

AN ABSTRACT OF THE THESIS OF

LEROY HUGH WULLSTEIN for the Ph.D. in MICROBIOLOGY
(Name) (Degree) (Major)

Date thesis is presented November 24, 1964

Title THE NON-ENZYMATIC FORMATION OF NITRIC OXIDE FROM SOIL AND
CLAY SYSTEMS

Abstract approved Redacted for Privacy
(Major Professor)

Prevailing concepts relating to the non-enzymatic gaseous loss of nitrite from soil are thought to be inadequate as regards to the mechanisms involved. The conventional explanation for nitric oxide formation i.e., the acid decomposition of nitrite, is held to be insufficient in substrates of only mild or slight acidity. A criticism is presented wherein it is maintained that certain past practices in methodology have created rather than eliminated confusion. Positive suggestions are proposed which may help to eliminate confusion in future experiments.

Evidence was obtained from soil and clay systems which attest to a mechanism of loss whereby nitrite may react with certain metals to yield nitric oxide as a primary gaseous product. A conventional half-cell reaction is presented wherein a transition-like metal is oxidized and nitrite is reduced to nitric oxide with the formation of water. It is implied that hydrogen ions may be necessary--acid conditions favor the reduced state of the metals which is a more active state with respect to the formation

of nitric oxide--for such reactions, but that they are not sufficient in substrates of only mild acidity.

An apparent corollary to this study is that the very conditions which favor classical denitrification from nitrate also favor metal-nitrite reactions. Under such conditions both nitrate and the metals involved would be expected to undergo reduction resulting in an increased possibility for nitric oxide formation. This possibility places in question the status of nitric oxide as a direct biochemical intermediate in the microbial formation of nitrogen gas.

It is suggested that a mechanism involving transition-like metals may account for significant losses of mineral nitrogen from field soils capable of accumulating nitrite under conditions of moderate or slight acidity.

THE NON-ENZYMATIC FORMATION OF NITRIC OXIDE FROM SOIL
AND CLAY SYSTEMS

by

LEROY HUGH WULLSTEIN

A THESIS

submitted to

OREGON STATE UNIVERSITY

in partial fulfillment of

the requirements for the

degree of

DOCTOR OF PHILOSOPHY

June 1965

APPROVED:

Redacted for Privacy

Professor of Microbiology

In Charge of Major

Redacted for Privacy

Chairman of Department of Microbiology

Redacted for Privacy

Dean of Graduate School

Date thesis is presented November 24, 1964

Typed by Ruth G. Baines

ACKNOWLEDGEMENTS

The author is deeply appreciative of the sincere guidance and enthusiastic support given by Dr. C. M. Gilmour, Major Professor.

In more ways than they realize this student is indebted to his Graduate Committee, Drs. W. B. Bollen, W. W. Chilcote, J. L. Young and C. T. Youngberg.

Without the companionship and patience of my wife this work would never have been so satisfying.

TABLE OF CONTENTS

	Page
INTRODUCTION	1
HISTORICAL	3
The Current Trend	3
The Criticism	6
Nitrite Concentration	6
Direct Methods Versus Indirect Methods	7
Temperature and Desiccation	8
Sterilization and Assimilation	8
PROCEDURES AND METHODS	11
Preparation and Collection of Soil Samples	11
Percent Moisture	11
Moisture Capacity	11
Substrate pH	12
Mineral Nitrogen	12
Extraction Procedures	12
Mineral Nitrogen	12
Metallic Ions	13
Precipitation of Metallic Ions	13
Determination of Metallic Ions	13
Recovery and Determination of Nitrogen Oxides	13
Substrates and Substrate Preparations	17
Modified Respirometer Flask	17
Nitrification Experiment	18
EXPERIMENTAL RESULTS AND DISCUSSION	20
Detection of Nitric Oxide	20
Soil-Sand Studies	21
Clay Studies	23
Role of Clay Extracts	26
Role of Soil Extracts	29
Effect of pH	32
Evaluation of Soil N Losses	32
Redox Potential	36
Effect of Calcium	39
Nitrification and N Loss	40

	Page
CONCLUSIONS AND SUMMARY	42
BIBLIOGRAPHY	45

LIST OF FIGURES

	Page
I. Flow diagram of extraction procedure	15
II. Modified respirometer flask	19
III. Effect of increasing soil/sand ratios on the formation of nitric oxide	22
IV. Nitric oxide formation from clay and clay extract	25
V. Nitric oxide formation from clay extract and treated clay extract (H_2S)	28
VI. Effect of calcium on the formation of nitric oxide from clay	38

LIST OF TABLES

	Page
I. Semi-quantitative estimates for elements detected in clay extract	27
II. Nitric oxide formation from soil extracts	31
III. Effect of pH and $\text{FeSO}_4\text{-Fe}$ on the evolution of nitric oxide	33
IV. Nitric oxide formation from soils amended with nitrite	35
V. Influence of ferric versus ferrous iron in NO evolution	37
VI. Observed nitrogen losses with urea and ammonium sulfate	41

THE NON-ENZYMATIC FORMATION OF NITRIC OXIDE FROM SOIL AND CLAY SYSTEMS

INTRODUCTION

Numerous investigators have reported significant losses or deficits of mineral nitrogen in soil amended with both organic and inorganic carriers. Allison's now classical review The Enigma of Soil Nitrogen Losses (3, p.213-250) leaves little doubt that such losses are real and constitute a serious economic problem to American agriculture. Academically, however, the primary concern and properly so, is one of elaborating the mechanisms responsible for or contributing to the losses encountered. In some instances modes or pathways of losses have been proposed and substantiated by concrete evidence. In other cases fundamental theoretical considerations have been proposed and substantiated by concrete evidence. In other cases fundamental theoretical considerations have been lacking or if entertained at all have been only weakly supported. Thus the "enigma" remains to be eliminated.

Generally soil microbiologists have agreed that the microbial reduction of nitrate and/or nitrite to nitrogen gas (denitrification) constitutes the major pathway of gaseous nitrogen loss from agricultural soils. On the other hand, there is increasing evidence that what appear to be strictly non-enzymatic routes of loss may be responsible for a greater percentage of loss than previously

supposed. The various mechanisms usually considered to be involved in both enzymatic and non-enzymatic pathways have been summarized and discussed recently (19, p. 47-51). While these will not be recapitulated here it is worthwhile to emphasize that the most puzzling types of deficits are those involving the nitrite ion when found in well aerated soils of only slight or moderate acidity. In the conventional sense then it would seem that the mechanism(s) in operation is non-enzymatic and yet entails something more than direct acid decomposition. It was the investigation for just such a mechanism that led to the experiments which are to be presented in this thesis.

HISTORICAL

Since Allison (3, p.213-250) has adequately reviewed the nitrogen loss field up to 1955, and since Gilmour and Wullstein (19, p.47-51) discussed the most pertinent work published between 1955 and 1962 with the exception of some of Clark's work (14, p.501-508), it would be redundant to give another literature review at this time. However, a consideration of the most recent trend, as alluded to in the introduction, will be taken up. This is to be followed by a criticism of certain past practices which, in the writer's opinion, have prevailed too long in our methodology.

The Current Trend

In 1960 Clark and his co-workers (15, p.50-54) presented data indicating rather large nitrogen deficits could occur in soil amended with either urea or potassium nitrite. The position was taken that the observed losses were non-enzymatic. That is, nitrite produced oxidatively or added directly to soil was lost as gaseous nitrogen by non-biochemical means. The soil systems studied were considered to be oxidative and the largest deficits occurred in those of only moderate or slight acidity. Prior to this work it had been generally accepted that the non-enzymatic gaseous loss of nitrite required relatively strong acidic substrates i.e., below pH 5.0 or 4.5, depending upon the authority (1, p.166; 2, p.313-314; 35, p.160-163). Thus there was good

reason to suspect that some unknown factor(s) or mechanism was involved in the losses reported by Clark. While he later suggested (14, p.501-508) that organic matter was related to such losses, he did not propose any chemical mechanism or pathway through which organic matter might react with nitrite. He was of the opinion, however, that amino groups were not reacting with the nitrite to produce nitrogen gas, presumably because the pH values of his substrates were 4.5 or higher.

Chao and Kroontje (13, p.44-47) discussed the relation of free energy changes to inorganic nitrogen oxidations. They stressed the importance of pH as it affects the form of a given nitrogen compound, which in turn has great bearing on the energy of formation of a given compound. They concluded that "In acid solutions nitrite will thermodynamically undergo three reactions: It may decompose to nitric oxide and nitrate, oxidize to nitrate, or be reduced to nitrous oxide." It will be remembered however that reactions which appear to be thermodynamically spontaneous do not necessarily proceed without the presence of some critical factor e.g., catalysis (37, p.13). With respect to nitrite in acid solutions, two specific points should be kept in mind. First, it is extremely important to know the form in which the nitrite exists or is added and second, it is not enough to know that a given solution or substrate is approximately acidic, that is, the acidity should be defined as accurately as possible (21, p.428-432). For example, it is well established that

prepared nitrous acid (HNO_2) spontaneously decomposes in open acidic solutions to yield nitric oxide, and therefore it seems some investigators have proposed just such a mechanism to explain their observed nitrogen losses. Indeed, where nitrous acid is formed one might expect it to decompose spontaneously. However, a very common practice has been to add potassium and sodium nitrite to soils of moderate acidity and to attribute a portion of the concomitant gaseous losses to the decomposition of nitrous acid with no mention being made of the conditions necessary to form the acid from the salts. Actually there is very little data on this point, although Wullstein and Gilmour (36, p. 428-430) observed little or no nitric oxide production when potassium nitrite was added to quartz sand which had been amended with hydrochloric acid (pH 4.0). Additional data that demonstrates that even stronger acidity is required to decompose potassium nitrite in acid/sand systems under standard conditions and in the absence of other reactants will be presented. It is also noteworthy that the sophisticated experiments of Cady and Bartholomew (11, p. 546-549) supported their hypothesis that the "conventional equation $3\text{NO}_2^- + 3\text{H}^+ \rightarrow \text{NO}_3^- + \text{H}^+ + 2\text{NO} + \text{H}_2\text{O}$ " does not sufficiently define the reactions involved in the formation of nitric oxide. More recently Allison (4, p. 408) stressed that additional fundamental research is needed on the reactivity of nitrite in soil.

Clearly, a new trend has arisen wherein the data has

accentuated the possibility that some unknown factor(s) or reaction(s) which seems to be necessarily involved in the decomposition of nitrite under conditions of moderate or slight acidity exist or occurs in some soils. The focal point in this trend has been the nitrite ion itself, and correctly so, for it is a key intermediate between the non-gaseous and gaseous states whether it arises via nitrification, denitrification, nitrate reduction direct addition, or by some anomalous route.

The Criticism

It is felt that a constructive criticism of some past practice in methodology is in order at this time. For even to the initiated there is considerable confusion in the literature. While some discrepancies are likely to persist without explanation, it should be made apparent that many differences in experimental technique can be resolved. The points to be made are brought out in an anonymous manner but are far more applicable to certain investigations of the last one or two decades than to the earlier studies which did not have the benefit of modern technology and financing.

Nitrite concentration: One of the most common practices has been that of amending substrates with preposterous amounts of nitrite. Instances are easily cited wherein investigators have added nitrite to substrates at rates equivalent to three hundred, five hundred, on up to several thousand ppm nitrogen. There can be justification for such high applications of certain

other nitrogen carriers but rarely for nitrite. Besides being a relatively active ion it is known to be toxic in low quantities to various life forms; between ten and 100 ppm as nitrogen may be toxic to certain plants (7, p. 17). Even under fertilization soils are rarely found that contain over a hundred ppm nitrogen in this form. Since assay methods for nitrite are sensitive enough to permit more conservative applications, it appears there is little or no basis for adding nitrite-nitrogen in excess of a hundred or perhaps two hundred ppm. Amendments of less than one hundred ppm would be even more realistic most of the time.

Direct Methods Versus Indirect Methods: Classically nitrogen losses have been based on indirect methods which measure the disappearance of added nitrogen and which are ordinarily expressed as deficits. Yielding, where leaching is unimportant and where assimilation is negligible or accountable for, that the larger portion of a given deficit is explained best as gaseous nitrogen loss such expressions obviously give little or no evidence as to the real nature of the gases evolved. In spite of this major limitation there has been a tendency to base theory on such data. Deficits are perhaps the most serious single pitfall confronting anyone who attempts to evaluate the literature. Indeed any theory which does not take into account the real nature of gaseous nitrogen products is highly questionable and may be better termed speculation. Since modern advances and newer knowledge are available to most investigators it is generally inexcusable to

forego gas analysis by such accepted methods as gas chromatography, infra-red spectrophotometry or simple absorption and assay in aqueous solution.

Temperature and Desiccation: In some investigations excessively high reaction temperatures have been employed. Certainly concepts based on experiments carried out at temperatures as high as fifty and seventy degrees centigrade should be carefully evaluated before applying any conclusions therefrom to systems operating under approximately standard conditions.

A related factor is that of desiccation or evaporation. Madhok and Uddin (22, p. 275-280) demonstrated that evaporation can greatly enhance the loss of nitrite from soil and sand substrates. Clark (14, p. 505-506) reemphasized this factor in the drying of samples containing nitrite prior to analysis. Still there has been a persistence in conducting experiments in open systems where effective moisture control becomes most difficult.

Sterilization and Assimilation: Certain studies have too quickly attributed losses to non-enzymatic pathways. This is particularly true where sterilization has been omitted. In its stead some investigators have trusted in the degree of reduction of certain ions e.g., nitrate, to be a faithful indicator of anaerobiosis and, as the reasoning goes, therefore a good indicator of denitrification. However, there is evidence that denitrification can occur in soil under "aerobic conditions"* (9,

*Authors' quotation marks

p. 129-137; 10, p. 359-363). Some of the most recent findings (20, p. 55-58) indicate a strain of Pseudomonas stutzeri is capable of using nitrate-oxygen and molecular oxygen simultaneously as hydrogen acceptors in "well aerated"* cultures. Superficially this latter work might be said to support that of Meiklejohn (23, p. 558-573), who was among the first to observe nitrogen reduction and concomitant gas production in "aerobic"* cultures. Meiklejohn, however, termed this observation "aerobic denitrification", which should be considered a misnomer by any conventional standard since denitrification, as such, implies the strictly anaerobic reduction of substrate nitrogen. If Meiklejohn was not experiencing aeration difficulties, then it appears she was observing something other than conventional denitrification. It is pertinent to mention here that Skerman and McRae (33, p. 215-230) and later McGarity et al. (26, p. 303-316) published data which gave no credence to the concept of "aerobic denitrification." The point is that much doubt and a good deal of argument would be eliminated if those concerned with the non-enzymatic reactions would include appropriate sterilization experiments in their studies.

A closely associated problem is that of assimilation. It can be demonstrated for example that assimilation values can reach significant proportions in nitrogen balance sheets (19, p. 50). Thus, when deficits are employed which are based only on mineral nitrogen determinations, it becomes difficult to assess

* Authors' quotation marks

accurately that portion of the deficit represented in cellular tissue. Even where total nitrogen values are obtained it is not a simple matter to achieve an accountable balance since the technique (Microkjeldahl) commonly employed has numerous modifications, and generally is not sensitive enough to assay less than fifty to one hundred ppm nitrogen in soil systems containing several thousand ppm nitrogen (10, p. 362).

Where both the sterilization factor and gas analyses are omitted, proper interpretation becomes a dubious challenge, if not impossible.

PROCEDURES AND METHODS

Preparation and Collection of Soil Samples

Soil samples were collected from the upper 6 inches, air dried, passed through a 10-mesh sieve and stored in closed glass containers at room temperature. The soils were obtained from sites which were considered to be free of major disturbances in recent times. Based upon the vegetative composition some of the collection sites were considered to be in near pristine condition (30, p. 1-166). The actual collection sites for the Woodburn, Lapine and Red Hill soil cannot be said to be undisturbed but have been judged to have received no known commercial fertilization in the case of the Lapine and Red Hill soils, and none in the last six or seven years in the case of the Woodburn soil. This information was commuted to the writer by members of the Farm Crops and Soils Departments, Oregon State University.

Percent Moisture

Determinations were made on 100-gram portions of soil. A rapid-drying "Moisture Teller" instrument was used for obtaining oven dry soil. In all analyses soil quantities were expressed on a water-free basis.

Moisture Capacity

Calculations were based on the amount of water held against

gravity. Soil placed in gooch crucibles was allowed to absorb water from beneath until saturated and then permitted to drain to a constant weight in a moisture saturated atmosphere.

Substrate pH

Measurements were made on 1:4 substrate suspensions in distilled water after shaking for 10 minutes and allowing a settling interval of 20 minutes. Readings were made with a model G Beckman pH meter.

Mineral Nitrogen

Nitrite and ammonium nitrogen (nesslerization) were determined by the American Public Health Association procedure (5, p. 246-247; p. 240-241). Nitrates were assayed for by the method of Eastoe and Pollard (16, p. 266-269). Nitrite interference in the latter test was prevented by the addition of sulfamic acid according to the technique of Bremner and Shaw (8, p. 28). Chlorides posed no serious problem since, when present, their concentration was below that shown to interfere (25, p. II-5). Photometric readings were made with a Bausch and Lomb "Spectronic 20" colorimeter. Appropriate standard curves can be found in the appendix.

Extraction Procedures

Mineral Nitrogen: The methods employed by McGarity were followed (25, p. 1-2).

Metallic Ions: The general scheme adopted is given in Figure I. Centrifugation was accomplished with a model RC-2 Servall centrifuge.

Precipitation of Metallic Ions

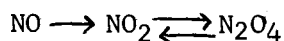
Copper and manganese were precipitated from the appropriate extracts with hydrogen sulfide and potassium hydroxide respectively, (32, p. 205-296; p. 429-430).

Determination of Metallic Ions

Metals were determined either by flame photometry or spectrophotometrically using a model DB Beckman spectrophotometer. Analyses were performed by personnel of the U. S. Bureau of Mines and the Wah Chang Corporation.

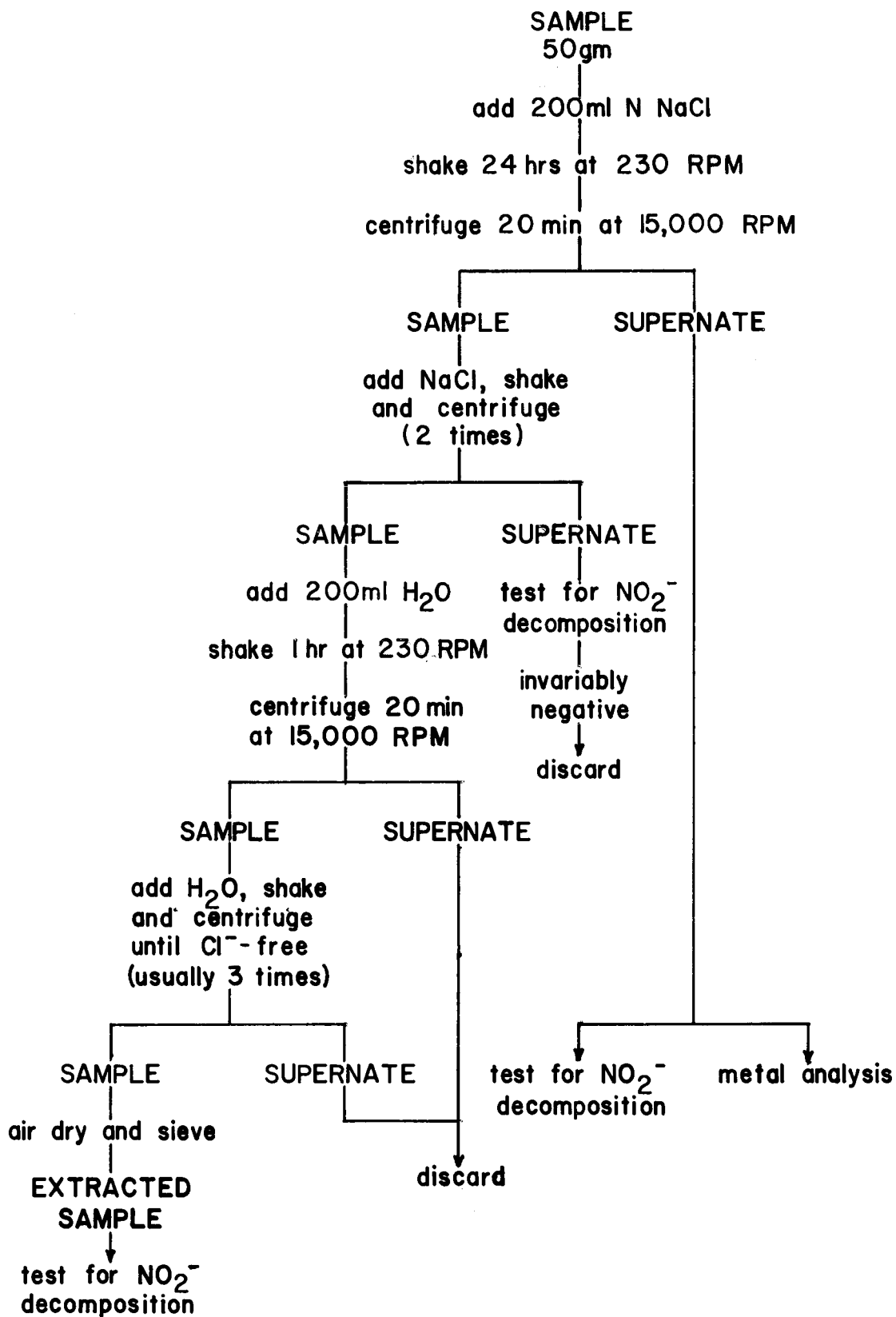
Recovery and Determination of Nitrogen Oxides

Nitric oxide (NO) formed in an aerobic atmosphere is normally oxidized to nitrogen dioxide (NO₂) and its dimer (N₂O₄) as shown in the following equation (18, p. 564-579).



Under standard conditions the above equilibrium is towards the right. With strict anaerobiosis the nitric oxide would not, of course, be oxidized. The oxides formed in the systems studied were absorbed in aqueous caustic solution (0.1% KOH) contained in vials placed within the reaction flasks (12, p. 1415-1422). The oxides thus absorbed form nitrite and nitrate (mostly nitrite)

Figure I. Flow diagram of extraction procedure



in the absorbent which were determined by volumetric assay to give a quantitative estimate of the sum of nitrogen oxides formed in the systems. Thus, by making use of base absorption phenomena, it was possible to employ a relatively simple, inexpensive and direct technique.*

In the systems under investigation it was demonstrated by differential absorption (29, p. 320) under anaerobic conditions-NO is only slightly soluble in water while NO₂ is quite soluble-that nitric oxide was the primary oxide produced. Gas chromatography data corroborated this conclusion, and furthermore demonstrated that nitrous oxide (N₂O) was not produced in measurable quantities under the conditions employed. It was then decided to adopt an expression of gaseous nitrogen loss based on the percent mineral nitrogen, added to the various substrates and recoverable as mineral nitrogen in the absorbent. Clearly this expression should not be construed to mean total gaseous nitrogen loss but may be regarded simply as a quantitative index of the nitrogen oxides formed. The importance of a precise differentiation of such indices was emphasized in the Historical section.

*By direct technique it is meant that the method employed measures the amount of gaseous product formed from a given substrate. In contrast an indirect technique is understood to be one which measures the extent of nitrogen deficit which may occur in a given substrate. See Historical section for further discussion.

Substrates and Substrate Preparations

Soil and clay substrates treated with nitrite were amended with aqueous potassium nitrite at rates approximating 50 percent of moisture capacity and less than 100 ppm nitrogen. A washed and ignited 10-20 mesh sand was employed. The clay was a kaolinite (#17) obtained from Ward's Natural Science Establishment Incorporated.

All soil samples were prepared for metal analysis by pulverization for 5 minutes in a mechanical mill "Shatterbox".

The extracted Woodburn soil was acidified to an appropriate pH value by suspending the soil in a solution of hydrochloric acid (pH 3.0) and stirring for 1 hour with an electric stirrer. The suspension was then evaporated to dryness at 60°C., sieved, and stored for future use.

In order to maintain an aerobic atmosphere above the substrates and to keep evaporation to a minimum, experiments were carried out in electrolytic respirometer flasks (26, p. 303-316).

Sterilization techniques included both autoclaving at 140°C. for 8 hours and gamma irradiation (6 megarads), using a procedure similar to that employed by Eno (17, p. 299). The actual irradiation was performed by the U. S. Bureau of Mines.

All studies were conducted at 30°C. ± 2°C. Additional details are presented with the individual experiments.

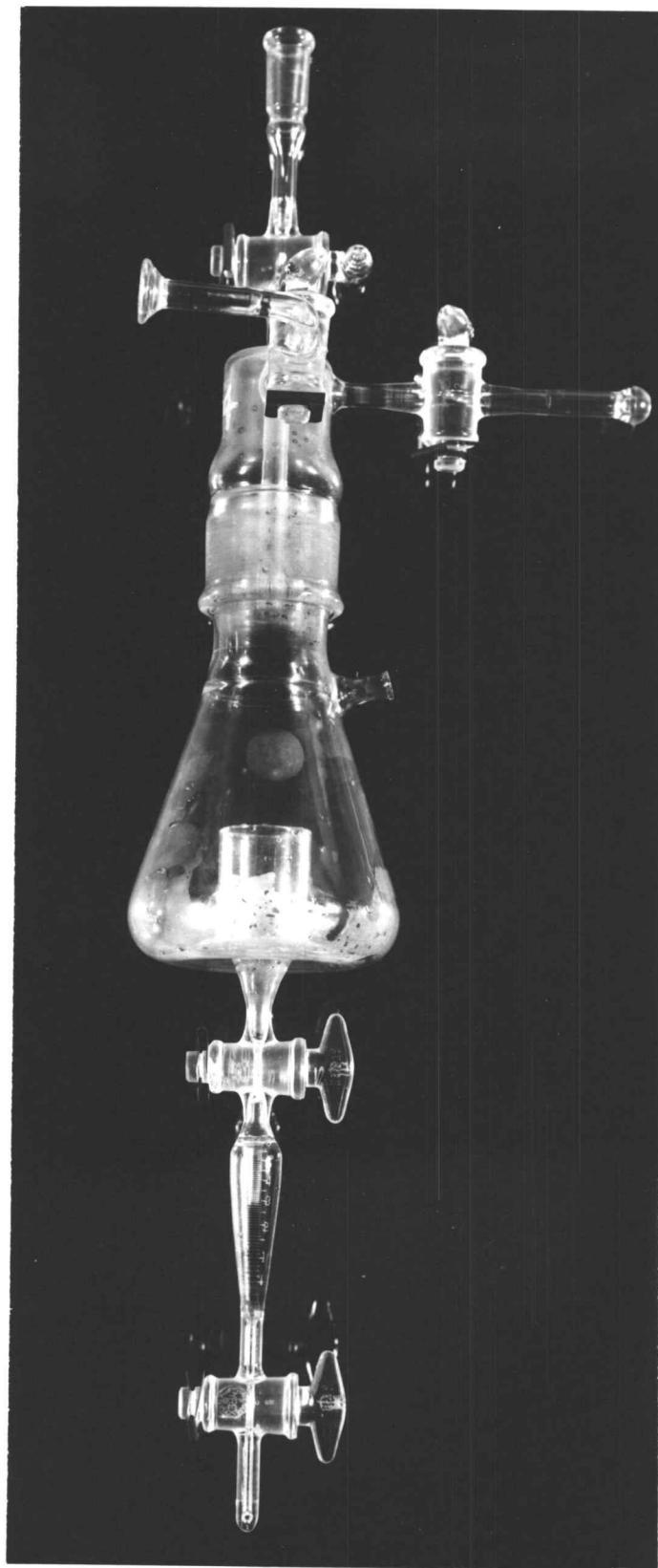
Modified Respirometer Flask

To facilitate frequent samplings of absorbent in the nitrification experiment, and to keep the number of required flasks to

At a minimum, a modified respirometer flask was constructed which allowed the taking of as many as five 2-ml samples in the course of six weeks' time. Figure II shows the modified unit which has, in addition to the standard flask, a sampling assembly welded into the flask base, connecting it to the center well. The assembly is constructed of a central, graduated, 10-ml centrifuge tube with a single stopcock placed between the tube and the flask proper, and another just below the tube. The upper stopcock allows the transfer of absorbent from the center well to the calibrated tube while measured aliquots are dispensed through the lower stopcock. A small opening in the upper portion of the adapted centrifuge tube, together with a slight positive internal flask pressure, ensures the free flow of liquid through the system. After each sampling the assembly should be rinsed with distilled water to remove any remaining absorbent.

Nitrification Experiment

A nitrification experiment was carried out in the modified respirometer flask to test the capacity of the Woodburn soil for producing nitric oxide from ammonium sulfate and urea amended at rates equivalent to 200 ppm nitrogen.



Upper stopcock

Modified
centrifuge
tube

Lower stopcock

Figure II. Modified respirometer flask

EXPERIMENTAL RESULTS AND DISCUSSION

Detection of Nitric Oxide

During the course of the denitrification studies in soils amended with potassium nitrite, appreciable nitrogen deficits were observed. Since neither gas chromatography techniques (26, p. 303-316) nor substrate nitrogen determinations (mineral and total nitrogen) resolved the nature of the deficits the situation became temporarily imponderable. However, during some of these experiments it was noticed that the carbon dioxide absorbent sometimes turned a pale yellow color. This clue, of course, provided the needed insight into the problem. A review of the analytical techniques related to base absorption phenomena suggested at least a part of the deficit encountered could likely be accounted for as nitric oxide. This was tested for and substantiated by methods previously discussed. As a corollary it was observed that when the absorbent was omitted from the reaction flasks, nitric oxide could be detected by gas chromatography. This observation emphasized the oversight of the earlier experiments. It was then decided, because of the nature of the accountable deficit i.e., the production of nitric oxide from only mildly acidic soil under an aerobic atmosphere, to investigate the mechanism(s) involved. To help accomplish this an appropriate soil (Woodburn silt loam) was selected for intensive study.

Soil-Sand Studies

To test further the activity of this soil, and to establish if denitrification might be responsible for the observed losses, increasing increments of irradiated soil were mixed with quartz in definite proportions as shown in Figure III. The mixtures were amended with 5000 μg of nitrite-nitrogen, permitted to react for 6 days and then tested for nitric oxide production. It is evident (Figure III) that the losses encountered were directly proportional to the soil/sand ratios and most probably were not enzymatic. A similar experiment using autoclaved soil gave almost identical results, permitting the final conclusion that the observed losses were certainly non-enzymatic. It was now, and only now, logically possible to infer without reasonable doubt that the soil itself possessed some organic or inorganic fraction or combination thereof which was mediating directly or indirectly the formation of nitric oxide. Even in the closed system employed, evaporation occurs to a small extent, as can be witnessed by the small moisture droplets which condense on the sides of the flask. Thus it is reasonable to suppose that a small quantity of the nitric oxide formed may be a resultant of activation by evaporation (22, p. 275-280). In any case the volume of condensation observed was estimated to be less than 1.0 ml. Additional moisture loss that might be inferred from an increase volume of absorbent was negligible since the latter increased less than 0.5 ml and since such increases also reflect the absorption of gases other than

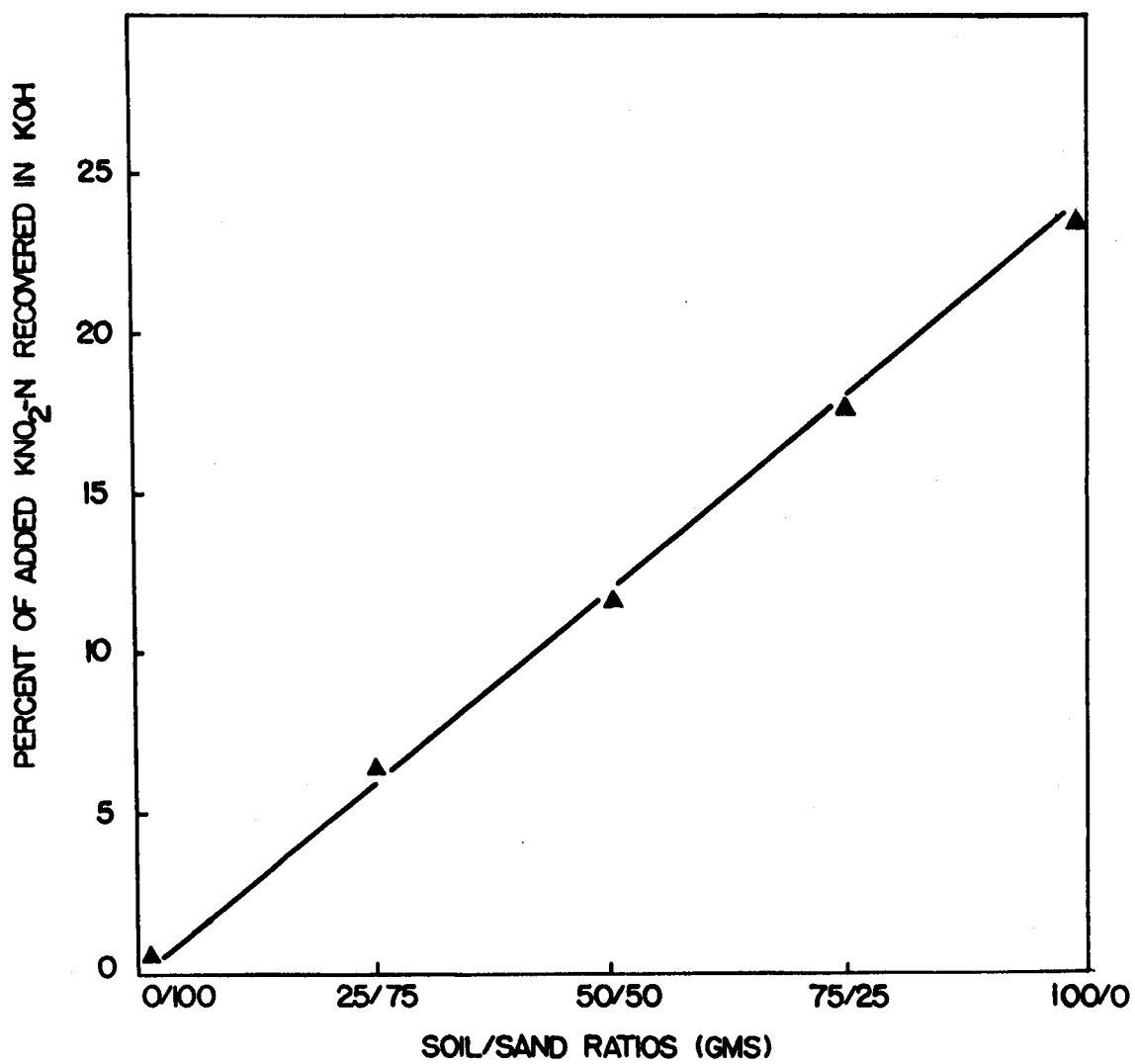


Figure III. Effect of increasing soil/sand ratios on the formation of nitric oxide.

water vapor.

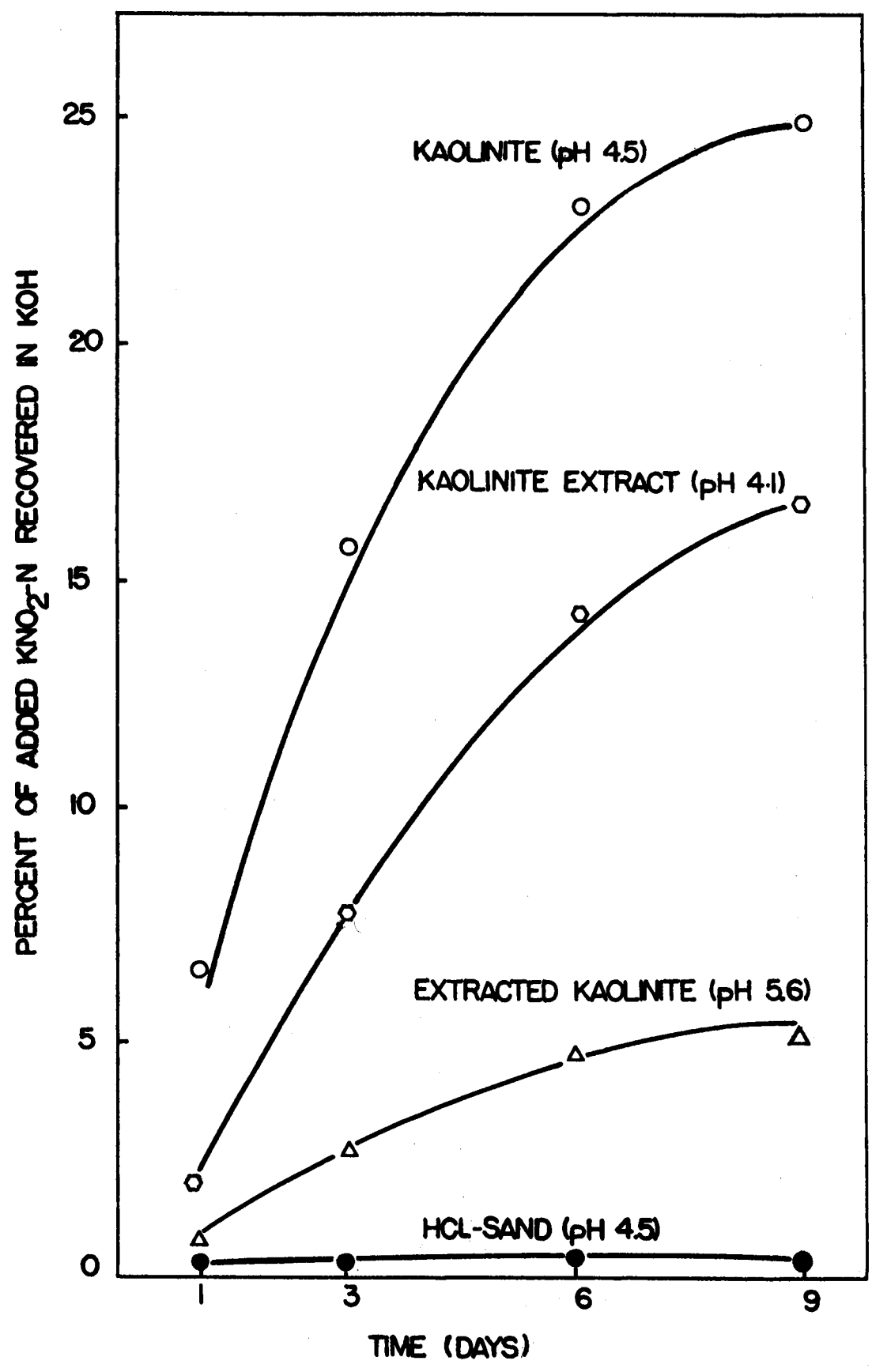
Clay Studies

At this juncture attention was given to a clay system using the kaolinite mentioned previously. A detailed characterization of the test clay was not available. A series of three reaction mixtures were prepared with 90-gm portions of sand mixed with 10 gm, 10 ml, and 10 gm of non-extracted clay, clay extract, and extracted clay, respectively. Nitrite was added to these mixtures at a rate equivalent to 20 ppm nitrogen. The control mixtures were comprised of 100 gm of sand, acidified with 10 ml of hydrochloric acid (pH 4.5) and nitrite. Sufficient preparations were made up to allow sampling at 1, 3, 6, and 9 days.

The comparative effects of the extraction are given in Figure IV. After 9 days the difference in activity (nitric oxide formation) between the non-extracted clay (25%) and its extracted counterpart (5%) can be largely accounted for in the activity of the extract (17%). The question of acidity is obvious since the non-extracted clay had a pH of 4.5 while the extracted fraction gave a value of 5.6. This implies that the effect is pH oriented. It is equally important to note that the acidified sand at pH 4.5 yielded little nitric oxide at only 0.4 of a pH unit higher than that of the extract. Thus it is noted that while acid conditions may be requisite, extractable factors are strongly implicated.

In order to ascertain semi-quantitatively the elements extracted, the clay was analyzed by spectrographic techniques.

Figure IV. Nitric oxide formation from clay and clay extract



Significantly, as shown in Table I, copper and aluminum were the only elements extracted which are known to react chemically with nitrite under standard conditions to yield nitric oxide.* Obviously the overall analysis indicates the presence of various clay oriented cations.

Role of Clay Extracts

To test the possibility that one or both metals might be involved, the clay extract was bubbled with hydrogen sulfide to precipitate the copper as copper sulfide, which was then filtered off and discarded. As indicated in Figure V, the treated extract (10 ml) added to sand (100 gm) amended with nitrite (20 ppm N) showed little activity, suggesting that copper was reacting in some manner essential to the formation of nitric oxide from the original extract (Figure IV). A critical corollary was the observation that both the treated (pH 4.0) and non-treated (pH 4.1) extracts may be considered to have had equivalent acidity. Clearly, hydrogen ions alone appeared to be insufficient for effecting the loss from the treated extract and, by inference, insufficient in the non-treated extract. Of secondary interest was the apparent unreactivity of the aluminum in the treated extract. While this observation does not eliminate the possibility of a complementary ion effect between aluminum and copper in the non-treated extract, it is nevertheless a reasonable inference that the chemical reactivity of the extractable copper was involved in 68 percent of the total loss observed from the

*Most comprehensive inorganic texts discuss metal-nitrite reactions. See: Sneed (34, 214 p.); Mellor (24, p. 418-419); Basalo and Pearson (6, 426 p.).

Table I. Semi-quantitative estimates^{1/} for elements detected in clay extract solution

<u>Element</u>	<u>Percent</u>
Al	0.165
Be	ND ^{2/}
Ca	0.006
Cu	0.006
Fe	ND
Ga	ND
Mg	0.006
Mn	ND
Na	10
Si	0.017
Ti	ND
All others	ND

1/ Spectrographic analysis by the U. S. Bureau of Mines

2/ ND = Not detected

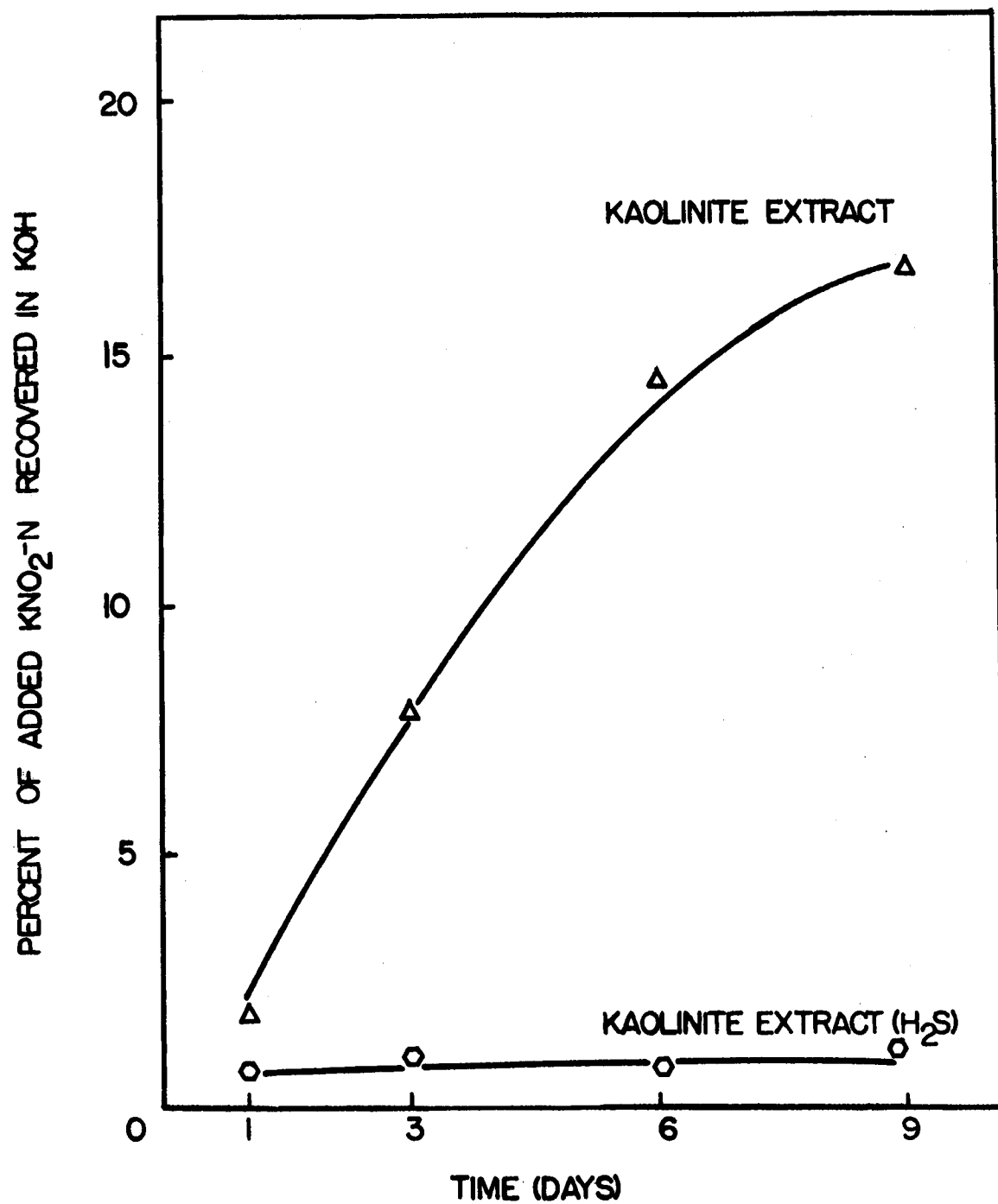


Figure V. Nitric oxide formation from clay extract and treated clay extract (H_2S).

non-extracted clay (Figure IV).

It is recognized that clay or soil systems represent more complexity than exists in true solutions, and thus, strictly speaking, conclusions drawn regarding the extracts may not necessarily apply to the clay or soil proper. Since the extracts under study may be thought of as closely approximating true solutions, their acidity as determined by glass electrodes may be considered much more realistic than any such value obtained for clay or soil suspensions. The reader is advised to consult Jenny for a comprehensive treatment and review of the soil acidity problem (21, p. 428-432).

A final point regarding the clay study is the difficulty in accounting for the loss (5%) which occurred from the extracted clay (Figure IV) although the existence of appropriate non-extracted metals should not be forgotten.

Role of Soil Extracts

The above study was complemented with a similar investigation of the Woodburn soil. In addition to testing the extract and extracted soil, the latter was acidified with hydrochloric acid to an appropriate acidity (pH 5.7) and observed for nitric oxide formation. Hundred-gram portions of soil were used and amended with nitrite at a rate equivalent to 50 ppm nitrogen. The extracts (10 ml) were added to sand (100 gm) and then nitrite

added as per the soil samples.

As in the clay it can be observed (Table II) that the extraction treatment resulted in decreasing the nitrite loss (in this case 50 percent). Significantly, 46 percent of the loss from the original soil was accounted for by the extract. Analysis of the extract (Table II) indicated that manganese (0.05%) was the only non-alkaline metal present in a detectable amount. It is not to be inferred that the observed manganese represents exchangeable manganese since the chloride ion may have influenced the manganese oxidation state (Personal communication). Manganese removal was accomplished by precipitation as previously outlined. The extract was then acidified to pH 4.8 with hydrochloric acid. These treatments resulted in an extract capable of forming little nitric oxide. It is also noteworthy that acidifying the extracted soil resulted in little or no increase in nitrite loss. Thus, nearly one-half the nitrite loss from the non-extracted soil may be attributed to the reactivity of extractable manganese. The control preparations again gave little activity. It can now be reemphasized that under the conditions employed acidic solutions and extracts seem to be incapable of promoting appreciable losses in the absence of active metals. Any possible nitrite reaction with ammonium (4, p. 404-409) cannot be seriously considered a major mechanism accounting for the observed losses from the clay and soil extracts, since the former contained only 2.0 ppm and the latter but 5.0 ppm $\text{NH}_4\text{-N}$.

Table II. Nitric oxide formation from soil and soil extracts

Substrates	% KNO ₂ -N added to substrates recovered in absorbent	Initial pH substrates	Metals in* extract		
			Fe	Cu	Mn
Soil	24.0	5.8	ND	T	0.05%
Soil (extracted)	12.0	6.9			
Soil (extracted, acidified)	13.0	5.7			
Extract	11.0	4.8	ND	T	0.05%
Extract (Mn removed)	< 0.002	4.8			
Sand (control)	< 0.002	5.0			

*Analyses by flame photometry U. S. Bureau of Mines

ND = not detected; T = trace

Effect of pH

To define more exactly the relationship between pH and nitrite decomposition, two sets of acidified sand preparations were made without and with iron added as ferrous sulfate*. Reactions were permitted to continue for 5 days and the absorbent then assayed in the usual manner. It is evident from Table III that in the absence of the iron a pH value of less than 3.0 was necessary to promote a significant production of nitric oxide. In the presence of the metal, however, considerable activity was observed at all pH values. These data support the previous contention that there has been an erroneous tendency to assume that nitrite, regardless of its chemical form, is spontaneously decomposed by hydrogen ions in acid soils. In spite of the inherent difficulties in making soil pH determinations and comparisons the evidence presented here is in agreement with that of Cady and Bartholomew (11, p. 546-549) who maintained that conventional explanations for nitrite decomposition have been incomplete.

A slight acidity effect apparently occurred in the presence of iron between pH 3.0 and 4.0. This effect may be due to slightly increased iron precipitation with the increase in pH.

Evaluation of Soil N Losses

Several soils of only mild or slight acidity were next amended with nitrite in the usual manner, with the primary

*Reduced iron is known to react with nitrite in acid solution to yield nitric oxide under standard conditions (34, p. 61).

Table III. Effect of pH and FeSO₄-Fe on the evolution of nitric oxide

pH	Percent added KNO ₂ -N recovered in absorbent <u>1/</u>	
	Sand H ₂ SO ₄ <u>2/</u>	Sand+5000 μg FeSO ₄ -Fe <u>3/</u>
2.0	25.0	30.0
3.0	< 0.001	30.0
4.0	< 0.001	25.0
5.0	< 0.001	25.0

1/ 10 ml nitrite added at rate equivalent to 50 ppm N

2/ 100 gm sand plus 10 ml acid @ appropriate pH

3/ FeSO₄ taken to appropriate pH with H₂SO₄ and KOH

All solutions were made up to equivalent concentrations of Fe.

purpose of obtaining some idea of the variation of nitric oxide formation in different soils. The results given in Table IV show a range of nitric oxide formation from 1.0 percent at pH 6.8 for the Walla Walla to 20 percent at pH 5.8 for the Red Hill soil. It may appear that the observed losses are directly proportional to pH, but it should be evident that no such simple relationship sufficiently explains the loss values obtained for the Ritzville (7.0 percent) and Lapine (17.0 percent) where both soils gave a common pH value of 6.3. Also, the decrease in pH in going from the Walla Walla to the Red Hill is but one unit and the latter soil would hardly be considered strongly acidic. Though there may be a broad correlation with soil pH values and nitric oxide formation it is difficult to imply, at least between pH 7.0 and 5.5, that simple acid decomposition was the mechanism responsible for the observed losses. Similarly no close correlation was obtained between the metals analyzed for and nitric oxide formation. Yet a comparison of the metal analyses (Fe and Mn only) for the Red Hill soil and the others suggests that where relatively large differences are accountable for relatively wide differences in nitric oxide production may be observed. No doubt factors other than quantity or concentration will affect the reactivity of the metals involved. Thus it would appear that with increased acidity or decrease in redox potential there would be expected an increase in the concentration of active metals i.e., an increase in the reduced state of a given metal. As reported

Table IV. Nitric oxide formation from soils amended with nitrite

Soil Series ¹	% added KNO ₂ -N recovered in absorbent	Initial pH	Metals ²		
			Fe	Mn	Cu
Walla Walla	1.0	6.8	3.8	370	35
Ritzville	7.0	6.3	1.7	490	48
Lapine	17.0	6.3	3.3	410	53
Red Hill	20.0	5.8	8.8	1330	44

¹ Soils autoclaved prior to testing

² Values given in ppm except for iron which is reported in percent

in the literature (24, p. 418-419) the more reduced states are generally more reactive in the formation of nitric oxide from nitrite.

Redox Potential

To test the above implication first hand, a simple experiment was performed wherein ferrous and ferric iron were added in aqueous solution at identical rates (2,500 $\mu\text{g-N}$) and allowed to react for 5 days, then observed for nitric oxide formation. It is evident from the data given in Table V that the ferrous iron solution at a higher pH was responsible for about 20 percent greater production of nitric oxide than the ferric solution.

Thus it is probable that nitric oxide formation, as considered here, is a rather complex phenomena dependent not only on the concentration of transition-like metals but also on their redox potential. Furthermore, as will be discussed in conjunction with the data given in Figure VI, other ions (calcium) seem capable of interacting in a given system to affect nitric oxide production. Accordingly, additional research is needed to clarify and better define the role played by such parameters and factors as kind and concentration of metals involved; pH and Eh relationships; interfering or complementary ion effects; possible indirect biochemical influences e.g., organic acids as they may reduce trace metals. These factors and perhaps others such as cation exchange capacity and zeta potential, as they are interrelated to one another

Table V. Influence of ferric versus ferrous iron in NO evolution

<u>Metal form</u>	<u>% added KNO₂-N recovered in KOH</u>	<u>pH of solution</u>	<u>Solution concentration ppm Fe</u>
FeSO ₄ ·7H ₂ O	32.0	3.3	250
Fe ₂ (SO ₄) ₃ *	23.0	2.5	250

*Reagent grade contained 0.12% ferrous iron

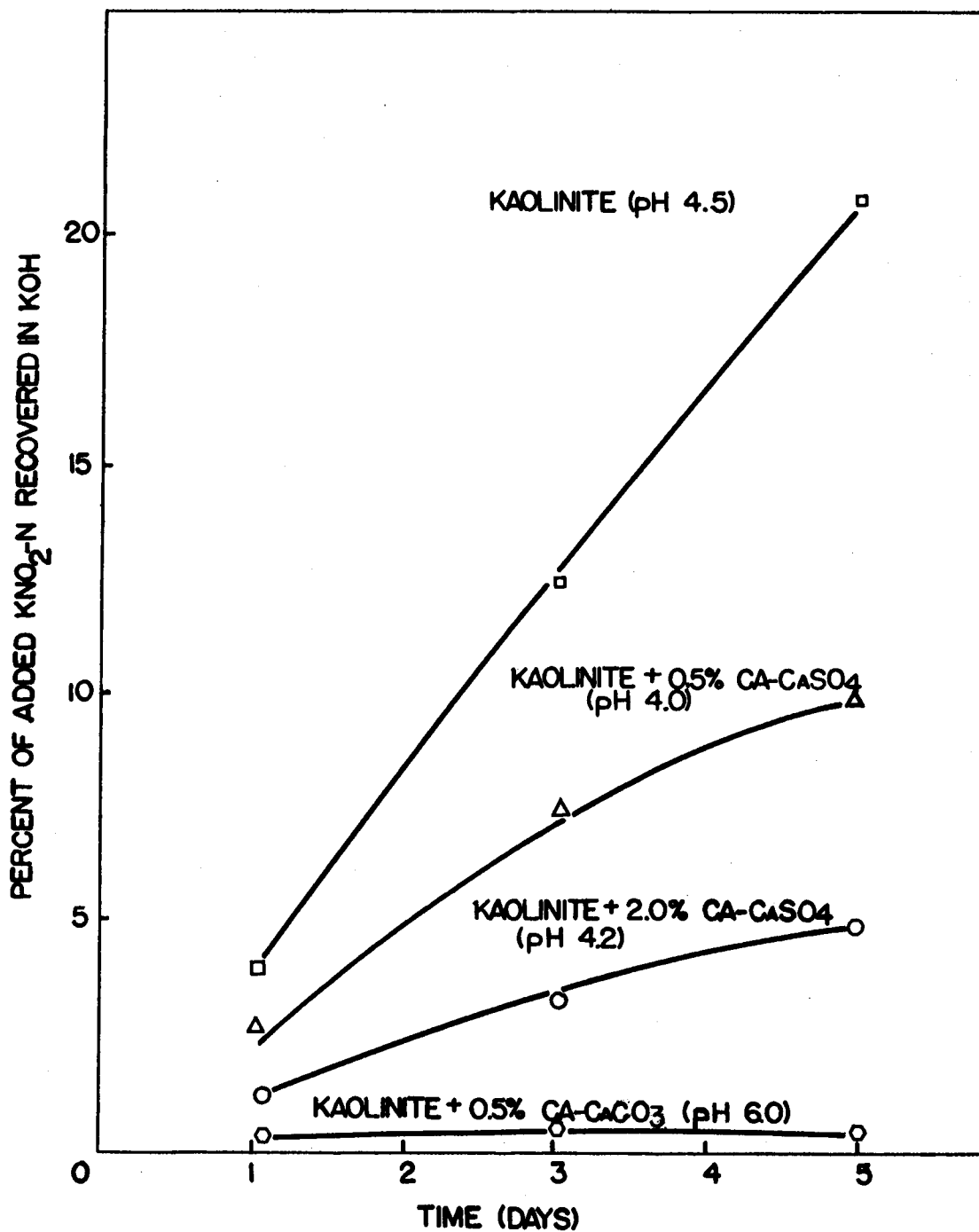


Figure VI. Effect of calcium on the formation of nitric oxide from clay.

will have to be better defined before we can expect to achieve consistent success in predicting potential losses from our soils. This is not to say that we cannot project certain expectations based on current knowledge. For example, where certain common fertilizers which contain rather high proportions of trace elements are applied one would not be surprised to find considerable nitric oxide formation, provided, of course, sufficient nitrite were present. On the other hand, as will be brought out presently, good possibilities exist for controlling such losses.

Effect of Calcium

A pertinent experiment was performed in an attempt to show that the addition of calcium as either the sulfate or carbonate to the kaolinite clay effectively reduces nitric oxide production. The mixtures were prepared as in the previous clay studies except for the addition of calcium as just noted. It will be seen (Figure VI) that over the course of the study little nitric oxide formation occurred with the addition of calcium carbonate (0.5% Ca on clay weight basis. This effect might be ascribed to the rise in pH of the initial substrate from pH 4.5 to 6.0 with the addition of the calcium carbonate. But the latter explanation seems oversimplified since calcium sulfate at 0.5 percent calcium gives an initial substrate pH of 4.0 or at 2.0 percent with a substrate pH of 4.2 yielded only 10 percent and 5 percent nitric oxide, respectively. It is suggested that in addition to any possible pH effect the calcium is

complexing with the active metal, in this case copper, and/or the nitrite to reduce the systems total capacity to form nitric oxide. Such complexes are sometimes referred to as "Werner complexes" or "ligands" (6, p. 1-2). The implication of using calcium as a practical agent for the control of losses similar to those observed here is quite evident.

Nitrification and N Loss

A final experiment was conducted to test the capacity of the Woodburn soil to produce nitric oxide when amended with urea or ammonium sulfate. It is obvious from the data given in Table VI that little of the oxide was formed after 4 weeks in the presence of either carrier.

The data simply show that a given soil may form nitric oxide when amended with nitrite but may not have the capacity to do so from other nitrogen carriers. Finally such data emphasize once again the need for a conservative viewpoint when considering any theory of gaseous nitrogen loss.

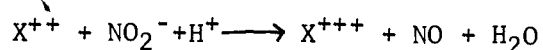
Table VI. Observed nitrogen losses with urea and ammonium sulfate

Nitrogen carrier	Solution concentration ppm N	% added $\text{KNO}_2\text{-N}$ recovered in KOH
Urea	200	< 0.001
Ammonium sulfate	200	< 0.001

CONCLUSIONS AND SUMMARY

It was emphasized that prevailing concepts relating to the non-enzymatic gaseous loss of nitrite from soil do not fully explain either the extent of such losses or the mechanisms involved. This is particularly true in well-managed soils of slight or moderate acidity.

Evidence obtained from the soil and clay systems studied attests to a mechanism of loss whereby nitrite may react with certain metals to yield nitric oxide as a primary gaseous product. The empirical chemical reaction underlying the mechanism proposed here was substantiated first by Priestly (31, p. 328-410) later confirmed and studied by numerous chemists as already noted. A conventional half-cell reaction wherein the metal is oxidized and nitrite is reduced to nitric oxide with the formation of water may be written as:



where X is a transition-like metal such as manganese, copper, iron etc. It is implied that hydrogen ions may be necessary - acid conditions favor the reduced state of the metals which is a more active state with respect to the formation of nitric oxide - for such reactions, but that they are not sufficient in substrates of only milk acidity.

Although it is difficult to establish with certainty the site of the observed reactions in solid systems it should not be

forgotten that in soil the metals involved are intimately associated with organic matter as well as with mineral colloids or indeed complexes of the two fractions. Thus it may be that as the cation exchange capacity of a soil varies so too may the activity of the reactants involved. If indeed this is the case then one would expect nitric oxide formation to vary inversely with the zeta potential of the system providing, of course, the requisite conditions necessary for the reaction were present. While it cannot be resolved at this time whether the more strongly adsorbed metal ions may react to form nitric oxide, it is clear that the reactants present in the liquid extracts were capable of doing so. It may be concluded therefore that the soil solution, or liquid phase, likely plays a critical if not an absolute function in mediating the chemical reaction proposed here.

Another point for serious consideration, as mentioned earlier, is that of redox potentials, since they can be reliable parameters of the reactivity of transition-like metals (27, p. 54-60; 28, p. 20-29). The implications of these latter reports are far-reaching, since they have demonstrated that with anaerobic conditions intermediate fermentation products e.g., organic acids, can participate directly or indirectly in the reduction of such metals as iron. It appears therefore that the very conditions which favor classical denitrification can favor metal-nitrite reactions since both nitrate and the metals may be expected to undergo reduction under such circumstances. This possibility

places in question the status of nitric oxide as a direct biochemical intermediate in the microbial formation of nitrogen gas.

It may also be that what often has been anomalously termed "nitrite instability" or sometimes is referred to as the spontaneous decomposition of nitrite as an explanation for nitrogen deficits in some soils may prove to be spurious terminology. It is suggested that a mechanism involving transition-type metals may account for significant losses of mineral nitrogen from field soils capable of accumulating nitrite, under conditions of moderate or slight acidity.

BIBLIOGRAPHY

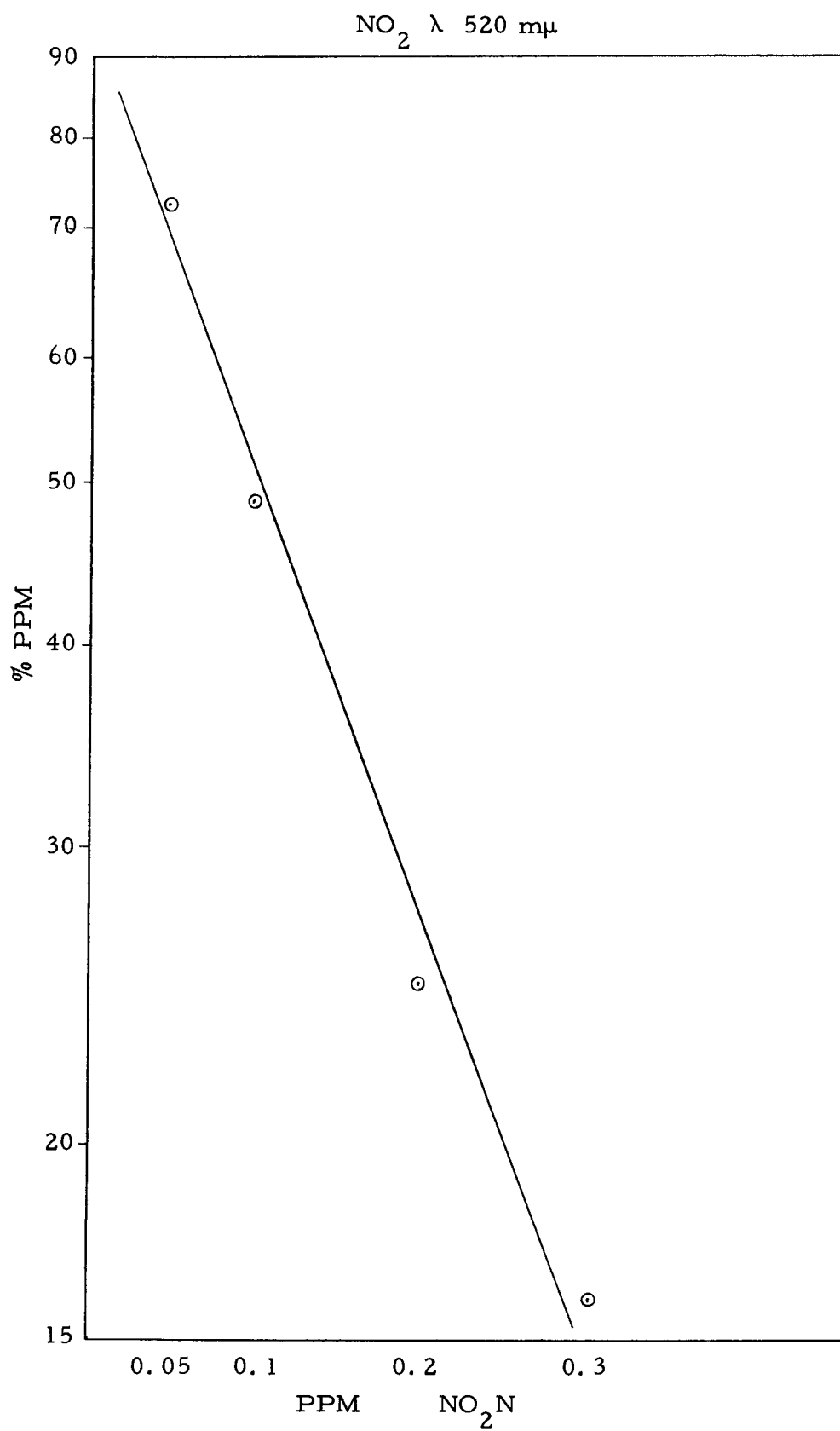
1. Allison, F. E. and Janet Doetsch. Nitrogen gas production by the reaction of nitrites with amino acids in slightly acidic media. Soil Science Society of America, Proceedings 15:163-166. 1950.
2. Allison, F. E., Janet Doetsch and Luann K. Sterling. Nitrogen gas formation by interaction of nitrites and amino acids. Soil Science 74:311-314. 1952.
3. Allison, F. E. The enigma of soil nitrogen balance sheets. Advances in Agronomy 7:213-250. 1955.
4. Allison, F. E. Losses of gaseous nitrogen from soils by chemical mechanisms involving nitrous acid and nitrites. Soil Science 96:404-409. 1963.
5. American Public Health Association. Standard methods for examination of water and wastewater. 10th ed. 1955. 522p.
6. Basolo, Fred and Ralph G. Pearson. Mechanisms of inorganic reactions. New York, Wiley, 1960. 425 p.
7. Bollen, W. B. Microorganisms and soil fertility. Corvallis, Oregon, College Press, 1959. 21p
8. Bremner, J. M. and K. Shaw. Denitrification in soil. I. Methods of investigation. Journal of Agricultural Science 51:22-39. 1958.
9. Broadbent, F. E. Denitrification in some California soils. Soil Science 72:129-137. 1951.
10. Broadbent, F. E. and B. F. Stojanovic. The effect of partial pressure of oxygen on some soil nitrogen transformations. Soil Science Society of America, Proceedings 16:359-363. 1952.
11. Cady, F. B. and W. V. Bartholomew. Investigations of nitric oxide reactions in soil. Soil Science Society of America, Proceedings 27:546-549. 1963.
12. Chambers, F. S. and T. K. Sherwood. Absorption of nitrogen dioxide by aqueous solutions. Industrial and Engineering Chemistry 29:1415-1422. 1937.

13. Chao, Tyng-Tsair and Wybe Krootje. Inorganic nitrogen oxidations in relation to associated changes in free energy. Soil Science Society of America, Proceedings 27:44-47. 1963.
14. Clark, Francis E. and William E. Beard. Influence of organic matter on volatile loss of nitrogen from soil. In: Proceedings of the Seventh International Congress of Soil Science, Madison Wisc., U. S. A., 1960. Vol. 3, no. 6, p. 501-508.
15. Clark, Francis E. and William E. Beard and Donald H. Smith. Dissimilar nitrifying capacities of soils in relation to losses of applied nitrogen. Soil Science Society of America, Proceedings 24:50-54. 1960.
16. Eastoe, J. E. and A. G. Pollard. A modified phenoldisulfonic acid method for determining nitrates in soil extracts etc. Journal of Science and Food Agriculture 1:266-269. 1950.
17. Eno, Charles F. and Hugh Popenoe. The effect of gamma radiation on the availability of nitrogen and phosphorus in soil. Soil Science Society of America, Proceedings 27:299-301. 1963.
18. Ephraim, Fritz. Inorganic chemistry. London, Gurney and Jackson, 1962. 805p.
19. Gilmour, C. M. and L. H. Wullstein. Some aspects of fertilizer nitrogen loss in soil. In: Proceedings of the Thirteenth Annual Fertilizer Conference of the Pacific Northwest, 1962. p. 47-51.
20. Gilmour, C. M., R. P. Bhatt and J. V. Mayeux. The comparative role of nitrate and molecular oxygen in the dissimilation of glucose. Nature 203:55-58. 1964.
21. Jenny, Hans. The soil acidity merry-go-round. Soil Science Society of America, Proceedings 25:428-432. 1961.
22. Madhok, R. R. and Fazal Uddin. Losses of nitrous nitrogen from soils on desiccation. Soil Science 61:275-280. 1946.
23. Meiklejohn, J. Aerobic denitrification. Annals of Applied Biology 27:558-573. 1940.
24. Mellor, J. W. A comprehensive treatise on inorganic and theoretical chemistry. VIII. New York, Longmans, Green, 1928. 1110p.

25. McGarity, J. W. Some factors affecting the nitrogen economy of a humid subtropical soil and a semi-arid temperate soil. Ph.D. thesis. Corvallis, Oregon State University, 1958. 302 numb. leaves.
26. McGarity, J. W., C. M. Gilmour and W. B. Bollen. Use of an electrolytic respirometer to study denitrification in soil. Canadian Journal of Microbiology 4:303-316. 1958.
27. Motomura, Satoru. Dissolution of iron compounds in soils by milk vetch extracts. Soil Science and Plant Nutrition 7:54-60. 1961.
28. Motomura, Satoru. Effect of organic matters on the formation of ferrous iron in soils. Soil Science and Plant Nutrition 8:20-29. 1962.
29. Pauling, Linus. College chemistry. 2d ed. San Francisco, Freeman, 1955. 685p.
30. Poulton, Charles Edgar. Ecology of the non-forested vegetation in Umatilla and Morrow counties, Oregon. Ph.D. thesis, Pullman, State College of Washington, 1955. 166 numb. leaves.
31. Priestly, Joseph. Experiments and observations on different kinds of air and other branches of natural philosophy. Birmingham, Pearson, 1790. p. 328-410.
32. Sandell, E. B. Colorimetric determinations of trace metals. 2d ed. New York, Interscience, 1950. 673p.
33. Skerman, V. B. D. and I. C. MacRae. The influence of oxygen on the reduction of nitrate by adapted cells of Pseudomonas denitrificans. Canadian Journal of Microbiology 3:215-230. 1957.
34. Sneed, M. Cannon and Robert C. Brasted. Comprehensive inorganic chemistry. V. Princeton, New Jersey, D. Van Nostrand, 1959. 214 p.
35. Wijler, J. and C. C. Delwiche. Investigations on the denitrifying process in soil. Plant and Soil 5:155-169. 1954.

36. Wullstein, Leroy H. and C. M. Gilmour. Non-enzymatic gaseous loss of nitrite from clay and soil systems. *Soil Science* 97:428-430. 1964.
37. Yost, Don M. and Horace Russel Jr. *Systematic inorganic chemistry*. New York, Prentice Hall, 1944. 423p.

APPENDIX



NO_3 λ 425 m μ

