cycloid Analyzer modified as noted
4. Electronic Control Unit
5. Brown Recorder
6. Inlet system and reaction vessel. See Fig. 2 for details.

FIGURE III



ELECTROLYSIS CELL

Direct assay of the H_2O^{18}: H_2O^{16} ratio is not feasible with our present instrument because its "memory" for water is several hours. That is, the quantity of water absorbed on the walls of the instrument is very large compared with the quantity of water vapor contained in the instrument at 10^{-5} to 10^{-6} torr. However, since this is basically the most desirable method to use it would be desirable to build a mass spectrometer in which the water memory has been sufficiently reduced to permit direct analysis of water; in fact, a mass spectrometer which is suitable for the direct assay of water has been designed (69) and was at one time commercially available (Consolidated Engineering Corporation, Recording Isotope-Ratio Mass Spectrometer Model 21-330). Another problem in determining the relative abundance of oxygen-18 in water by measuring mass numbers 18 and 20 is that mass number 20 also includes the singly charged neon ion and the doubly charged argon ion. The possible interference from neon and argon might be overcome by measuring mass numbers 17 and 19. However, there is a spurious mass 19 peak due to H_3O^{16+} (from the reaction $HO^{16+} + H_2O^{16} \longrightarrow H_3O^{16+}$) which is proportional to the square of the pressure of water vapor and at 10^{-5} mm pressure is a considerable fraction of the total peak at mass 19. The mass spectrometer designed by Washburn et al. (69) allows for adjustment of the repeller voltage, the electron beam current, and sample pressure to reduce the formation of H_3O^+ ions. With this specially designed instrument, ratios of D/H+D in water could be measured within 0.002 atom % in about 1-1/2 hours after introducing a new sample. It is likely that further modifications might be made to decrease still more the "memory" for water.

The feasibility of the determination of O^{18}/O^{16} in H_2O by measuring mass numbers 44, 45 and 46 after equilibration of the H_2O with bicarbonate and carbon dioxide is shown in Table III. Various anions (23) catalyze the reaction,

$$H_2O + CO_2 \longleftrightarrow H_2CO_3$$

but the half-times of such a catalyzed exchange even though suitable for measuring the isotope ratios of a water sample, are still several minutes and would therefore, limit the possibilities of measuring the kinetics of a reaction occurring in the reaction vessel attached to the inlet system. Also, in a life detection system, these anions may actually be poisons for the very life processes we are attempting to detect. Perhaps the most serious objection to this indirect procedure for measuring oxygen-18 abundance in the water of reaction mixtures which contain purified enzymes, cell homogenates or soil extracts is the possible interference from ethyl alcohol (mass number 46).* It is also difficult to use the method at pH values below 6.5 because of the rapid loss of carbon dioxide and the accompanying change in pH. At pH values above 8.0 the half-time of the exchange catalyzed by such anions as selenite and arsenite is 20 minutes or longer, thus limiting their usefulness.

The utility and practicability of utilizing carbonic anhydrase to allow monitoring of an exchange reaction is illustrated in Fig. IV. The ease of measurement with this system is obvious, and the good results are

*If the C^{13} abundance is known, the presence of ethyl alcohol could be detected and corrected for by comparing the relative heights of the peaks 44 and 45.

TABLE III

Half-times of the reaction

$$H_2O^{18} + CO_2 \rightleftharpoons H_2CO_3$$

under various conditions*

Catalyst	None	Arsenate 0.02 M	Arsenite 0.01 M	Carbonic 0.5 μg	Anhydrase 5μg
pH					
6.0	8 min.	2 min.	- min.	2.4 min.	< 10 sec.
7.0	68	18	13	8	< 30 sec.
8.0	160	40	18	13	< 30 sec.

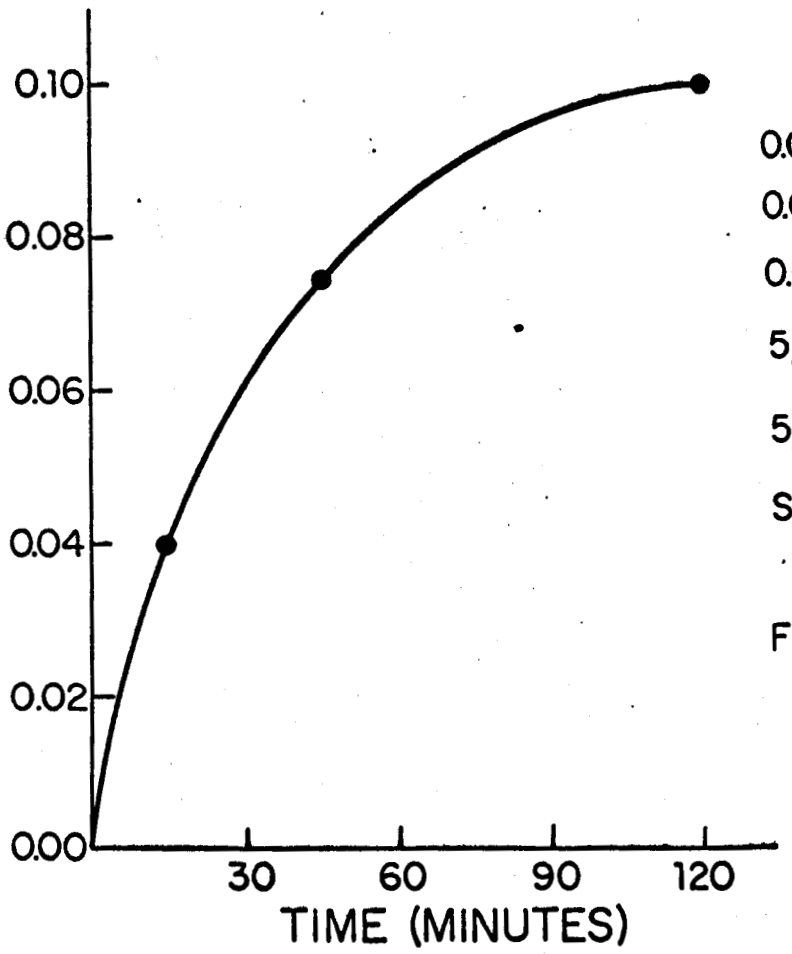
* The final volume of the reaction mixture was 0.20 ml.
The final concentration of bicarbonate was 0.05 M.

FIGURE IV

MODEL SYSTEM
DETECTION OF PYROPHOSPHATASE ACTIVITY

$$KH_2PO_4^{18} + H_2O \overset{\text{PPi-ase}}{\rightleftharpoons} KH_2PO_4 + H_2O^{18}$$

$$H_2O^{18} + CO_2 \rightleftharpoons H_2CO_3^{18} \rightleftharpoons H_2O + CO_2^{18}$$



CONDITIONS:

0.05M $KH_2PO_4^{18}$ (pH7.0) 55A% EXCESS

0.05M $KHCO_3$ (pH7.0)

0.005M Mg^{++}

5μg PYROPHOSPHATASE

5μg CARBONIC ANHYDRASE

SAMPLE VOLUME = 50μl – See Accompanying Sketch

FINAL A% EXCESS AT EQUILIBRATION WOULD BE 0.20

apparent. The points shown in the figure represent measurements taken during the course of the reaction occurring in the vessel at the teflon inlet membrane. Reactions with half-times of ten minutes or greater are most convenient for measurements with our present instrument because when switching from one peak to another there is a delay of about 10 seconds in machine response. Given an instrument which would measure accurately the greatly different signals of 2 different mass numbers utilizing a double ion collector system, reactions with a half-time of a few seconds could be followed because the half-time of diffusion of a gas from the solution to the filament is only a few seconds (Table IV).

Measurement of the relative abundance of oxygen isotopes in water by measuring the oxygen produced electrolytically now appears to be the best of the three possibilities for most applications. The electrolysis cell utilized by us is illustrated in Fig. III. The sample (~ 20 μl), distilled from the exchange vessel is placed in the cell, and a small amount of electrolyte is added. By adjusting the potential applied across the gold electrodes, O_2 is produced at a constant rate and signal strength is reasonably steady.

The evolved gases are passed over the membrane and the peaks at mass numbers 32($O^{16}O^{16}$) and 34($O^{16}O^{18}$) are measured.

If R = [32]/[34]: N = % abundance of O^{18} = $\frac{[34] \times 100}{2([32] + [34])} = \frac{50}{R + 1}$.

At this point it should be stressed that the present machine will detect ratio differences of 1 part in 100. (We expect an accuracy of 1 part in 1000 to be readily obtainable with small, light-weight instruments. Accuracies of an order of magnitude higher have been obtained in conventional

TABLE IV

TEFLON WINDOW CHARACTERISTICS

HALF-TIMES OF DIFFUSION
FOR VARIOUS GASES
(in seconds)

Window	He	O_2	CO_2
0.001"	3	10	15
0.0005"	< 3	3	5

instrument. An accuracy of one part in 1000 would allow the detection of an exchange of 0.1 μatom oxygen per ml.)

One should note that complete equilibration of the suggested concentration (0.1M) of phosphate having 100 A% O^{18} label causes an increase of the A% O^{18} in the water of only 0.72%. Thus the elegance of the present method, which lies in the relative ease with which one can measure the appearance of O^{18} in water, is offset by the large dilution of 0.1 molar anion into 55 molar water, entailing a substantial loss of sensitivity.

If, instead of the appearance of O^{18} in water, one could measure the decrease of O^{18} in the anion, the sensitivity would be about 100 fold higher; the method could also gain in generality. Presently, it appears that this approach would imply prohibitive technical complications.

To make a rough estimate of the sentivity of the method, at this point we assume that the exchange rate is of the same order as the metabolic consumption or production of oxygen. This is in accord with our preliminary data using whole cells and phosphate. Assuming (a) an average value of terrestrial metabolic rate of 10 μM of oxygen per mg. protein per hour, (b) ability to detect the exchange of 0.1 μatom O^{18} per ml., and (c) an incubation time of five hours, the limit of detectibility is 2 μg protein per ml.

2. Exchange reactions with whole organisms and cell-free extracts.

Utilizing whole organisms that had been deprived of either PO_4 or NO_3 it was found that there was detectable exchange with PO_4^{18} and NO_3^{18} as illustrated in Table V. To show that the enzymatic material of a cell-free preparation is also active, corresponding data for these organisms are listed

TABLE V

Preparation	Anion	Respiration Rate	Exchange Rate
Saccharomyces whole cells (PO_4 starved)	PO_4	0.85 $\mu MO_2/\mu l$ hr	0.035 $\mu AO/\mu l$ hr (0.41 μAO/mg Prot. hr)
Cell-Free	PO_4	0.48 μMO_2/mg Prot. hr	96.6 μAO/mg Prot. hr
Hydrogenomonas whole cells (PO_4 starved)	PO_4	3.74 μMO_2/mg hr	0.055 μAO/mg hr
Cell-free	PO_4	0.97 μMO_2/mg Prot. hr	2.76 μAO/mg Prot. hr
Scenedesmus whole cells (NO_3 starved)	NO_3	0.088 $\mu MO_2/\mu l$ hr	0.197 $\mu AO/\mu l$ hr (2.24 μAO/mg Prot. hr)

as well in Table V. As can be seen, there is appreciable exchange occurring with both PO_4 and NO_3, and in both whole cell and cell-free preparations. It is interesting to note that in some cases the cell-free preparation is significantly faster than the whole cell preparation, presumably because of greater enzyme site availability. The time courses for these exchanges are illustrated in Figures V, VI, VII. These curves all fail to extrapolate out to complete exchange which indicates that the preparation loses activity before complete exchange is reached. Figure VI demonstrates this particularly well; here, two different dilutions of the same sample which were run concurrently show the same time dependence and shape.

Conditions of Figures V-VII:

In all cases (except Saccharomyces cell-free, see below) a sample of cells or cell-free preparation suspended in minimal media was added to a buffered solution of KNO_3^{18} (84 A% O^{18}) or $Na_2HPO_4^{18}$ (94.4 A% O^{18}) so that the final concentration of anion was 0.1M and the pH 7.0 (in Saccharomyces whole cells pH was 4.5). The mixture was placed in the carterwell of a Warburg flask, and the rate of respiration was determined by standard manometric techniques. Aliquots were removed periodically, distilled, and analyzed on the mass spectrometer by electrolysis to determine the rate of exchange.

The Saccharomyces cell-free material was prepared by shaking a slurry of whole yeast cells in a Nossal shaker and centrifruging down the heavy material. The supernatent was added to solutions of KH_2PO_4 (25 A% O^{18}, final concentration 0.05M) and $MgCl_2$ (final core 0.05M) buffered to pH 7.2. The respiration rate was determined on a polarograph, and the exchange rate was measured using the carbon dioxide-carbonic anhydrase equilibration method.

FIGURE V

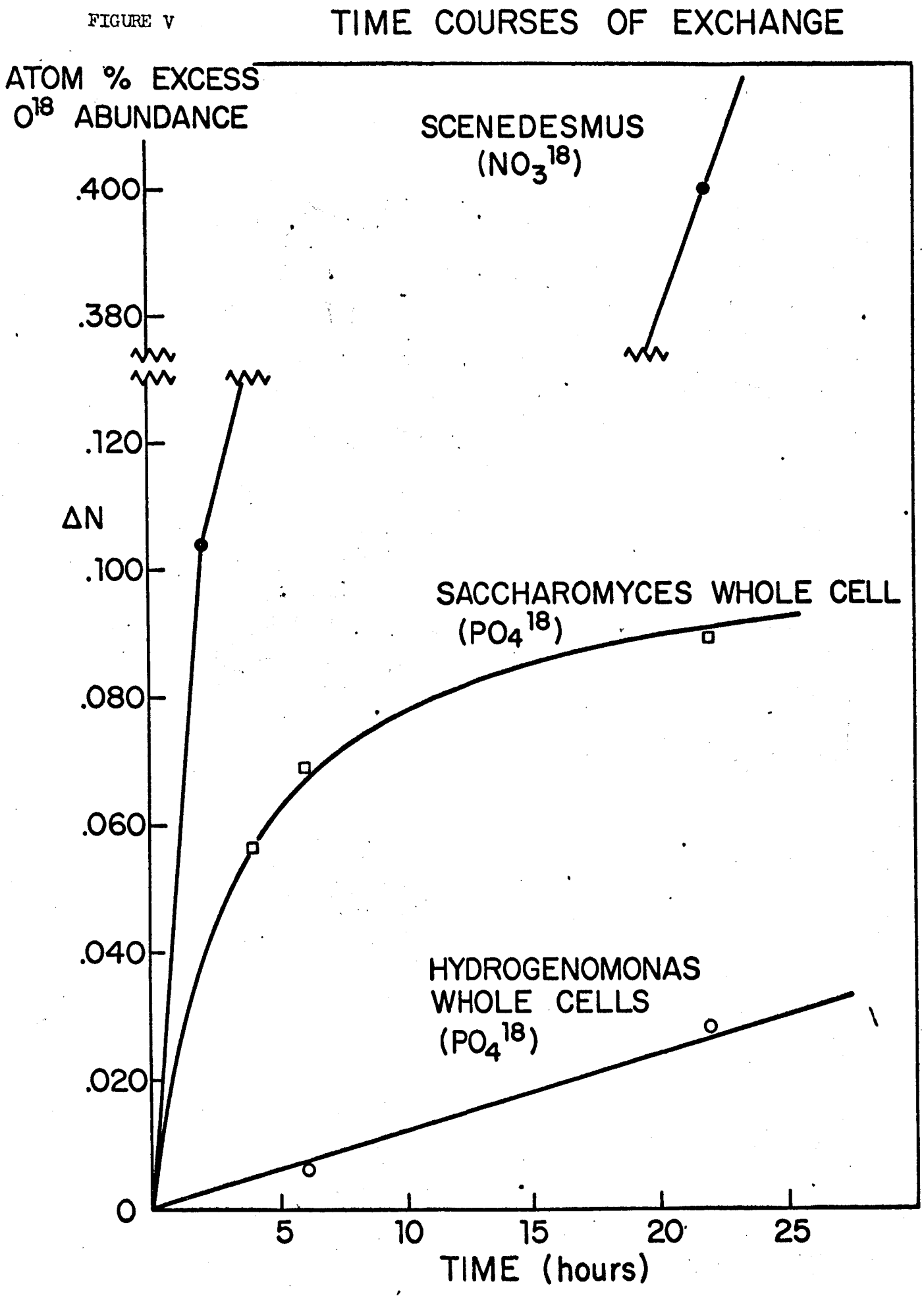

TIME COURSES OF EXCHANGE
ATOM % EXCESS
O18 ABUNDANCE
SCENEDESMUS
(NO3 18)
.400
.380
.120
ΔN
.100
SACCHAROMYCES WHOLE CELL
(PO4 18)
.080
.060
.040
HYDROGENOMONAS
WHOLE CELLS
(PO4 18)
.020
0
5
10
15
20
25
TIME (hours)

FIGURE VI

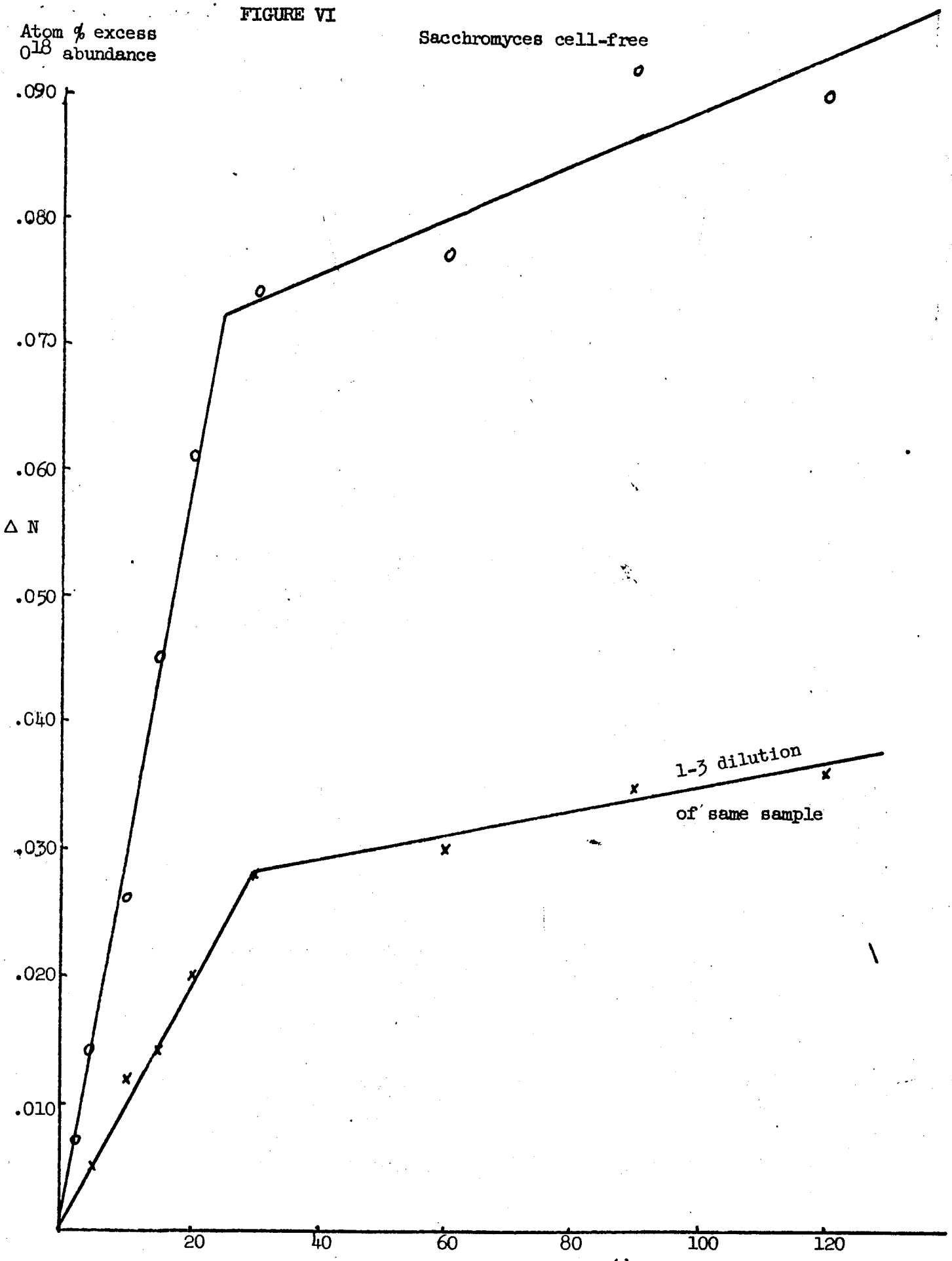
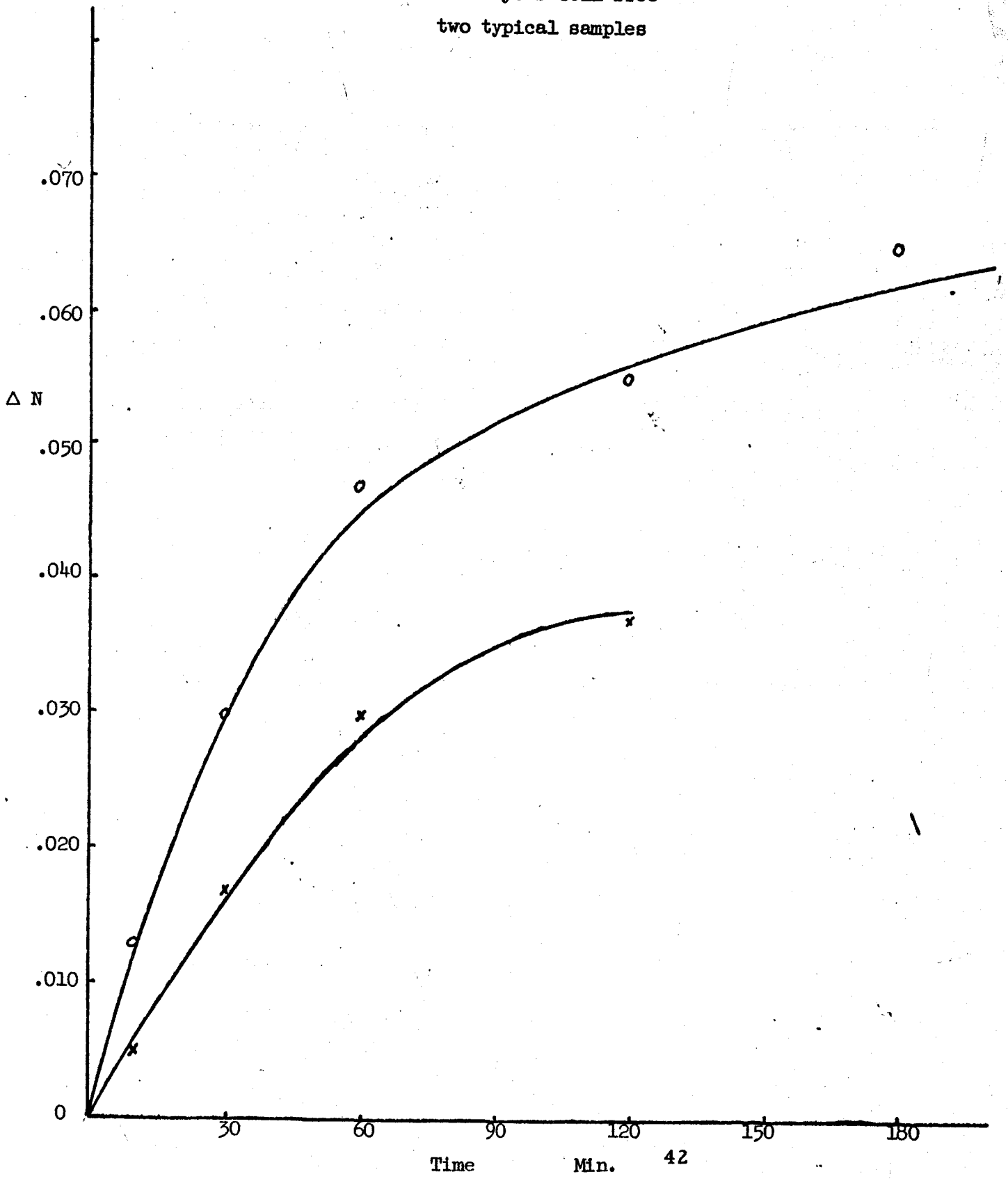

Atom % excess
O^{18} abundance
Saccharomyces cell-free
.090
.080
.070
.060
Δ N
.050
.040
.030
.020
.010
1-3 dilution
of same sample
20
40
60
80
100
120
Time
Min.

FIG. VII

Sacchromyces cell-free
two typical samples



3. Exchange reactions catalyzed by soil enzymes and/or microorganisms.

Several soil samples have been tested for the ability to catalyze exchange of oxygen-18 from labeled phosphate into water.

In Figure VIII is illustrated the exchange that was found with two random soil samples, indicating the time course of exchange with O^{18}-phosphate. These two curves indicate the function of the system and its feasibility as a life detection system. The data collected so far are tabulated in Table VI. It should be noted that not all samples showed exchange, measurable with our present equipment and that their rates varied according to season. The samples collected in February show some exchange with PO_4, but mostly with NO_3, while the summer samples showed mostly exchange with PO_4, and little or none with NO_3. Whether this difference is due to different organism predominance, or to different metabolic status of the same organisms remains to be determined. It should, however, be noted that most samples gave positive results with one or the other anion. Soil samples which were "primed" with sterile glucose and yeast extract showed a two or three fold greater exchange. This greater exchange undoubtedly reflects the increased microbial activity due to the priming material.

These results (Table VI) represent only our first attempts at measuring exchange reactions catalyzed by soil enzymes. It seems unlikely that the first set of conditions tried were optimum. We expect to be able to improve these conditions and consequently to increase the observed exchanges. We are particularly intrigued with the possibility of deionizing

and concentrating a hot water extract of soil and using this material as a primer of soil microbial activity and thereby increase the exchange reactions. Such a procedure, if successful, is of course, to be preferred to challenging possible Martian organisms with possible "strange" substances such as glucose and/or yeast extract.

In conclusion, we present herein data supporting our initial proposal for a workable, general, sensitive life detection system.

Figure VIII

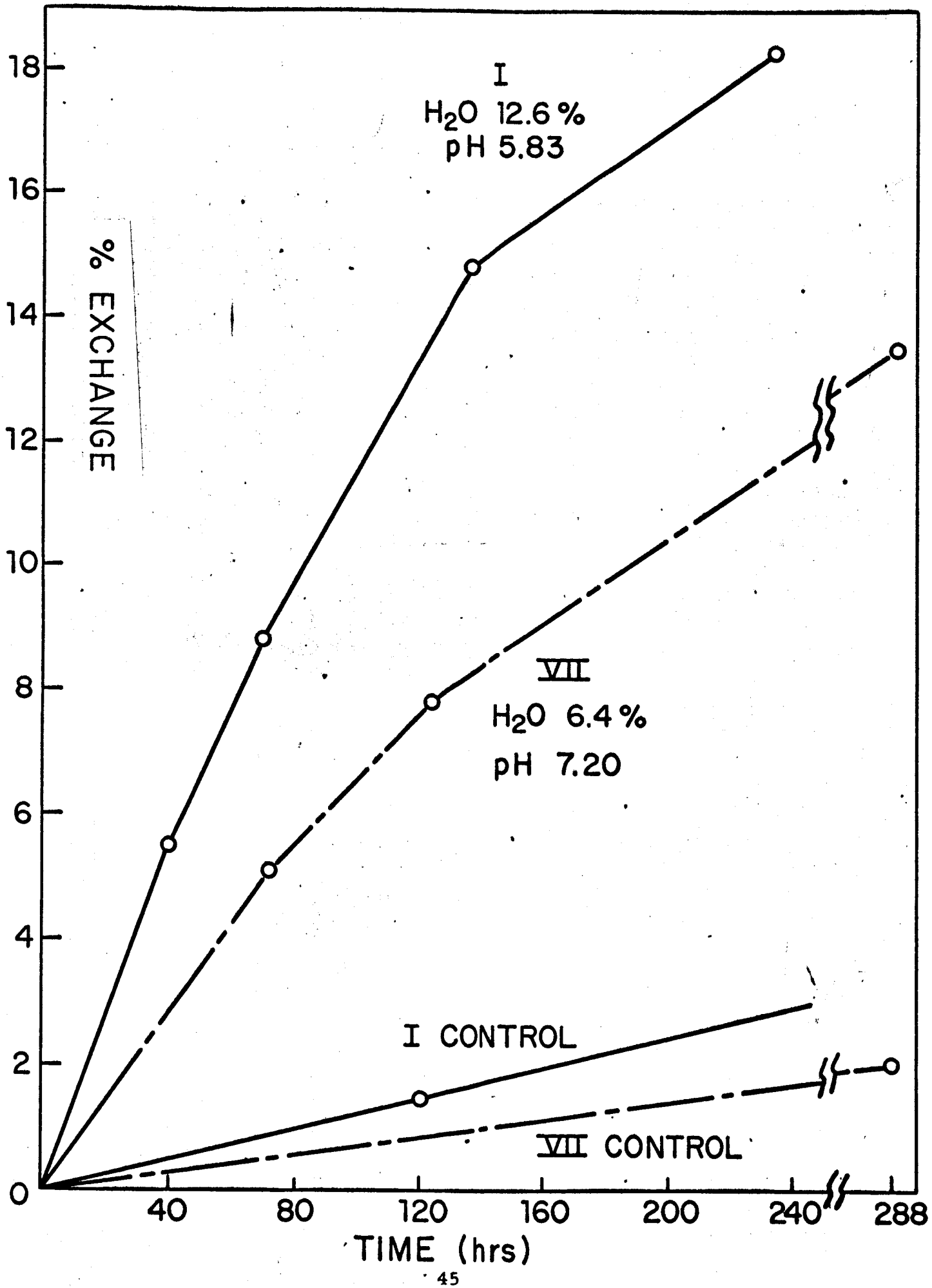

I
H2O 12.6%
pH 5.83
VII
H2O 6.4%
pH 7.20
I CONTROL
VII CONTROL
% EXCHANGE
TIME (hrs)
0
2
4
6
8
10
12
14
16
18
40
80
120
160
200
240
288

TABLE VI

February Samples

N = Atoms % excess

	SOIL	ANION	PRIMER	TIME	Δ N FOUND	Δ N THEO.	% EXCHANGE
1.	A	HPO_4^{18} = (0.10M)	-	24 hrs	0.007 ± 0.003 *A% XS	0.187 A% XS	3.7
2.	B	"	-	"	0.000 ± 0.001	0.187	0.0
3.	C	"	-	"	0.000 ± 0.002	0.187	0.0
4.	D	"	-	"	-0.001 ± 0.001	0.187	0.0
5.	E	"	-	48 hrs	0.004 ± 0.004	0.187	2.1
6.	F	"	-	48 hrs	0.002 ± 0.001	0.187	1.1
7.	A	"	-	48 hrs	0.002 ± 0.004	0.187	1.1
8.	B	"	-	48 hrs	0.003 ± 0.002	0.187	1.6
9.	A	"	-	90 hrs	0.010 ± 0.001	0.187	5.3
10.	B	0.2	-	90 hrs	0.009 ± 0.003	0.374	2.4
11.	D	0.2	-	90 hrs	0.012 ± 0.001	0.374	3.2
12.	D	0.2	-	90 hrs	0.009 ± 0.002	0.374	2.4
13.	E	0.2	Glucose + Yeast Extract	90 hrs	0.015 ± 0.004	0.374	4.0
14.	E	0.2	"	90 hrs	0.023 ± 0.001	0.374	6.1
15.	F	0.2	"	90 hrs	0.025 ± 0.002	0.374	6.7
	Controls						
16.	A **	0.2	-	90 hrs	0.000 ± 0.002	0.374	0.0
17.	H_2O	Lyophilized from Soil			-0.001 ± 0.001	0.000	0.0
18.	D	NO_3^{18-} (0.1M)	-	168 hrs	0.022 ± 0.004	0.212	10.4
19.	F	" (0.1M)	-	168 hrs	0.017 ± 0.001	0.212	8.1
20.	A	" (0.2M)	0.3% Glucose + 0.3% Yeast Extract	88 hrs	0.202 ± 0.003	0.423	47.7
	Control						
21.	A **	NO_3^{18-} (0.2M)	0.3% Glucose + 0.3% Yeast Extract	88 hrs	0.000 ± 0.001	0.423	0.0

TABLE VI (Cont'd)

Summer Samples

N = Atom % excess

Sample Soil	Anion	pH	$\%H_2O$ in Soil	ΔN 24 hrs	ΔN 48 hrs	ΔN 40 hrs	(.198) ΔN Control	hrs	Total % Exchange
II	NO_3	5.20	8.5	.001	--	.003	.000	90	0.6
III	PO_4	6.26	2.3	.017	.034	--	.003	48	5.0
IV	NO_3	5.80	1.8	.002	--	.002	.003	90	0.4
V	PO_4	6.80	2.9	.008	.021	--	- .002		3.1
VI	PO_4	6.85	6.3	.002	.020	--	- .001		2.9
VIII	PO_4	6.83	3.8	.024	.038	--	- .002	48	5.6
XI	PO_4	6.75	5.9	.013	.018	--	.003	48	2.6
				ΔN 120 hrs	ΔN	hrs			
XXI	PO_4	5.30	11.3	.030	.024	192	.004	144	3.5
XXI	NO_3			.006	.007	192	.004	144	1.5
XXII	PO_4	6.20	6.1	.022	.038	192	.008	144	5.6
XXII	NO_3			.005	.008	192	.005	144	1.7
XXIII	PO_4	5.69	3.0	.033	.044	192	.004	144	6.5
XXIII	NO_3			.018	.008	192	.005	144	1.7
				ΔN 24 hrs	ΔN	hrs	ΔN Control	hrs	
Spinach	PO_4			.028	.165	96	.014		49.5
Swiss Chard	PO_4			.218	.230	96	.015		68.0
Bee	PO_4			.267	(estimated 78% exchange)		.008	24	78.0

See last page of Table VI for experimental details

TABLE VI (Cont'd)

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* The numbers shown are the averages of four to six readings on the sample.

** The soil and the solutions were autoclaved separately, then combined under aseptic conditions for the incubation period.

Conditions: 1.5g of soil was moistened with 0.5 ml of phosphate (pH 7.0, 51.6 A% XS) nitrate (pH 7.0, 80 A% XS) or sulfate (pH 7.0, 13.9 A% XS). None of the samples with sulfate showed detectable exchange. Therefore, the results with sulfate are not shown. At the end of the incubation period water was recovered from the sample by reduced pressure distillation at room temperature, one tenth volume of 1M Na_2SO_4 (as an electrolyte) added and the water electrolyzed in the inlet vessel. Where glucose and yeast extract were added the solutions were autoclaved and the final concentration of each was 0.3%. The theoretical values were calculated from the known A% XS of the anions and the exchangeable oxygen in the soil (Table VII).

TABLE VI (concluded)

These soil samples were collected at random from local sources, and for each determination a 0.500 g sample was weighed out, 200 µl of 0.1 M $Na_2HPO_4^{18}$ (94.4 A% xs) or /0.1 M $NaNO_3$ (84 A% 18) added, one tube sterilized at 123°C for 18 min. and after the times noted, a small water sample collected under vacuum distillation. Each water sample was then electrolyzed and the $O_2^{16}/O^{16}O^{18}$ ratios measured.

For each soil sample the pH and the moisture content were determined, (1:1 soil-H_2O, and the filtrate measured) (samples dried overnight @ 70°C in a vacuum oven).

The plant material was ground, pressed through cheese cloth, and 200 µl of the extract mixed with 200 µl of 0.1 M Na_2HPO_4, and samples collected and determined as above.

For the bee experiment, two honey bees were ground, equal parts placed in two flasks, 200 µl 0.1 M Na_2HPO_4 (94.4 A% XS) added and the samples run as above.

TABLE VII

EXCHANGEABLE OXYGEN IN THE SOIL

Weight of Soil	1.50g
Soil moisture 16%	
Weight of water in Soil	0.24g
H_2O^{18} added	0.50g
A% XS of H_2O^{18} added = 1.175	
A% XS of H_2O recovered = 0.610	
H_2O equivalent of total exchangeable oxygen present = 0.50 x 1.175/0.610 = 0.96g	
H_2O equivalent of non-water exchangeable oxygen = 0.96 - (0.50 + 0.24) = 0.22g	

The 0.22g water equivalent of exchangeable oxygen not accounted for as water removed at 105°C is presumed to be tightly bound water of hydration and silicate oxygen.

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APPENDIX (for Part I)

Bromate

Reference:

(1) Anbar, et al. Ea = 14,600 (0° - 60°) pH 1.0-2.3 (BrO_3^-) .15 - 1.0

(38) Hoering, et al. Ea = 14,230 (20 - 35°)(H^+) .01 - 1.00 (BrO_3^-) .04 - .10

$R = k(H^+)^2(BrO_3^-)$

	1		38 (calcd)	
k_o	4.8×10^{-2}	$l^2/mole^2$ min.	(obs.)k_o	4.92×10^{-4} $l^2/mole^2$ sec.
k_{25}	4.65×10^{-1}	"	(obs.)k_{25}	4.44×10^{-3} "
k_{70}	1.05×10	"	(calcd)k_{70}	1.04×10^{-1} "

T °C	pH	1.	4.
0	2	4.33×10^{5}	7.03×10^{5}
0	7	4.33×10^{15}	7.03×10^{15}
0	10	4.33×10^{21}	7.03×10^{21}
25	2	4.47×10^{4}	7.80×10^{4}
25	7	4.47×10^{14}	7.80×10^{14}
25	10	4.47×10^{20}	7.80×10^{20}
70	2	1.98×10^{3}	3.33×10^{3}
70	7	1.98×10^{13}	3.33×10^{13}
70	10	1.98×10^{19}	3.33×10^{19}

(38) Cl^- catalysis: $R = (BrO_3^-)(H^+)^2(k_1 + k_2(Cl^-))$

@ 30° ⟶ $k_2 = 3.6 \times 10$ $k_1 = 5.6 \times 10^{-1}$ $l^2/mole^2$ min.

so that @ $(Cl^-) = .01$ the rate would only double.

k_2 Ea = 13,900.

Note: Hoering (18) later reported Ea = 13,600, but has no reference or data to support it.

Reference: 38

For oxidation of X^- by BrO_3^-,

$R = (H^+)^2(BrO_3^-)\ [96.6\ (I^-) + 5.00\ (Br^-) + 1.5 \times 10^{-3}\ (Cl)]$

time in seconds at 25°C.

If $(Cl^-) = .01$

$R = 1.5 \times 10^{-5}(H^+)^2(BrO_3^-)$

i.e., slower than the exchange.

If $(Br^-) = .01$

$R = 5 \times 10^{-2}(H^+)(BrO_3^-)$

i.e., 8 times faster than the exchange.

Reference: 35

$NaBrO_3$ in H_2O for 9 hrs at 95-100° gave 14% exchange

$NaBrO_3$ in H_2O for 48 hrs at 95-100° gave 61% exchange

$NaBrO_3$ in NaOH for 9 hrs at 95-100° gave 24% exchange.

Chlorate

Reference: 39

$Ea = 27{,}100\ (85 - 100^{\circ})$

$R = k(H^{+})^{2}\ (ClO_3^{-})$

$k_o = 1.24 \times 10^{-9}\ l^2/mole^2\ sec$ (calcd.)

$k_{25} = 8.17 \times 10^{-8}\ l^2/mole\ sec$ (calcd.)

$k_{70} = 3.29 \times 10^{-5}\ l^2/mole^2\ sec$ (calcd.)

Concentration range (H^{+}) .09 - .80 (ClO_3^{-}) .50.

T °C	pH	T 1/2 in min.
0	2	2.79×10^{11}
0	7	2.79×10^{21}
0	10	2.79×10^{27}
25	2	4.23×10^{9}
25	7	4.23×10^{19}
25	10	4.23×10^{25}
70	2	1.05×10^{7}
70	7	1.05×10^{17}
70	10	1.05×10^{23}

Reference: 36

$ClO_3 = 0.59$ M

T 1/2 = 13 hrs.

Observed exchange

HCl = 0.14 M at 25°

(calcd. from k above T 1/2 = 3.38×10^{4} hrs.

calcd. from R below T 1/2 = 4.51×10^{3} hrs.)

$ClO^3 = 1.0$ M

HCl = 1.95 M at 25° T 1/2 = 3.4 hr.

(calcd. from k above T 1/2 = 1.35 hr.

calcd. from R below T 1/2 = 1.64 hr.)

Chlorate (Cont'd.)

Reference: 39

The oxidation of halides by $ClO_3^- (4H^+ + 2Cl)_3^- + 2Cl^- \longrightarrow 2ClO_2 - Cl_2 + 2H_2O$)

(ClO_2 is removed by reaction with arsenous acid)

at 25°C.

a. $R = 4.5 \times 10^{-5}(H^+)^2(ClO_3^-)(Cl^-)$

b. $R = 1.6 \times 10^{-9}(H^+)^2(ClO_3^-)(H_2O)$

therefore a. is 5 times faster than b.

(at (Cl) = .01) time in seconds

$R = 9.3 \times 10^{-5}(H^+)^2(ClO_3^-)(Br^-)$

$R = 2.0 \times 10^{-3}(H^+)^2(ClO_3^-)(I^-)$.

Reference: 71

K ClO_3 - no exchange in 65 hr. at 20° with 1.175 N - NaOH

at 100°, 1.06 N - NaOH for 43 hrs. max. is 10% exchange.

In 1.028 N H_2SO_4, at 100° in 37.5 hrs., 100% exchange.

In H_2O, no exchange in 289 hrs. at 20° or 22.5 hrs. at 100°.

Reference: 35

$NaClO_3$ in H_2O for 5 hrs. at 95-100° gave 0% exchange

$NaClO_3$ in NaOH for 23 hrs. at 95-100° gave 7% exchange.

Nitric Acid

Reference: 1

$Ea = 21.3$ k cal mole (70 - 100°)

$R = k(H^+)(HNO_3)$

$k_o = 9.9 \times 10^{-9}$ 1/mole min (calcd.)

$k_{25} = 2.6 \times 10^{-7}$ 1/mole min (calcd.)

$k_{70} = 3.6 \times 10^{-4}$ ($\pm$ 0.4) 1/mole min (observed at (HNO_3) 1.00 - 6.76)

$K_o = 48$ $K_{25} = 27.5$ $K_{70} = 15$

Table of T 1/2

°C	pH	T 1/2
0	2	10^{14} min.
0	7	10^{24} min.
0	10	10^{30} min.
25	2	2.2×10^{12}
25	7	2.2×10^{22}
25	10	2.2×10^{28}
70	2	8.66×10^{8}
70	7	8.66×10^{18}
70	10	8.66×10^{24}

Catalytic effects:

$Ea(Cl) = 21.8$

$$R = [H^+][HNO_3]\ (k_1 + k_2(Cl^-))$$

@ 100° therefore at .01 M, $k_1 = 4.8 \times 10^{-3}$ $k_2 = 3.2 \times 10^{-1}$

$k_1 + k_2\ (.01) \cong$ twice the rate.

63. $NaNO_3$ in H_2O for 5 hrs. at 95-100° gave 1% exchange

$NaNO_3$ in NaOH for 12 hrs. at 95-100° gave 0% exchange

$NaNO_3$ in HNO_3 for 12 hrs. at 95-100° gave 3% exchange.

Nitrous Acid

Reference: 14

0° No buffer

$$R = k(H^+)(HNO_2) \qquad k = 230 \text{ l/mole sec. (obs.)}$$

$$K_{HNO_2} = 3.2 \times 10^{-4} \qquad (NO_2^-) = .01 \text{ (initial)}$$

pH	R	T 1/2
2	2.23×10^{-2}	1.03×10^{-3} min.
7	7.2×10^{-11}	3.20×10^{6} min.
10	7.2×10^{-17}	3.2×10^{12} min.

Reference: 12

0° Acetate buffer

$$R = k/K\ (H^+)^2(NO_2^-)(OAc^-)$$

$$k = 5 \times 10^{-3} \frac{l^2}{\text{moles}^2 \text{ sec.}} \qquad \text{(obs.)} \quad K = 3.2 \times 10^{-4}$$

$$(OAc) = (NO_2^-) = .01 \text{ (initial)}.$$

Concentration range pH 4.6 - 6.0 (NO_2^-) .02 - .17 M

pH	R	T 1/2
2	4.84×10^{-3}	4.76×10^{-2} min.
7	1.56×10^{-11}	1.48×10^{7} min.
10	1.56×10^{-17}	1.48×10^{13} min.

Nitrous Acid (Cont'd.)

Reference: 2

25° Phosphate buffer

$$R = k(H^+)^2(NO_2^-)$$

$$k = 2.6 \times 10^8\ l^{-2}/mole^2\ min.\ (obs.)$$

Concentration range pH 4.3 - 8.0 (NO_2^-) .005 - .5

pH	R	T 1/2
2	2.60×10^2	5.33×10^{-5} min.
7	2.6×10^{-8}	5.33×10^5 min.
10	2.6×10^{-14}	5.33×10^{11} min.

Reference: 10

In unbuffered solution, addition of perchlorate and nitrate have no effect, but chloride and bromide enhance the rate of exchange, effectively doubling the rate by using concentrations of halide approximately equivalent to that of the nitrous acid.

Benzoic Acid

Reference: 9

Ea = 16,400 (73 - 101°). Concentration range (H-) 0.4 - 3.0 M

$k_o = 3.2 \times 10^{-5}$ l/mole min. (calcd.)

$k_{25} = 3.9 \times 10^{-4}$ (calcd.)

$k_{70} = 1.5 \times 10^{-2}$ (calcd.)

Temp (°C)	pH	T 1/2 (Min.)
0	2	4.3×10^{4}
0	7	4.3×10^{9}
0	10	4.3×10^{12}
25	2	3.6×10^{3}
25	7	3.6×10^{8}
25	10	3.6×10^{11}
70	2	9.2×10
70	7	9.2×10^{6}
70	10	9.2×10^{9}

Acetic Acid

Reference: 49

Ea for k^1 = 15300 (25°-123°)

Ea for k^3 = 23260 (101-123°)

Concentration range 10^{-11} - 4 M

$k_0^1 = 1.01 \times 10^{-6}$ $l^2/mole^2$ sec. (calcd.)

$k_{25}^1 = 1.00 \times 10^{-5}$ $l^2/mole^2$ sec. (obs.)

$k_{70}^1 = 3.15 \times 10^{-4}$ $l^2/mole^2$ sec. (calcd.)

$k_0^3 = 6.41 \times 10^{-3}$ $l^2/mole^2$ sec. (calcd.)

$k_{25}^3 = 1.19 \times 10^{-1}$ $l^2/mole^2$ sec. (calcd.)

$k_{70}^3 = 2.05 \times 10$ $l^2/mole^2$ sec. (calcd.)

$K_0 = 1.87 \times 10^{-5}$ $K_{25} = 1.76 \times 10^{-5}$ $K_{70} = 2.28 \times 10^{-5}$

$$R = f[k^1(H^+) + k^2 + k^3 \frac{(OH^-)}{(H_2O)}]$$

$$k^2 = 0 \qquad f = \frac{[HOAC]}{[OAc^-]}$$

Temp. (°C)	pH	T 1/2 (min)
0	2	2.29×10^4
0	7	3.74×10^9
0	10	3.75×10^9
25	2	2.31×10^3
25	7	1.91×10^8
25	10	1.91×10^8
70	2	7.35×10
70	7	1.43×10^6
70	10	1.42×10^6

PART II - CHEMOSYNTHESIS

Chemosynthesis:

Studies were initiated to investigate the biochemistry of the energy metabolism of the chemosynthetic bacteria, a form of life which thrives entirely on the energy of combustion of inorganic compounds. The interest in such investigations arose because of the possible similarities of the extraterrestrial life forms to the terrestrial chemosynthetic microorganisms capable of building up their bodies by reducing atmosphereic carbon dioxide. The results obtained during the past year have been reported in the following publications:

1. N. E. R. Campbell, and M. I. H. Aleem, 1965. The Effect of 2-chloro, 6-(trichloromethyl) pyridine on the chemoautotrophic metabolism of nitrifying bacteria. I. Ammonia and hydroxylamine oxidation by Nitrosomonas. Antonie van Leeuwenhoek, 31: 124-136.
2. N. E. R. Campbell and M. I. H. Aleem, 1965. The Effect of 2-chloro, 6-(trichloromethyl) pyridine on the chemoautotrophic metabolism of nitrifying bacteria. II. Nitrite oxidation by Nitrobacter. Antonie van Leeuwenhoek, 31: 137-144.
3. M. I. H. Aleem, 1965. Path of carbon and assimilatory power in chemosynthetic bacteria. I. Nitrobacter agilis. Biochim. Biophys. Acta, 107: 14-28.
4. M. I. H. Aleem, G. E. Hoch and J. E. Varner, 1965. Water as the source of oxidant and reductant in bacterial chemosynthesis. Proc. Natl. Acad. Sci. (U.S.), Sept., 1965.

5. M. I. H. Aleem, 1965. Generation of reducing power in chemosynthesis. II. Energy-linked reduction of pyridine nucleotides in the chemoautotroph, Nitrosomonas europaea. Biochim. Biophys. Acta. (In Press).

6. G. E. Becker, M. I. H. Aleem and A. Nason, 1965. Further studies of nitrite oxidase particles from Nitrobacter agilis. Bacteriol. Proc., p. 95.

7. M. I. H. Aleem, 1965. Chemosynthesis and assimilatory power in Thiobacillus novellus. Bacteriol. Proc., p. 10.

8. M. I. H. Aleem, 1965. Thiosulfate oxidation and electron transport in Thiobacillus novellus. J. Bacteriol., 90: 95-101.

9. M. I. H. Aleem and E. Huang, 1965. Carbon dioxide fixation and carboxydismutase in Thiobacillus novellus. Biochem. Biophys. Res. Comm., 20: 515-520.

10. M. I. H. Aleem, 1965. Generation of reducing power in chemosynthesis. III. Energy-linked reduction of pyridine nucleotides in Thiobacillus novellus (autotrophic). J. Bacteriol. (In Press).

The results obtained may be divided into two broad aspects: 1) Nitrogen metabolism and, 2) Sulfur metabolism.

NITROGEN METABOLISM: The studies were limited to the nitrifying bacteria, Nitroscmonas and Nitrobacter. In case of Nitrosomonas the over-all primary energy yielding reaction is as follows:

$$NH_4^+ + 1\ 1/2\ O_2 \longrightarrow NO_2^- + H_2O + 2H^+ + 84\ \text{K. cal.}$$

The K cal. represent the free energy made available by the above reaction. The organism fulfills its growth requirements for carbon dioxide assimilation from the energy trapped from ammonia oxidation involving hydroxylamine as an intermediate. Since CO_2 fixation by the autotrophic bacteria is a reductive endergonic process, these organisms must, of necessity, generate reduced pyridine nucleotides and ATP coupled to their primary energy-yielding reactions. In the case of Nitrosomonas the coupling of the oxidations of ammonia to hydroxylamine ($E_o' = 0.89$ volt) or of the latter to nitrite ($E_o' = 0.450$ volt) with the reduction of pyridine nucleotides ($E_o' = -0.32$ volt) would not be achieved unless energy is invested to drive the electrons, denoted by ammonium or hydroxylamone, to the level of pyridine nucleotides:

$$2NH_4^+ + 2PN^+ + H_2O \longrightarrow 2PNH_2 + 2NH_2OH \quad \Delta F = +40 \text{ K. cal.}$$

$$NH_2OH + 2PN^+ + H_2O \longrightarrow 2PNH + 3H^+ + NO_2^- \quad \Delta F = +110 \text{ K. cal.}$$

The results reported in Ref. 5 indicate that the reduction of pyridine nucleotides in Nitrosomonas is energy-dependent. The ATP-dependent reversal of electron transfer occurred from ferrocytochrome c and the pyridine nucleotide reduction took place concomitantly with the oxidation of the reduced cytochrome c. The reversed electron flow was markedly sensitive to atabrine and amytal and involved flavoprotein systems. By blocking ATP synthesis with oligomycin in the presence of hydroxylamine or succinate as electron donors, the trapped energy in the cytochrome region could also be utilized for the pyridine nucleotide reduction. Uncoupling agents of oxidative phosphorylation such as 2,4-dinitrophenol or m-chlorocarbonylcyanide

phenylhydrazone completely inhibited the ATP-dependent pyridine nucleotide reduction; these uncouplers also inhibited the reduction of pyridine nucleotides driven by the high-energy intermediates generated within the respiratory chain.

Studies were also conducted concerning the metabolic control of the Nitrifying bacteria, Nitrosomonas and Nitrobacter using 2-chloro, 6-(trichloromethyl) pyridine (Ref. 1 and 2). This chemical was found to be a potent inhibitor of the ammonium oxidizing systems. The hydroxylamine or nitrite oxidizing systems were not affected to a great extent. The inhibition of ammonium oxidation was completely reversed by copper. The metal was also effective in reversing the inhibition of the cytochrome oxidase in these organisms. On the bases of these results it would appear that copper might be involved in the nitrogen oxidation process.

The results concerning the energy metabolism of the chemoautotroph Nitrobacter have been reported in Ref. 3, 4, and 6. The pertinent reactions catalyzed by the organism are as follows:

(1) $NO_2^- + n\ ADP + nP_i + 1/2\ O_2 \xrightarrow{\text{cytochrome electron transport chain}} NO_3^- + nATP$

(2) $NAD^+ + 2H^+ + 2\bar{e} + Energy \longrightarrow NADH + H^+$

(3) $NADH + H^+ + ADP + Pi + 1/2\ O_2 \longrightarrow NAD + ATP + H_2O$

(4) $x.ATP + y.NADH_2 + CO_2 \longrightarrow cell\ stuff + x.ADP + x.P_i + yNAD^+$

Nitrite oxidation is coupled with the reduction of cytochrome c which is oxidized by molecular oxygen (through cytochrome oxidase) with the formation of ATP, or by pyridine nucleotide (through the lower half of the electron transport chain) with the utilization of ATP.

It has been found that the oxygen atom incorporated during the oxidation of nitrite to nitrate arises from water. However, although a slight but far from complete exchange of oxygen atoms occurs between nitrite and water, no exchange has been found to occur between nitrate and water. In addition, water also served as a source of hydrogens for the pyridine nucleotide reduction. In view of these results it may be proposed that nitrite or its activated form is hydrated prior to dehydrogenation. The over-all stoichiometry of the reaction is presented by the following equation:

$$H_2O.NO_2^- + A \rightleftharpoons NO_3^- + AH_2.$$

Where "A" represents a flavin or a pyridine nucleotide. The ATP and reduced pyridine nucleotide thus generated (reaction 4) drive the endergonic CO_2 reduction involving reactions of the carbon reduction cycle (see Ref. 3).

SULFUR METABOLISM. Studies were undertaken to investigate the biochemical mechanisms involved in thiosulfate oxidation catalyzed by a facultative autotroph, Thiobacillus novellus. It has been possible for the first time to demonstrate the complete oxidation of thiosulfate to sulfate catalyzed by cell-free extracts of the bacterium without the addition of various artificial electron carriers. The extracts oxidized thiosulfate according

to the following equation:

$$Na_2S_2O_3 + 2\ O_2 + H_2O \longrightarrow Na_2SO_4 + H_2SO_4$$

The thiosulfate oxidase activity resides in the soluble cell-free fractions which could catalyze the enzymatic transfer of electrons from thiosulfate to molecular oxygen through cytochrome *c*, *a* and a_3 - like components. The cell-free extracts also catalyze formate oxidation involving similar electron transport pathway (see Ref. 8). The level of thiosulfate or formate-activating enzymes as well as the respective oxidases is abolished in the cell-free extracts obtained from heterotrophically grown organism. Similarly the activity of carboxydismutase as well as of the carbon dioxide reducing enzymes disappears under heterotrophic growth conditions although the level of phospho-enol-pyruvate carboxylating enzyme remains virtually unchanged under the autotrophic or heterotrophic growth conditions. In the autotrophic cells and extracts either thiosulfate or ATP and NADH provide energy and reducing power for CO_2 reduction and assimilation and the pattern of carbon dioxide fixation resembles the autotrophic carbon dioxide reduction cycle (Ref. 7 and 9).

The cell-free extracts of Thiobacillus novellus would also oxidize sulfite to sulfate. Assuming that sulfur oxidation is coupled to pyridine nucleotide reduction, such a process would require an input of energy as indicated by the following equation based on the free energy values of Gibbs and Schiff (1960):

$$SO_3^{-2} + H_2O + PN^+ \longrightarrow SO_4^{-2} + PNH + H^+ \quad \Delta F = 33.7 \text{ K. cal.}$$

The results reported in Ref. 10, provide the first experimental demonstration of an energy-dependent reduction of pyridine nucleotides in a member of the genus Thiobacillus. The process of ATP dependent reversal of electron transfer occurs with a remarkable efficiency in this organism. The exogenous electron donor employed in this system was either thiosulfate or mammalian ferro-cytochrome c. In the latter case, oxidation of ferro-cytochrome c occurred under anaerobic or non-respiring conditions with concomitant reduction of pyridine nucleotides. The values calculated for the molar ratios of ATP utilized to the pyridine nucleotide reduced and of cytochrome c oxidized to the $NADP^+$ reduced were 1:1 and 2:1 respectively. Assuming that the energy-linked reversed electron transfer is effected from the cytochrome c level, then by analogy to the animal mitochondrial system, it would be expected that at least 2ATP equivalents should be required per equivalent of pyridine nucleotide reduced. It seems probable therefore, that one high-energy compound is generated within the electron transport chain below the level of cytochrome c with thiosulfate as an electron donor. The actual site of this energy coupling step is not known at present but indications are that either a cytochrome component of b type or a non-heme iron is involved in the process. The energy-dependent reduction of pyridine nucleotides in T. novellus is inhibited by atabrine or amytal implicating the possible participation of a flavin in reversed electron flow. The process is also inhibited by low concentrations of uncouplers of oxidative phosphorylation such as carbonyl cyanide m-chlorophenylhydrazone and 2,4 dinitrophenol.

Evidence has also been obtained that the reduced pyridine nucleotides are essential for providing the reducing power to effect the energy-dependent reduction of carbon dioxide in Thiobacillus novellus.