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SPACE SCIENCES LABORATORY

The Third Semiannual Progress Report On

ENZYME ACTIVITY IN TERRESTRIAL SOIL IN
RELATION TO EXPLORATION OF THE MARTIAN SURFACE

NASA Grant NsG 704/05

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ENZYME ACTIVITY IN TERRESTRIAL SOIL IN RELATION TO EXPLORATION OF THE MARTIAN SURFACE

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and J.J. Skujins

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I. INTRODUCTION

Our objective is to develop qualitative and quantitative tests for various enzyme activities in soil and to adapt the most sensitive of these to procedures compatible with telemetry from Mars probes. In addition we elucidating enzyme reactions in environments of limited moisture.

The first part of this report reviews the presently available information on enzymatic reactions in terrestrial soil. The emphasis is placed on characterization of free, extracellular enzymes in soil and the metabolic activities of soil microorganisms.

The experimental part reports on our progress in the evaluation of urea as a possible substrate for the detection of catalytic, i.e., enzymatic, breakdown of urea in a Martian environment. Emphasis is placed on the detection of urease activity because of the possible primordial origin of urea as an organic substance, because of the relative stability of urea as an enzyme substrate and because of the ubiquity of soil urease.

A new method for the detection of phosphatase activity in soil has been developed.

The Martian environment has a limited moisture content and any biological reactions possibly take place at interfaces and on surfaces in an environment of restricted water availability. A study of surface effects in the hydrolysis of insoluble chitin by adsorbed chitinase has been initiated to investigate some of the factors influencing reactions at interfaces.

Acknowledgement. The participants in the currently reported phase of this project included:

Mr. I.G.N. Davidson,

Mr. A.H. Pukite,

Mr. J.R. Ramirez-Martinez.

II. ENZYMES IN SOIL*

I. Introduction

All biological transformations in soil are catalyzed by enzymes in or secreted by soil organisms. As a part of their physiological activity many soil organisms release extracellular enzymes; for example, protinases and cellulases hydrolyze large macromolecules and the degradation products become available as nutrients. It can be assumed, a priori, that some extracellular enzymes exist in the soil in active state outside the living cells. However, not all of the known enzymatic reactions in soil, in the presence of various inhibitory agents for microbial proliferation, can be ascribed to the microbial extracellular enzymes.

Many reactions in soil are catalyzed by typical intracellular enzymes. Upon the death of cells and the collapse of cell wall and membrane integrity some protoplasmic constituents are released into soil. Although most of the released material may be easily metabolized by other living organisms, some enzymes may persist in soil for a certain period in an active state, and at least some enzymes may be quite resistant to denaturation in a soil environment. In considering biochemical activities in soil, the soil may be looked upon as a biological entity, i.e., as a "tissue". This concept has been advanced by Quastel in 1946 (1), and previously (in 1902) by Visotskii (2) who compared the soil solution to the blood of animals. However, almost all attempts to isolate enzymes in pure form from this soil "tissue" have been unsuccessful. This may be due to the strong binding of proteins by clays and humus

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*Submitted for publication in "Soil Biochemistry", Marcel Dekker, N.Y., in preparation.

constituents. Only in recent years reports have appeared regarding successful isolations of soil enzymes.

Clearly, it is of profound interest to elucidate these enzymatic activities in soil. Among the questions which may be asked are: what are the precise origins of these enzymes in soil; what is their distribution on a macro scale and what is their localization with respect to other soil constituents; what is their significance, in addition to microbial activity per se, in decomposition of organic matter and in humus formation; what is the significance of soil enzymes in plant nutrition, i.e., are there significant soil enzyme - plant root interrelationships? It is also known that some inorganic soil constituents exhibit catalytic properties; for example, some iron and manganese compounds catalyze decomposition of H_2O_2, which is similar to catalase activity. Thus it is also of interest to distinguish between enzymatic activity in soil and the eventual catalytic activity due to inorganic matter present in the soil.

The main methodological problem with enzymatic studies in soil has been to achieve an effective inhibition of microbial activity and at the same time leave the soil enzymes unaffected. It is also desirable not to disturb other chemical and physical soil properties in any way. The most widely used method for this purpose has been the addition of toluene or other bacteriostatic agents to soil as microbial inhibitors; methods utilizing high energy radiation sterilization were introduced in the 1950-ies. The experimental separation of the metabolic activities of microorganisms and extracellular enzymes, however has as yet not been solved.

Much attention has been devoted to enzymological examination of the soil in the last decade and a considerable amount of empirical data has

been collected. However, descretion must be exercised in evaluating the published work in soil enzymology. Such work ranges from attempts to separate the microbial activity from true extracellular enzyme activity in soil to observations on soil as a biological whole without an intent to exclude the contribution by living, and proliferating organisms.

Experimental approaches to the enzymatic examination of soil have involved several lines of thought. Aside from the point of view of "basic" science with an intent to examine actual enzymes existing outside the cells in soil, methods have also been employed which give an insight into the general physiology of soil with respect to life processes therein, and particularly with respect to soil fertility. A new impetus to soil enzymology has been given of late by the need to apply knowledge in this field to extraterrestrial life detection, particularly in planetary exploration.

II. Historical

With the advent of animal and plant biochemistry and enzymology it was also recognized that in the soil, aside from the microbial activity *per se*, many organic matter transformations could possibly be catalyzed by enzymes existing in the soil originating from but outside living tissue. Among the first investigators describing the presence of enzymes in soil was A.F. Woods who wrote in 1899 (3):

> I have also determined by experiment that the oxidizing enzymes, especially the peroxidase may occur in the soil and, as a rule, are not destroyed by the ordinary bacteria of decay. These enzymes enter the soil through the decay of roots and other parts of plants which contain them.

König *et al* (4) used biological inhibitors (cyanide) in order to show the enzymatic nature of catalatic activity in soil. May and Gile

in 1909 (5) studied catalase activity in soil and their conclusions regarding a correlation of catalatic activity in soil with organic and inorganic fractions and with microbial activity have been repeatedly verified. Presence of "oxydases" (peroxydases) in the soil was indicated by Cameron and Bell in 1905 (6), and soils were examined by Fermi in 1910 (7) for a proteinase, "gelatinase", and other enzymes known at that time.

During these early years most of the attention was directed towards catalase activity, apparently from an ease of detection and a limited knowledge about other enzymes. Determination of catalaze activity was listed as one of the methods for the examination of biochemical activities of soil in 1924 (8). Presence of a deaminase activity in soil was demonstrated in 1927 (9) and in the 1930-ies Rotini found soil phosphatases (10) and urease (11). In the early 1940-ies J.P. Conrad re-examined the urease activity and H.T. Rogers correlated the soil phosphatase activity with rhizosphere phenomena.

The difficulties encountered in distinguishing enzyme activities from associated phenomena were soon recognized. Fermi (7) realized that in order to fully elucidate enzymatic activities in soil, it would be desirable to extract the enzymes and to demonstrate activities in the absence of microorganisms. Penkava (12) pointed out the existance of non-enzymatic, inorganic calalysis among soil constituents and studied the catalaze-like activities of iron and manganese compounds in soil. Also, methods employed to inhibit microbial activity presented difficulties which were recognized in 1914 by Buddin (13); he pointed out an incomplete effectiveness in the use of toluene for soil sterilization. The foregoing problems in the enzymological examination of soil have been by no means solved.

Since 1950 new advanced methods were introduced in soil enzymology and a spate of information regarding various enzymatic reactions in soil has been collected. Most of this information is, however, difficult to evaluate in terms of importance to agriculture and in terms of nutrient cycles in soil. E. Hofmann and G. Hoffmann with co-workers in Germany have been among the most fruitful in this area. J. Drobnik in Czechoslovakia and I.S. Kiss in Rumania, among others, have also made significant contributions to an understanding of the significance of enzymatic reactions in soil. Correlatiors of enzymatic reaction in soil with practical aspects of agriculture and soil fertility have been examined by H. Koepf in Germany and especially by A.S. Galstyan, V.F. Kuprevich, and others in the USSR. High energy radiation sterilization of soil for enzymatic studies was introduced in 1956 and pursued by A.D. McLaren and collaborators at the University of California.

Valuable new insights in understanding enzyme reactions in soil have also been achieved by examining the behaviour of added enzymes on soil mineral and organic constituents, and by applying the information of enzyme reactions at interfaces to soil enzyme studies, for example, by D.L. Lynch and A.D. McLaren in the USA, G. Durand in France, S. Aomine and S. Koboyashi in Japan, among others. This short listing by no means presents all important contributors to soil enzymology.

Although the presence of several free enzymes has been detected in soil extracts previously, the first purified, solid preparation of an extracted soil enzyme, urease, was obtained by Briggs and Segal in 1963 (14).

Excellent review articles on enzymatic activities in soil have been written by Kiss (15) and by Durand (16).

III. Methodology

Conditions during storage and treatment of soil during experimental examination greatly affect the apparent enzymatic activities in soil. Aside from specific methods used for the assay of each individual enzymatic activity in soil, various sterilizing agents and sterilization procedures, buffering systems, temperature and agitation of soil during assay have specific influence on the experimental results.

A. Soil Sterilization

An ideal sterilizing agent for extracellular enzyme detection in soil would be one which would completely inhibit all microbial activities in soil but would not lyse cells and would not affect the extracellular enzymes in any way. Unfortunately such a sterilizing agent is not as yet available. Various agents used for microbial inhibition have certain shortcomings and the results should be interpreted accordingly. Of course, in many cases complete microbial sterilization is not desired, but only inhibition of microbial proliferation.

1. Chemical agents. A variety of sterilants, antiseptics and bacteriostatic agents have been used to inhibit microbial growth and physiological processes.

Toluene has been the most widely used microbial inhibitor, but as early as 1914 Buddin (13) pointed out the incomplete effect of the use of toluene in soil sterilization, and obtained considerable growth in toluene treated soils after several days of incubation.

It might be expected that use of toluene in soil would stop further synthesis of enzymes by living cells and would prevent assimilation of products of enzymatic reactions. Toluene has also been shown to be a

plasmolytic agent: in certain groups of microorganisms it apparently induces a release of intracellular enzymes.

During the examination of glycerophosphate hydrolysis in soil Rogers (17) noted that CO_2 release from soil was effectively inhibited by addition of toluene, while a high rate of glycerophosphate hydrolysis was taking place. E. Hofmann and his group in Germany have used toluene extensively as a microbial inhibitor. The general procedure is to add 1 to 2 ml toluene to 10 g of soil, then enzyme substrate and then to proceed with incubation followed by an appropriate analytical method.

The usefulness of E. Hofmann's method was severely criticized by Claus and Mechsner (18) who, similarily to Buddin, observed considerable growth of microorganisms in toluene treated soils. A stimulating effect of toluene toward soil bacteria has been noted also by Waksman and Starkey (19). Hofmann and Hoffmann have stressed the adequacy of toluene for the assay of enzymatic activities in soil as sufficient for their purpose (20). Although Drobnik (21) had noted variations in the effectiveness of toluene on the inhibition of soil microorganisms, he also observed that toluene prevented the assimilation of metabolic products by microflora and he suggested (22) that "toluene should not be rejected as an antiseptic agent for investigations of soil enzymes without further experimental check". Galstyan (23) made a study of enzyme kinetics in soil of a variety of enzymes and concluded that no assimilation of the enzymatic reaction products by soil microbes took place, and that any autolysis of microbial cells did not increase enzymatic activities. Kiss et al (24) successfully used toluene as a microbial inhibitor for a prolonged incubation (14 days at 35°).

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A critical examination of the effect of toluene on soil microorganisms has been made by Beck and Poschenrieder (25). They have shown that the inhibitory effect and the needed concentration of toluene is strikingly dependent on the pretreatment and moisture content of a particular soil. To suppress microbial growth in an air-dry, in a naturally moist, or in a dried and remoistened soil, at least 20 per cent of toluene is necessary. In a soil suspension 5 to 10 per cent of toluene is sufficient. Gram-positive bacteria, and *Streptomyces*, are considerably more resistant to toluene treatment than gram-negative bacteria. They showed that activities in a 1:50 soil suspension with 10% toluene present can be considered as enzymatic and not due to microbial growth.

An enlightening study has been presented by Jackson and DeMoss on the effects of toluene on *Escherichia coli* (26). In washed cell suspensions 0.15% toluene drastically decreased the viable cell count. At a 2.5 to 5% toluene concentration there were no viable cells and β-glucosidase was unmasked, although not released from the cells. At this concentration cell membranes lost selective permeability, but no disruption of cell walls occurred, although the cytoplasmic contents collapsed towards the center of the cell. Some protein and RNA material was released through the cell walls, there was no protein synthesis, but the cells still could oxidize certain substrates and the terminal respiratory chain appeared to be intact.

It is of interest to note that toluene has been used previously as an unmasking agent for an assay of several enzymes in microorganisms, for example, for β-galactosidase (27) and alkaline phosphatase (28). It is apparent that these enzymes are not released free by the action of toluene, rather, the cell walls become permeable to the substrates and products.

It is known that toluene inhibits some oxidoreductases, especially some carbohydrate oxidases, but it is without effect on most other enzymes. Some seemingly activating effects by toluene have been observed. For example, increase of urease activity in soil upon addition of toluene (11,29) may be ascribed to the above described unmasking action of toluene.

In 1928 Gray and Thornton (30) isolated several organisms from soil which decomposed toluene and other hydrocarbons. Later toluene decomposing *Pseudomonas* were isolated by Kitagawa (31) and *Pseudomonas* and *Achromobacter* by Claus and Walker (32). A study of toluene decomposition in soil by Swingle-Branson (33) showed that toluene at 0.1% concentration was used as a carbon source by soil microbes more than other hydrocarbons. It was shown that 8.7% of all microbes in pasture soil and 0.4% in cultivated clay loam decomposed hydrocarbons. Among the active microbes more than 50% were *Streptomyces*. It is evident that no significant biological decomposition occurs at 10 to 20% toluene concentration used in soil enzyme assays.

Other chemical agents. A large variety of chemicals have been used for the sterilization of soil (34), but only a few of them may be used successfully for studies in soil enzymology. Most of the chemicals are effective because of their action on protein and thus they also preferentially inactivate any extracellular protein, i.e., enzymes present in soil. For example, Haig (35) examined the usefulness of ethylene oxide and found that at the concentrations where it acted as a sterilant of microorganisms, it also completely inactivated soil urease and esterase (hydrolysis of ethylbutyrate), and reduced acetylesterase (hydrolysis of phenylacetate) activity by a half.

Other chemical agents that have been used for the microbial inactivation in the study of soil enzymes are chloroform, phenol, thymol and ether. Subrahmanyan (9) demonstrated that glycine deaminase activity in soil was lower in presence of ether than in a presence of toluene; chloroform showed intermediate effect. Several aseptic agents were examined by Rotini (11) coincidental with his studies on soil urease activity. Soil was incubated with urea and with bacterial inhibitors for 4 hrs. at 42°, and the residual urea in the soil was determined as xanthylurea (maximum available: 25 mg xanthylurea):

50 g soil + 30 ml of 0.1% urea in presence of:	mg xanthylurea recovered
1 ml water	13.6 - 14.2
1 ml phenol (5% aq.)	12.8 - 13.2
1 ml acetone	9.4 - 9.6
1 ml toluene	7.6 - 7.9
1 ml thymol (10% in alcohol)	5.8 - 6.1
1 ml chloroform	1.3 - 2.5

Rotini suggested that the decrease in residual urea after incubation with the above listed chemicals indicated an increased bacterial lysis (i.e., an increased cell wall permeability) and thus an increased accessability of substrate (urea) to urease.

2. Irradiation. The first attempt to observe radiation effects on enzymatic activities in soil was performed by Scharrer in 1928 (36,37) who used ultraviolet radiation to observe its effect on catalase activity in soil. He concluded that "by ultraviolet irradiation the catalytic activity in soil diminishes somewhat".

Dommergues (38) examined influence of infrared radiation on invertase activity in soil and concluded that any effect of infrared irradiation on soil enzymes is negligible.

Perhaps the most nearly ideal agent for the sterilization of soil is high energy ionizing radiation. Either an electron beam of sufficient intensity (5 to 10 Mev), hard X-ray or gamma radiation (Co^{60}) may be used.

The utilization of ionizing radiation for sterilization, including sterilization of soil was first explored by Dunn _et al_ in 1948 (39). McLaren _et al_ in 1957 (40) showed that soil can be sterilized by an electron beam of sufficient energy and intensity. A 2×10^6 rep dose was necessary to obtain sterile soil in one gram samples. Enzymatic activity (urease) was retained in the sterilized soil. By using gamma radiation Stotzky and Mortensen (41) found that a 8000 roentgen dose significantly decreased the fungal population. This decrease was partially ascribed to the inability of fungi to recover in competition with antagonistic bacteria. Popenoe and Eno (42) irradiated soil with Co^{60} gamma rays in 100-150 gram packages in doses up to 2.048×10^6 roentgens and found that complete sterility was not achieved. Gamma radiation effects on the nitrogen cycle, among other radiation effects in soil, was studied by Vela (43): a 0.25×10^6 r dose permanently inhibited nitrogen fixation but stimulated urease activity. Gallon quantities of soil in a U^{235} neutron and gamma ray field were irradiated by Stanovick _et al_ (44). Complete sterility was not achieved at 4×10^{10} rep dosages; apparently the soil was not uniformly irradiated.

Van de Graaff electron generator (3 Mev) was used as a radiation source for soil sterilization by Peterson (45,46), who obtained complete

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sterility at 3.3×10^6 rad doses. The sterilized soil exhibited respiratory activity. Other authors also have made measurements of CO_2 released from irradiated soils (41,42,44).

Bowen and Rovira (47) sterilized soil by gamma irradiation in test tube amounts at 2.5×10^6 rad doses and tested the sterile soil for its effects on plant growth. One soil (Urrbrae red brown earth) exhibited some phytotoxicity upon irradiation. Plant growth in another soil (Mount Compas sand) was not affected by irradiation. It has been shown by McLaren _et al_ (48) that the soils tested (Dublin clay loam and Columbia very fine sandy loam) did not exhibit any phytotoxicity when irradiated with 5 Mev electron beam at 4×10^6 rep doses. Bowen and Cawse (49,50) achieved complete sterility in various soils at 4 to 5 Mrad doses with Co^{60} gamma irradiation; they indicated that irradiation had a beneficial effect on plant growth.

Some increase of organic matter in soil solution upon irradiation has been observed (51) and it has been suggested that such an increase comes from lysed microorganisms. Work by Groenewoud (52) indicated that gamma ray sterilized humus exhibited negligible chemical changes.

Significant changes in acid-soluble and water-soluble phosphates in soils due to gamma-irradiation up to 3×10^6 rep doses were detected by Mack (53) and changes in soluble manganese by Bowen and Cawse (49). Eno and Popenoe (54) detected an increase in extractable phosphorus in peat soils, gamma-irradiated up to 2.048×10^6 r. Potassium chloride extractable nitrogen increased in mineral and peat soils with increasing doses of radiation. Gamma irradiation of soil apparently caused a release of phosphate from organic compounds (55). Mineral availability to plants in irradiated soil has been studied also by Cummins and McCreery (56).

All studies on the irradiation of soils have shown that, aside from sterilizing the soil, other effects on the physical and chemical properties have been mild and often negligible.

The effects of ionizing radiation on microbial cells in pure cultures has been studied in considerable detail. This work has been reviewed by Lea (57), Zelle and Hollaender (58), and by Bacq and Alexander (59). Similarily, effects of ionizing radiation on enzymes have been reviewed by Setlow (60) and Augenstine (61), among others.

In general, the number of live cells diminishes as a logarithmic function with respect to radiation dosage: $\frac{N}{N_o} = e^{-kD}$, where N/N_o is the ratio of the residual numbers to the initial numbers of microorganisms, D is the radiation dosage; the constant k is dependent on type of micro-organisms and environmental factors in soil.

Fungi are more susceptible to radiation damage than are bacteria, whereas the bacterial vegetative cells and nonsporulating bacteria are more susceptible than bacterial spores. Most of the enzymes are still active in radiation sterilized soil (2 to 5 Mrad doses). Phosphatase activity decreases somewhat in sterilized soil; it may be conveniently studied (48,62). Urease activity increases upon irradiation sterilization (40,43) but tryptic activity is completely inactivated in sterilized soils (40). It was evident that the inactivation of phosphatase in soil follows the equation $A/A_o = e^{-kD}$ (62), but the k value for phosphatase is such that a considerable amount remains active in soil after sterility has been achieved. Increase of urease activity in irradiated soils suggests that the selective permeability of cell walls has been destroyed by the high dosage of radiation and that either the enzyme is released

from the cells or the substrate (urea) and reaction products easily penetrate the cell walls. However, Voets et al (63) showed that urease invertase, phosphatase and proteolytic activities were not influenced by a 2 Mrad gamma irradiation of air-dry sandy loam.

The efficiency of radiation sterilization has been compared with methyl bromide and steam sterilization by Eno and Popenoe (64) and a detailed description of experimental technique of soil irradiation has been presented by McLaren et al (48). Significant differences in microbial sensitivity to radiation were found in different soils. This phenomenon might be ascribed to a different "protective capacity" of the respective soils, where the organic matter content might have a major role, and also to differences of water content in an "air dry" state. This phenomenon could also be caused by a different soil microbial population, i.e., one soil having a more sensitive population than the other one. The number of bacteria in soils approached less than one organism per gram at about 2 Mrep doses, and that of fungi at about 0.3 Mrep. Considerably higher doses (up to 5 Mrep) were necessary for total sterility of soils in larger quantities, as a consequence of the exponential character of the microbial inactivation by radiation. The dosage necessary for total sterilization appears to be independent of the number of microorganisms initially present (65). The 4 to 5 Mrep dose, necessary for total sterilization of soils in 100 gram or larger single quantities, and the dose of 2.5 to 3.5 Mrep sufficient for single gram soil quantities conforms with the reported values by Peterson (45,46), Bowen and Rovira (47), Bowen and Cawse (49), and Monib and Zayed (66).

It is known, however, that radiation sterilized microorganisms have merely lost their ability to divide and that most of the biochemical activities might be present in the cells for some time. That this phenomenon applies also to soil microbes has been indicated by Peterson (45,46) who has shown that metabolic O_2 uptake and CO_2 release takes place for several days after soils have been sterilized by irradiation.

Work in soil sterilization by ionizing radiation indicates that this method can be used for a total microbial inactivation in soil; that it is a differential sterilization method, i.e., most enzymatic activities remain in the soil after bacterial inactivation has been achieved; and that such irradiated soil generally exhibits otherwise negligible changes in chemical and physical properties.

3. Soil Storage. Althought it is often of interest to determine enzymatic activities in fresh soils, it should be recognized that considerable changes in microbial numbers and enzyme activities take place in soils during handling and drying, and it is difficult to obtain reproducible results. During air drying considerable losses in enzymatic activities may occur, but once air dried, further losses in activity are usually minimal, even for extended periods of time.

Already in 1951 Kuprevich (67) pointed out that for representative results fresh soil samples should be used. Air dried soil, however, is being used extensively in the study of enzymatic activities. Several investigators have examined the influence of soil drying on its enzyme content and it appears that response to air drying is specific to the enzymes. As expected, the activity changes are also dependent on the temperature of drying and storage.

Jackman and Black (68) showed that phytase activity in soil was greater when the measurement was made on moist soil as sampled than on the soil sample partly dried at room temperature before analysis; phytase activity also changed with time after sampling. Geller and Dobrotvorska (69) noted reduction of phosphatase activity after air-drying of soil. During one year of storage of soils in an air-dry state, invertase activity decreased 15 to 20% but the changes in β-glucosidase activity were negligible (70). Decrease of invertase activity during storage was observed also by Kleinert (71) who suggested activity determinations soon after air-drying of samples. The effects of air-drying and refrigerated storage on invertase and amylase activities were reexamined by Ross (72). Activities of enzymes hydrolyzing sucrose (invertase) and starch (amylase) were lowered significantly in all air dried soils, except for some naturally arid soils; the reductions in activities resulted mainly from the initial drying at room temperature which also reduced the numbers of viable bacteria. Although invertase activity initially decreased on storage at $-20^{o}C$, further change was very slight on prolonged storage. Decrease of amylase activity at this temperature was greater and increased with length of storage. Inactivation was due partly to the effects of freezing and thawing and was greater in dry than in moist samples of soils. Activities were changed least by storage at $+4^{o}C$. Ross suggests that soil storage in a refrigerator is most suitable and air-drying least suitable for assays of these enzymes.

According to Tagliabue (73) freezing of soil appeared to increase urease activity. On the other hand, Vasilenko (74) showed that air-drying decreased urease activity in soils.

No general rules, however, can be established for storage and drying procedures; conditions should be established individually for each soil and with a consideration of the behaviour of the enzyme to be assayed. Even so, Latypova and Kurbatov (75) observed decrease in catalatic activity upon drying, whereas Baranovskaya (76) indicated that no substantial changes in catalase activity took place during drying.

4. Heat treatment. Soil is an excellent protective agent for microorganisms against heat and steam sterilization. Similar protective action is also exhibited towards enzymatic activities in soil. Inactivation of enzymatic activities in soil by heat requires high temperatures and longer periods than similar inactivation in pure preparations and solutions. Steaming is usually more effective than dry heat sterilization.

One of the most resistant enzymatic activities in soil is that of invertase, as noted by Hofmann and Seegerer in 1951 (77) and by other investigators later. After repeated steaming, activity still remained in soil which was destroyed by prolonged heating at 150°C or by autoclaving. Heated at 160°C, soil retained 1 to 4% β-glucosidase activity (78). Hofmann and Hoffmann (79) showed also that after 30 hours at 150°C, measurable amylase activity remained in sandy and gravelly soils and 25% of the initial activity in clay soils. Amylase, however, may be destroyed easily by autoclaving (21).

Urease activity could be destroyed by dry heat at 150°C after one hour (67), after prolonged heating at 85°C (80), or after steaming at 100°C for 80 min (81). Rotini (11) examined urease inactivation in soil at temperatures above 50°C and demonstrated that in 15 hours urease is totally inactivated in soil at 110°C. In samples kept at 58°C urease

activity increased, apparently due to lysis of microorganisms; similarly, at this temperature urease activity increased even more in the presence of toluene.

Many investigators have shown that by dry heating, steam heating or autoclaving only biological catalase-like activity may be destroyed, and thus separated from the non-enzymatic H_2O_2 catalytic decomposers.

Effect of temperature on the inactivation of soil enzymes was reexamined by Galstyan (82): for many soil enzymes inactivation proceeds at 60° to 70°C, whereas complete inactivation occurs at 180°C. Generally, the inactivation of the enzymes in soil occurs at approximately 10°C higher temperature (especially in the range below 100°C) than in solution.

IV. Characteristics and Determination of Individual Enzymes

Enzymatic activities detected in soils are listed in Table I. It should be emphasized that only a few enzymes have been extracted from soil (see Section VII) and most of the investigators cited do not claim to have detected free extracellular enzymes in soil, rather they claim to have detected specific enzyme-like activities, often without specific reference to origin or localization in soil.

A. Oxidoreductases

1. Dehydrogenases

The measurement of dehydrogenase activity in soil has been introduced to obtain correlative information on various biochemical activities of microorganisms in soil. Due to the biochemical properties of dehydrogenases, a free dehydrogenase in soil is hardly expected and the experimental procedures do not involve use of bacteriostatic or sterilizing

Table I. A select listing of enzymatic activities detected in soils.

Enzyme	Reaction catalyzed	References
	1. Oxidoreductases	
Dehydrogenases	$XH_2 + A \longrightarrow X + AH_2$	Lenhard (83)(84)(85) Stevenson and Katznelson (86) Stevenson (87)(88) Schaefer (89) Kozlov and Mikhailova (90) Kozlov (91) Hirte (92) Galstyan (93)(94) Casida *et al.* (95) Peterson (96)
Catalase	$2\ H_2O_2 \longrightarrow 2\ H_2O + O_2$	May and Gile (5) König *et al.* (4) Balks (97) Kurtyakov (98) Kappen (99) Osugi (100) Rotini (101) Valy (102) Ambroz (103, 104) Katznelson and Ershov (105) Ukhtomskaya (106) Radu (107) Kuprevich (67) Kuprevich and Shcherbakova (108) Sharova (109) Baranovskaya (76) Galstyan (110) Seifert (111) Vlasyuk *et al.* (112)

Enzyme	Reaction catalyzed	References
		Smolik (113) Scharrer (36, 37, 114) Valasco and Levy (115) Mashtakov *et al.* (116) Shumakov (117) Johnson and Temple (118) Runov and Terekhov (119) Weetall *et al.* (120)
Peroxydases and polyphenol oxidases	A + H_2O_2 $\longrightarrow$ oxidized A + H_2O	Galstyan (121) Kozlov (91
Catechol oxidase (phenolase, tyrosinase)	o-diphenol + $\frac{1}{2}$ O_2 $\longrightarrow$ $\longrightarrow$ O-quinone + H_2O	Kuprevich (67)
Diphenyloxidase	p-diphenol + $\frac{1}{2}$ O_2 $\longrightarrow$ $\longrightarrow$ p-quinone + H_2O	Trojanowski and Matwijow (122)
Glucose oxidase	glucose + H_2O + O_2 $\longrightarrow$ $\longrightarrow$ gluconic acid + H_2O_2	Galstyan (123)
Urate oxidase (uricase)	uric acid + O_2 $\longrightarrow$ $\longrightarrow$ unidentified prod., incl. allantoin and CO_2	Durand (124) Martin-Smith (125)

2. Transferases

Enzyme	Reaction catalyzed	References
Transaminase	$R_1R_2\text{-CH-NH}_3^+ + R_3R_4CO \longrightarrow$ $\longrightarrow R_3R_4\text{-CH-NH}_3^+ + R_1R_2CO$	Hoffmann (126)
Transglycosylases and levansucrase	n $C_{12}H_{22}O_{11}$ + HOR $\longrightarrow$ $\longrightarrow$ $H(C_6H_{10}O_5)_nOR$ + n $C_6H_{12}O_6$	Drobnik (21) Hoffmann (126) Kiss and Peterfi (127) Kiss (128)

Enzyme	Reaction catalyzed	References
	3. Hydrolases	
Phosphatases (phosphomono-esterases)	Phosphate ester + H_2O → → R-OH + PO_4^{-3}	Rogers (17) Skujins *et al.* (62) Vlasyuk *et al.* (112) Kroll *et al.* (129) Drobnikova (130) Overbeck and Babenzien (131) Novogrudskaya (132) Mazilkin and Kuznetsova (133) Kramer and Erdei (134, 135) Kiss and Peterfi (136) Keilling *et al.* (137) Halstead (138) Ramirez-Martinez and McLaren (139)
Pyrophosphatase	pyrophosphate + H_2O → → 2 orthophosphate	Rotini (10)
Metaphosphatase (incl. poly-phosphatase)	hydrolysis of polymeta-phosphate to ortho-phosphate	Rotini and Carloni (140)
Phytase	*myo*-inositol hexaphosphate + 6 H_2O → *myo*-inositol + 6 H_3PO_4	Jackman and Black (68)
Nuclease		Rogers (17) Mazilkin and Kuznetsova (133)
Acetylesterase	Acetic ester + H_2O → → alcohol + acetic acid	Haig (35)
Lipase	Triglyceride + 3 H_2O → → glycerol + fatty acid	Pokorna (141)

Enzyme	Reaction catalyzed	References
Amylase (α- and β-)	hydrolysis of 1,4-glucosidic bonds of polyglucosans	Hofmann and Hoffmann (79) Ross (72, 142) Peterson (143) Overbeck and Babenzien (131) Kuprevich (67) Galstyan (23) Drobnik (21)
β-fructofuranosidase (invertase, saccharase, sucrase)	β-fructofuranoside + H_2O ⟶ ⟶ R-OH + fructose (β-fructofuranoside usually sucrose)	Scheffer and Twachtmann (144) Gettkandt (145) Vlasyuk _et_ _al_. (112) Ross (72, 142) Kuprevich and Shcherbakova (108) Nagata and Matsuda (146) Novogrudskaya (132) Nowak (147) Overbeck and Babenzien (131) Peterson (143) Shumakov (117) Peterson and Astafeva (148) Kleinert (71) Galstyan (23) Kiss (149) Hofmann and Seegerer (77)
α-glucosidase (maltase)	α-R-glucoside + H_2O ⟶ ⟶ R-OH + glucose	Hofmann and Hoffmann (150, 151) Kiss (152) Kiss and Peterfi (153, 154)

Enzyme	Reaction catalyzed	References
β-glucosidase (emulsin, cellobiase, gentiobiase)	β-R-glucoside + H_2O ⟶ ⟶ ROH + glucose	Hofmann and Hoffmann (78, 150) Galstyan (23) Galstyan and Vardanyan (155) Peterson (143)
α-galactosidase (melibiase)	α-R-galactoside + H_2O ⟶ ⟶ ROH + galactose	Hofmann and Hoffmann (151)
β-galactosidase (lactase)	β-R-galactoside + H_2O ⟶ ⟶ ROH + galactose	Hofmann and Hoffmann (151) Kiss and Peterfi (154)
Cellulase	Hydrolyzes β-1,4-glucan links in cellulose	Markus (156) Sörensen (157)
Lichenase	Hydrolyzes β-1,3-cellotriose links	Kiss *et al.* (158)
Xylanase	Hydrolyzes β-1,4-xylan links	Sörensen (157, 159)
Proteases	Hydrolysis of proteins to peptides and amino acids	Fermi (7) Hofmann and Niggemann (160) Hoffmann and Teicher (161) Katznelson and Ershov (105) Voets and Dedeken (162) Peterson (143)
Cathepsin and pepsin		Antoniani *et al.* (163) Ukhtomskaya (106) Ambroz (164) Vlasyuk *et al.* (112)
Trypsin		McLaren *et al.* (40)

Enzyme	Reaction catalyzed	References
Asparaginase	Asparagine + H_2O → → aspartate + NH_3	Drobnik (165) Galstyan and Tsyupa (167) Mouraret (166) Kuprevich (67)
Amidase (deaminase)	monocarboxylic acid amide + H_2O → monocarboxylic acid + NH_3	Subrahmanyan (9)
Urease	$CO(NH_2)_2 + H_2O$ → → $2\ NH_3 + CO_2$	Rotini (11) Galstyan and Tsyupa (167) Conrad (80, 168, 29) Kuprevich (67) Hofmann and Schmidt (169) Scheffer and Twachtmann (144) Drobnik (170) McLaren *et al*. (40) Porter (171) Vlasyuk *et al*. (172) Hoffmann and Teicher (173) Vasilenko (74) Van Niekerk (174) Stojanovic (175) Novogrudskaya (132) Galstyan (23, 176)
Cyanase (?)	$NH_4CNO + H_2O$ → → $2\ NH_3 + CO_2$	Rotini (177)

4. Lyases

Enzyme	Reaction catalyzed	References
Aspartic acid decarboxylase	aspartic acid → alanine	Drobnik (165)

agents. Dehydrogenase tests are utilized to obtain information on the biological activities of microbial population in soil, rather than on the enzyme *per se*.

The dehydrogenase test in soil consists of the measurement of the reduction of 2,3,5-triphenyltetrazolium chloride (TTC) to triphenylformazan. It was first introduced by Lenhard (83,84) to measure the activity of soil microorganisms. Generally, to 10 g of soil 0.1 g $CaCO_3$, a metabolite (although usually not a direct H-donor in a strict sense), and a 1% solution of TTC is added. The soil is incubated at 37°C anaerobically (waterlogged) for 24 hours; at the end of the incubation period triphenylformazan is extracted with water and its absorbance determined. Several modifications of this method have been described by Stevenson (87), Galstyan (93), and Kozlov and Mikhailova (90). It appears, that the procedure may be performed successfully also under aerobic conditions in soil as Casida *et al* (95) have indicated that the presence of atmospheric oxygen does not affect the TTC method.

High activity may be obtained also without any additions of metabolites in the experimental procedure and the results in such cases reflect endogeneous respiration, as has been shown by Casida's group and others (89, 92, 94). Generally, the activity does not reflect plate counts in non-amended soils, but by an addition of nutrients and metabolites, dehydrogenase activity increases with increasing microbial numbers.

Stevenson (87) has demonstrated that an apparent inhibitor for dehydrogenase activity may be leached from the soil. Addition of Coenzyme I increases dehydrogenase activity (94). Galstyan (94) also suggests that soils contain substrate-specific dehydrogenases. Ethyl

alcohol, for example, cannot be used as an electron donor by soil dehydrogenases.

The dehydrogenase activity in soil may be eliminated by treating the soil with chloroform. Although a non-biological TTC reduction occurs in soil samples above 65°C, the formazan release at the biological temperatures used in assays (30° to 37°C) is due to biological activity only (95).

Correlation between the 2,4-D addition to soils and its effects on various biological phenomena and dehydrogenase activity in soil has been examined by Lenhard (178).

Fertile, cultivated soils exhibit high dehydrogenase activity, in saline and in high pH soils the activity is negligible.

2. Catalase

Catalase activity may be measured by the release of O_2 from the soil after addition of H_2O_2, or the residual H_2O_2 may be titrated with $KMnO_4$ or other suitable reagents.

Initial work on the examination of catalaze activity in soils by König et al (4), May and Gile (5) and others was done by manometric methods. Usually, a 3% H_2O_2 solution is added to soil and the activity of catalase is related to the rate of O_2 produced at room temperature. A standard procedure was described by Kuprevich (67) and later an improved method by Kuprevich and Shcherbakova (108). Vlasyuk et al (112) made the assays on soil suspended in a pH 6.9 phosphate buffer, and Seifert (111) determined the O_2 release at 2°C. Most of the work in the USSR has been done following Kuprevich's method and its modifications.

An improved volumetric method for catalase determination in soil has been recently described by Weetall et al (120).

The titrimetric method was first introduced by Kappen (99) and later modified by Osugi (100), Rotini (101) and others. After an incubation of soil with 0.2% (103, 104) to 2% (105) solution of H_2O_2, the residual peroxide is titrated with $KMnO_4$ in presence of H_2SO_4. The permanganate method has been used also by Johnson and Temple (118); Verona (179) used $KI-Na_2S_2O_8$ titration to assay the residual peroxide.

Catalase activity in soils is associated with high organic matter content. The highest catalase activity is found in litter-accumulating surface layers and in humus-accumulating A horizons, and a sharp decrease is noted at deeper levels. Seasonal variations usually are not evident, although sometimes the catalatic activity increases towards autumn (119). It is also found that catalatic activity is stronger in alkaline and calcareous soils rather than in acid soils.

Catalatic activity in soils has been related to microbial numbers in soil and to vegetation and also associated with non-biological, inorganic or organic catalysts. Increase of catalatic activity in soils due to microbial proliferation has been indicated by Runov and Terekhov (119) and the positive correlation of catalatic activity with microbial numbers in soils has been asserted further by several authors (109,180, 181). Weetall et al (120) have devised a quantitative method for detection of soil microorganisms based on the catalatic activity of lysed organisms in soil.

Zemlyanukhin (182) suggested, however, that catalase activity in soil was dependent more on the presence of vegetation than on the

microbial numbers. Germinating seeds of various plants considerably increased the catalatic activity in soils; Verona (179,183) has termed this phenomenon the "seed effect".

It is evident that a large portion of the catalatic type of peroxide decomposition in soil is non-enzymatic. Autoclaving of soil inactivates the peroxide-decomposing capacity only partially. Up to 40% of the total "catalase activity" may be thermo-stable, i.e., non-biological. Sharova (109) suggests that most of the non-biological activity is due to manganese compounds in soil, Baraccio (180) ascribes this activity to iron compounds and colloids. The importance of non-biological peroxide decomposition in soil has been emphasized also by Vigorov (181) but he concludes that soil fertility is proportional to the amount of thermolabile catalase.

The contribution of various factors in soil on the peroxide decomposition was critically re-examined by Johnson and Temple (118) and the enzyme kinetics of catalase in soils were examined by Velasco and Levy (115).

3. Peroxidase and polyphenoloxidases

The activities of these enzymes have been little studied in soils, although polyphenoloxidases, including *p*-diphenol oxidase, appear to be instrumental in the humification process.

Presence of peroxidase in soil was indicated already in 1905 by Cameron and Bell (6). Phenoloxidase (catecholoxidase, tyrosinase) was noted in soil by Kuprevich in 1951 (67). Methods for peroxidase and polyphenoloxidase determinations in soil have been published by Galstyan (121,184), who used pyrocatechol in presence of oxygenated water and by

Kozlov (91). Galstyan indicates that activities of these enzymes are higher in carbonate containing than non-carbonate soils and they are dependent on numbers of soil microorganisms. The activities of these enzymes change with the type of vegetation (crop) and with the seasons.

The appearance of extracellular p-diphenyloxidase in the process of humification has been demonstrated by Trojanowski and Matwijow (122).

4. Glucose oxidase

Galstyan (123) described a method for β-glucose oxidase determination in soil and demonstrated that the activity of this enzyme is present in a variety of soils. Surface layers showed the highest activity and it decreased gradually to zero at 1 to 1.5 m depth.

5. Urate oxidase (uricase)

Presence of urate oxidase in soil was demonstrated by Durand (124). Martin-Smith (125) succeeded in extracting the active fractions from soil. There appeared to be two active uricolytic components, extractable with 0.1 M phosphate at pH 7 and pH 8.4, resp. The uric oxidases apparently are extracellular enzymes released by microorganisms in uric acid enriched soils. It is evident that further metabolism of the products of uric acid degradation by urate oxidase, namely, allantoine and allantoic acid takes place intracellularily by microorganisms (124). Both urate oxidase and uric acid may be adsorbed by clays, and the uric acid degradation occurs at a significant rate only if the substrate and enzymes are desorbed (185).

B. Transferases

It was noted in 1955 by Drobnik (21) that during hydrolysis of starch and maltose by soil enzymes not only glucose was released but also a new buildup of oligosaccharides took place by transglucosidation. Further evidence has been collected by Hoffmann (126,186) and by Kiss and collaborators (127,128,187) that such synthetic processes take place in a variety of soils although the rates are rather slow. Hoffmann (186) has shown that after an initial decrease of maltose as a substrate added to the soil, upon further incubation for eight hours synthetic polymeric products appear. Maltotriose and maltotetrose increase continuously during incubation under aseptic (toluene) conditions and other types of carbohydrates are formed from the 5th day on.

Hoffmann, as well as Kiss used chromatographic methods for the detection of oligosaccharides. Kiss and Peterfi (127) showed that due to an enzymatic action in soils not only fructose and glucose were formed from sucrose (i.e., invertase activity) but also various oligosaccharides appeared. In the presence of methanol a β-methylfructofuranoside was detectable. A presence of a levan sucrase in soils was indicated by Kiss (128) and a subsequent work (187) showed that levan sucrase activity was inhibited by m-dinitrophenol, whereas other phenol compounds showed a lesser inhibitory activity. These authors concluded that enzymatic processes in the formation of humic acids may influence the formation of levans, by establishing equilibrium conditions of the release and formation of monomeric and polymeric phenolic compounds. Thus enzyme reactions may influence the aggregation of soil particles by levans.

Experimental evidence by several authors (188) suggests that enzymes produced in soil by microorganisms, and also by plants, are instrumental not only in decomposition but also in synthesis of high molecular weight humic substances.

Although it is evident that specific transglucosidases and possibly transfructosidases have been detected in soils, it should be noted that much of the synthetic action may be due to invertase, as it has been shown that invertase possesses transfructosidase properties similar to other hydrolytic glucosidases (189).

Transaminase activity in soil was examined by Hoffmann (126). He demonstrated that in a toluene treated soil alanine was formed by a transamination reaction from a pyruvate in presence of leucine, valine, glutamic acid and aspartic acid.

C. Hydrolases

1. Phosphatases (phosphomonoesterases)

Already in 1932 Rotini had suggested (10) that transformations of organic phosphates in soil were caused by enzymes and the presence of phosphatase (phosphomonoasterase) activity in soil was demonstrated by Rogers (17) who suggested that the phosphatase in soil was excreted from plant roots.

In the earlier work on phosphatase activity determination in soil, methods were used in which the inorganic phosphate released from substrates was assayed and correlated with enzymatic activity. See, for example, Jackman and Black (68), Mortland and Gieseking (190), Rogers (17, 191), and in studies on crop-rhizosphere and soil phosphatase activity

interrelationships by Nilsson (192) and by Vlasyuk et al (112). Mortland and Gieseking (190) concluded that phosphatase activity in soils was inhibited by addition of clays, such inhibition being proportional to the base exchange capacity of the clay; however, Kroll and Kramer (193) pointed out that their assay method for phosphate did not take into account fixation of phosphate by soil clays. Kroll and Kramer used phenylphosphate as the substrate and the phosphatase activity determination was based on released phenol. This method was used by Kramer (194) and Kramer and Erdei (134,135) in their later studies on the correlation of phosphatase activity and soil fertility. The use of phenolphthalein phosphate as a substrate has been described by Krasilnikov and Kotelev (195). Drobnikova (130) studied the phosphatase activity in soil with respect to pH and assayed the inorganic phosphate released from the substrate; however, the phosphate fixation by soil was determined separately. Vlasyuk et al (112) studied the rhizosphere effect on phosphatase activity and also determined it by the amount of the released phosphate; ascorbic acid was used as the extracting agent. Phosphatase activity measurements based on the determination of nonreacted glycerophosphate have been described by Skujins et al (62) and similarly, on the released glycerol by Kiss and Peterfi (136).

Phosphatase activity in soils has been studied by a great many investigators. Nevertheless, the published reports are abundant in contradictory observations and interpretations. Most of the observations show that the maximum activity of phosphatases in soil are near the neutral pH values and not necessarily at the natural pH of the soils

examined. Several investigators suggest, however, that alkaline and acid phosphatases may be separately observed in soils (138), or that there are present even acid, neutral and alkaline phosphatases (196). In some soils the activity may increase with increasing pH (130). Keilling et al (137) have reported a positive correlation between "alkaline" phosphatase activity and the levels of nitrogen and carbon in soils. They found no correlation between phosphatase activity and nitrogen content or bacterial population in organic manures. However, other investigators (69,196) have shown that addition of manure, compost or glucose to soils increase phosphatase activity. Similarly, phosphatase activity is higher in soils containing higher amounts of organic matter (138). It is evident that phosphatase activity in soil is inversely proportional to biologically available phosphate. Addition of inorganic phosphate fertilizers almost invariably decreases the phosphatase activity. Even in organic soils the phosphatase activity is similarily associated with phosphate availability (197). Generally, though, addition of mineral and especially organic fertilizers increases the activity (198).

Krasilnikov and Kotelev (195) have demonstrated that phosphatase is produced by a large number of soil bacteria and it has been shown by several investigators (69,196,197) that phosphatase accumulates in soil as a result of microbial activity. Contribution to phosphatase activity in soils by fungi has been studied by Janossy (199) and by Casida (200). They have suggested that a rather high contribution of phosphatase activity in soils is due to soil fungi. However, Kotelev et al (201) indicate that in certain soils the phosphatase activity of soil and rhizosphere bacteria and actinomycetes was greater than that of fungi,

and Kramer and Erdei (134) show that the amount of carbohydrate consumed during composting has no direct relationship between phosphatase activity and total microbial activity.

Various aspects of phosphatase activity in soils have been reexamined by Drobnikova (130) and by Burangulova and Khazierv (202) who conclude that phosphatase activity is not identical in different soils and is dependent on their genetic and physico-chemical properties.

A detailed examination of factors involved in the determination of phosphatase activity in soils by Ramirez-Martinez (203) showed that it is of utmost importance to evaluate properly the analytical methods used in activity determinations. Considerable variations in the phosphatase activity in the same soil are introduced by performing assays on the soil collected and stored at various moisture contents and drying procedures. In all assay procedures some fraction of substrate or hydrolysis products are adsorbed by soil particles and the adsorption characteristics must be determined separately. It is advisable to use substrates which would give reasonable results in short incubation times. For example, a fluorometric assay of β-napthol, the hydrolysis product of β-napthyl-phosphate, is rapid (139). Most of the soils tested show the highest activity around neutral pH, and not necessary at the natural pH of the soils. Some soils may show the presence of an "alkaline" phosphatase. An important criterion for the detection of alkaline phosphatase is the use of the same buffer system throughout the pH range (e.g., Ostling and Vitama's Universal buffer) different optimum pH values for soil phosphatase may be found when different buffer systems are used. Ramirez-Martinez's work also shows that there is no significant correlation between microbial and phosphatase activities in soils.

2. Pyro- and polymetaphosphatases

A presence of pyrophosphatase activity in soils has been reported by Rotini in 1932 (10). Rotini and Carloni (140) also studied hydrolysis of polymetaphosphates. In a toluene treated soil the hydrolysis was decreased by 16% as compared with non-treated soil; soil heated at 105°C for 80 hrs. still retained 66% of the original hydrolytic activity.

3. Phytase

Phytase activity in soils was examined by Jackman and Black (68). Phytase was determined by assaying inorganic phosphate produced from added phytate after 20 hours incubation at 45°C in the presence of citrate, at pH 5. Ten drops of toluene were used per 5 g of soil suspended in 20 ml of buffer and substrate solution. Phytase activity followed the microbial activity in soils.

Phytase activity of isolated soil microorganisms was examined by Greaves *et al* (204); the composition of their substrate apparently was different from that used by Kotelev *et al* (201) in a similar work, or from that used by Jackman and Black. It is known that commercial phytate contains considerable amounts of lower esters. Evidence has been presented (205) that tetraphosphophytates may be dephosphorylated by phosphatases which are unable to attack the penta- and hexaphosphophytates. The published results on phytase activity in soils should be evaluated accordingly.

4. Nucleases

Degradation of nucleic acids in toluene treated soils was studied by Rogers (17). High rates of inorganic phosphate release was obtained at pH 7 and at 60°C, giving evidence for presence of ribonuclease,

nucleotidase or deoxyribonuclease in soils. Similar results, but with considerably lower rates of hydrolysis were obtained by Nilsson (192) at 28°. Mazilkin and Kuznetsova (133) examined bacterial flora of forest soils with respect to its contribution to phosphatase and nuclease activity. Generally, the activities were quite low, and only a few species showed ribonuclease and deoxyribonuclease activities.

5. Acetylesterase

The studies of acetylesterase activity in soils by Haig (35) indicated that the catalytic activity of the decomposition of phenyl-acetate was due to an extracellular enzyme. The acetylesterase activity was predominantely associated with a specific clay fraction of soil. Hydrolysis of ethyl butyrate was much slower but was evident after prolonged incubation. Haig concluded that since all assays were carried out with toluene as an antiseptic agent, the long incubation time required for ethylbutyrate (and urea) hydrolysis indicated microbial activity rather than extracellular enzyme activity. Sterilization of soil with ethylene oxide reduced acetylesterase activity by half, while urease activity and the ability to hydrolyze ethylbutyrate was completely destroyed.

6. Lipase

Lipolytic activity in several peats and muddy soils has been studied by Pokorna (141). The activity was higher in peats than in muds and a presence of a lipase in these soils was suggested.

7. Amylases

Presence of amylases in soil was first indicated by Kuprevich (67). Methods for amylase detection in soils have been developed by Drobnik (21)

and by Hofmann and Hoffmann (79): 20 ml of 2% soluble starch solution in a buffer are added to 10 g of air-dry soil, and the suspension is incubated with 1.5 ml toluene for 96 hours at 37°C. The released reducing sugar is determined by the Lehmann-Maquenne thiosulfate method, as modified by Schoorl-Regenbogen. It is evident (79) that soils contain mostly β-amylase rather than α-amylase. The amylase activity could be typically increased by addition of sodium chloride.

Other aspects of the amylase activity in soils have been examined by Peterson (143), Ross (72,142), Galstyan (23), and Markosyan and Galstyan (206). The amylolytic activity of soils, based solely on the presence and proliferation of soil microorganisms has also been studied by Augier and Moreau (207).

It is apparent that amylase (and invertase) is produced adaptively in soil (21). Amylase activity increases with increasing organic matter content in soil and it may also be correlated with cation-exchange capacity (79). In various soils, however, the maximum activity appears to be at the same pH values: 5.5 to 6.0.

8. β-Fructofuranosidase (invertase, saccharase, sucrase)

The activity of invertase in soils has been widely studied by many investigators. The basic method for the assay of invertase was published by Hofmann and Seegerer (77): 20 ml of 10% sucrose solution in a buffer, pH 5.5, is added to 20 g of soil, containing 2.5 ml toluene. After incubation at 37°C for 24 hours the reducing sugar released is determined with an appropriate method, usually by the Lehmann-Maquenne titration method or gravimetrically. A colorimetric method for soil invertase determination, based on the color developed by Fehling's solution, has

been developed by Gettkandt (145). A polarimetric method has also been used by Kiss (149,208).

Generally, the investigations have shown that invertase activity is closely associated with microbial numbers and metabolic activities in soils. Usually the highest activity may be found in neutral, calcareous soils; cultivated neutral soils have high activities, but the activity decreases in sandy and in acid soils. Decrease of invertase activity down the profile in many soils parallels the decrease in humus content (209). However, in individual cases no correlation between invertase activity, pH, and humus content has been noted, although the activity decreases with depth (210). Davtyan (211) noted that high invertase activity was associated with a low catalase activity and vice versa.

Although generally a correlation between the invertase and microbial activity in soils is evident, contradictory data have been presented by Nowak (147); he did not find such a correlation and concluded that the addition of toluene did not inhibit microbes sufficiently to separate enzymatic and microbial activities. Invertase activity also tends to increase under vegetation and decrease in the subsequent fallow (212); also germinating seeds increased invertase activity in soil similarly to catalase as shown by Verona (213). It is evident that irrespective of microbial contribution to invertase activity, as shown, for example, by Kiss (214), plant roots and possibly rhizosphere organisms (211) contribute considerably to soil invertase.

Kiss (214,215), and Kiss and Balint (216) have examined the factors influencing the activation and inhibition of invertase in soils. Invertase was inhibited by characteristic invertase inhibitors: $HgCl_2$,

aniline, p-toluidine, formaldehyde, but not by methylene blue or streptomycin and other antibiotics; also, various co-factors did not affect the activity. Invertase is strongly adsorbed by soil particles, addition of clays stabilizes invertase activity. However, invertase in living and partially autoclaved yeast cells added to the soil was partially inactivated and that in cell-free autolysates was inactivated completely upon addition to soil.

It is interesting to note that a decrease in the invertase activity in soils carrying hops has been associated with the accumulation of bacteriostatic substances contained in the crop's roots and adsorbed by the clay minerals (217).

9. α-Glucosidase (maltase)

α-Glucosidase hydrolyzes maltose by acting as an α-glucotransferase. It was first detected in soils by Hofmann and Hoffmann (150,151) by using α-phenylglucoside as the substrate and by assaying the formed reducing sugar with the Lehmann-Maquenne method. A polarimetric method was used by Kiss (152) who later used also paper chromatography (153,154) to detect the products of hydrolysis.

The α-glucosidase activity usually is considerably smaller than the invertase activity (152) and lesser than activities of other carbohydrases (151). A study of the inhibition of soil α-glucosidase showed that even at very high concentrations of biological inhibitors (dihydrostreptomycin, $AgNO_3$, $HgCl_2$) the inhibitory activity on α-glucosidase was only partial (153). It is apparent that soil exerts a protective effect on the enzyme. Addition of maltose to soils results in an increased production of α-glucosidase by soil organisms.

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10. β-Glucosidase (emulsin, cellobiase, gentiobiase)

β-Glucosidase has been detected in soil (78,150) with salicin, arbutin, and β-phenylglucoside as substrates. Galstyan (23) showed that no assimilation of hydrolysis products by microorganisms took place during the incubation of soils according to the method by Hofmann and Hoffmann in the presence of toluene. He confirmed this conclusion by showing that the β-glucosidase activity in soil has a zero-order reaction rate similar to urease, invertase and amylase. The maximum activity of β-glucosidase in several soils appears to be at pH 5.9 to 6.2 (206).

11. Galactosidases

α-Galactosidase (melibiase) and β-galactosidase (lactase) were both detected in soils by Hofmann and Hoffmann (150,151) with the respective phenylgalactosides as substrates. Kiss and Peterfi (154) examined the β-glucosidase activity by means of paper chromatography and noted that the relative activities of α-glucosidase and β-galactosidase in soils depend on the substrates used. α-Glucosidase had smaller activity than β-galactosidase with α-phenylglucoside and β-phenylgalactoside, resp., as substrates. However, with maltose or lactose the activities of the respective enzymes were reversed, i.e., β-galactosidase had larger activity than α-glucosidase in the same soil.

12. Cellulase

Markus (156) observed significant differences in cellulase activity between toluene treated and non-treated soils in total activity and in response to pH. Presence of an extracellular cellulase in soil has been suggested by Sörensen (157).

13. Lichenase

Lichenase activity was found in 9 out of 10 tested soils by Kiss *et al* (158). Only one of these soils showed cellulase activity. These

authors suggest the use of lichenin as a substrate for the determination of the cellulase activity in soil; however, this suggestion should be reevaluated in view of the available information regarding lichenase specificity (218).

14. Xylanase

Sörensen (157,159) incubated soil with pH 6.2-6.5 phosphate and a xylan solution for 24 hours at 37°C; the released reducing sugar was determined with Somogyi reagent. The amount of xylanase in the soil appears to be primarily a function of the amount of xylan in the soil giving rise to an accelerated excretion of the adaptive enzyme, xylanase by microorganisms.

15. Inulase

Presence of inulase in soil has been indicated by Kiss and Peterfi (219) who suggest that inulase and other carbohydrases, with an exception of invertase, are released in the soil solely by microorganisms.

16. Proteinases

In 1910 Fermi (7) extracted a proteolytically active fraction from soil, with phenol, which hydrolysed gelatine. A fraction having activities similar to pepsin and cathepsin was isolated by Antoniani et al (163).

For most of the investigations in the proteolytic activities in soil, gelatin, casein and peptone wave have been employed (103,104,112). Ambroz tried ovalbumin, gelatin and casein (164) and found that gelatin was hydrolysed in all soils tested, whereas casein hydrolysis was less active in acid soils and was absent in acid peats. McLaren et al (40) demonstrated the presence of a trypsin activity in soils by assaying with a specific substrate, benzoylargenineamide.

Several methods for proteolytic activity determination in soils have been described. Hofmann and Niggemann (160) based their method on the rate of liquefaction of gelatin. Hoffmann and Teicher (161) incubated 10 g of soil with 20 ml of 2 per cent gelatin solution and 1.5 ml toluene for 20 hours at 37°C; the released amino acids were determined photometrically by complexing with Cu^{++}. A similar method based on hydrolysis of gelatin has been described by Voets and Dedeken (162) who bioassayed only the release of arginine with *Leuconostoc mesenteroides*.

As with invertase, proteolytic activity decreases with depth in profile, and increases with increasing humus content (161,220). Proteinase activity in general is higher in grassland and in humus rich soils than in cultivated, mineral, or fallow soils (161,221). Proteinase activity varied during the vegetative period; it was also correlated with the moisture content (222). The proteolytic activity decreases considerably in soils during storage. Tryptic activity is destroyed by irradiation-sterilization, although other enzymes can still be detected at a nearly unreduced level in these soils (40).

17. Asparaginase

Presence of asparaginase in soils was first indicated by Kuprevich (67). Drobnik (165) used Conway diffusion technique for the asparaginase detection. An extensive and detailed study of asparaginase activity in soils has been presented by Mouraret (166).

18. Amidases (deaminases)

Deaminases in soils were studied by Subrahmanyan in 1927 (9). An especially strong deaminating activity was observed towards glycine.

This activity was present in most soils and he apparently was able to extract the active fraction.

19. Urease

Urease in soils was first examined by Rotini (11) and in early 1940-ies by Conrad (80,168). In the last decade several new methods have been developed for the determination of urease activity: titration (144,169) and Conway's microdiffusion method (40) for released ammonia, and xanthhydrol (67,172) or p-dimethylbenzaldehyde (171) methods for the residual area.

Urease activity in the soil appears to be correlated in general with the number of microbes in soil (223,224) and the activity is increased with increasing organic matter content (225). There exists free urease in soils, which may be extracted (14); thus it might be possible to distinguish the activities of a "free" urease and the same associated with microbial metabolism (223). Free urease exists also in manures (226).

The maximum activity of soil urease in most soils is at pH 6.5 to 7.0 (169). In alkaline soils urease activity decreases considerably, and the activity is decreased also in carbonate rich soils, apparently due to the detrimental effect of Ca^{++} on urease producing organisms (176). By adsorption of urease on clays, the activity shows a pronounced shift towards a higher pH value (227).

Urease activity is considerably higher in the rhizosphere and it is dependent on the particular plant species (172,211); considerable seasonal variation also may be noted (175).

Upon air drying of soil a part of the urease activity is irreversibly inactivated (74). Urease is also inactivated by prolonged heating of soil, but a subsequent reactivation might occur, which has been ascribed to the metabolic activities of the surviving and germinating spores (81,228). Urease in soil is very stable towards sterilization of soil by high energy irradiation (40,174) and the urease behavior in a soil, sterilized in this manner, may be conveniently studied in absence of microbial activity.

Toluene in amounts normally added to soil in enzymatic studies, increases urease activity. It has been suggested that this effect may be due to the proliferation of microorganisms (229), although it is reasonable to assume that urease is released from microorganisms in presence of toluene, a plasmolytic agent. On the other hand, a study of urease activity of intact and disrupted bacteria has shown that for most species the urease activity is the same in both (230).

The adsorption of the substrate for urease, urea, is negligible in the soil (231).

Ammonium cyanate, NH_4CNO, is an isomeric form of urea. Rotini (177) has indicated by his studies that a specific enzyme, cyanase, would decompose any added or isomerically formed ammonium cyanate in soils.

D. Lyases

In his studies on the enzymatic decomposition of asparagine in soils, Drobnik has indicated (165) that an asparatic acid decarboxylase might exist in soils which decarboxylates aspartate to form alanine.

It should be noted here that the release of CO_2 from soil might be caused by various factors other than biological respiration processes.

Several aspects of the biological and nonbiological decarboxylation processes in soil and the principles of detection have been described, for example, by Hofmann and Hoffmann (232) and by Beckmann and Scharpenseel (233), among others. Studies on carbon dioxide development in soils prompted Stotzky and Norman (234) to suggest that the initial degradation and oxydation of glucose in soils was not accomplished by cell-free enzymes as suggested by Drobnik (21) but rather by microorganisms.

V. Origin of Soil Enzymes

Studies in soil enzymology performed in presence of bacteriostatic and plasmolytic agents, e.g., toluene, show the activities of metabolizing (but non-dividing) microorganisms and of enzymes released by plasmolysis, as well as the activities of any accumulated extracellular enzymes in soil and any catalytic activities that may be exhibited by the inorganic soil constituents. One of the primary questions in soil enzymology is the elucidation of the problem of release and accumulation of extracellular enzymes in soil. The biochemical activities of microorganisms *per se* fall in the realm of soil microbiology rather than enzymology.

There are three apparent sources of free enzymes in soil: 1) enzymes released as extracellular enzymes by proliferating microorganisms and enzymes eventually released in soil upon death (i.e., due to changing permeability of cell walls) of microbes, 2) enzymes similarily released by soil animals, and 3) enzymes released by plant roots and other plant residues.

Many investigators have tried to correlate enzymatic activities in soil with microbial numbers and activities, or with prevailing vegetation.

Any positive correlation between those factors has been interpreted as an indication of the microbial or plant origin of the enzymes in soil. However, many other factors may enter the relationship between enzymatic activity, vegetation, and microbial activity. For example, enzymatic amounts in the rhizosphere may be quite different than those in non-rhizosphere soil due to a different microbial population. The population in turn is regulated by the respective prevailing vegetation.

Numerous microorganisms produce extracellular enzymes. Most of these enzymes catalyze breakdown of high molecular weight compounds. The large polymeric molecules are unavailable for direct assimilation by the microbial cells and the extracellular microbial enzymes fulfill the same function in microbial nutrition as the various enzymes released in digestive tracts of animals: the organic polymers are degraded to lower molecular weight compounds which may be assimilated through cell walls.

Often it is difficult to decide even in pure culture whether an enzyme is truly extracellular or whether it has been released upon autolysis of cells. Studies on *Aspergillus oryzae* (235), among others, have shown that enzymes are released to the medium in a certain sequence: first, the carbohydrases and phosphatase, then the proteases and esterases, and finally, catalase. Some enzymes were released during the initial growth phase, but others at a later phase, when the mycelial weight was declining. For the purposes of the present discussion it is of interest to note that catalase, which may be considered as a typical endocellular enzyme, has been found free in the medium.

Release of various extracellular carbohydrases by microorganisms in synthetic and natural media has been extensively studied and the

results have received excellent reviews by Phaff (236). Amylases, cellulases, pectic enzymes and also proteolytic enzymes are released by numerous bacteria and fungi; dextranase has been demonstrated in several Penicillium cultures by Kobayashi (237), production of xylanase by Streptomyces (238), and other pentosanases by fungi and Bacillus has been demonstrated by others (239,240). Extracellular production of various lignin decomposing enzymes, polyphenol and diphenol oxidases ("laccase") by wood rot and soil inhabiting hymenomycetes has been extensively examined by Fahraeus and coworkers (241), and by Lindberg and Holm (242), Van Vliet (243), Trojanowski and Matwijow (122) and others.

Extracellular chitinase production by Streptomyces was demonstrated by Jauniaux (244). Chitinase producing soil bacteria have been examined by Gehring (245) and Clarke and Tracey (246). Extracellular, soil inhabiting Streptomyces β-1,3-glucanase and chitinase in combination lyse fungal hyphae walls (247).

Non-phosphorolytic oligo- and polysaccharide synthesizing enzymes (transferases) are produced extracellularly by a number of soil inhabiting bacteria and fungi. Levan sucrases, for example, have been isolated from the culture liquid of Bacillus asterosporus and Azotobacter chroococcum (248) and extracellular Aspergillus, Penicillium, Myrothecium and Bacillus subtilis transglycolases, which synthesize oligosaccharides, have been extensively examined (235).

Many soil studies are based on invertase activity. In microorganisms, invertase normally occurs as a cell-surface enzyme. However, Wickerham (249) and Dworschack and Wickerham (250) have shown that several species of Saccharomyces and Hansenula anomala produce extracellular invertase.

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Extracellular invertase was also produced in an early growth phase by Myrothecium verrucaria (251). It appears, though that plants may be the major contributors of invertese activity in soils. For example, Knudson (252) found that invertase is secreted by plant roots. This observation has been later verified by several investigators, notably by Krasilnikov (253) and Ratner and Samoilova (254).

Enzymes involved in phosphate metabolism may also appear extracellularily. Ribonucleases (255) and alkaline phosphatase (256) are excreted by Bacillus subtilis under certain conditions, and Weimberg and Orton (257) have shown that acid phosphatase may exist extracellularily on the surface of cell walls in Saccharomyces mellis.

Extracellular release of phosphatases and other esterases by Fusarium has been demonstrated by Meyer et al (258), and Jacquet et al (259) have shown that a number of bacteria release phosphatases.

Sterile barley roots showed striking invertase and phosphatase activity, whereas urease activity depended on the rhizoplane organisms (260). Kuprevich (261) has indicated that plant roots excrete a series of enzymes, namely: catalase, phenolase, tyrosinase, urease, asparaginase, protease, lipase, invertase, amylase, and cellulase. However, his methodology for the maintenance of sterility has received criticism (260). Secretion of α-amylase by Bacillus subtilis and B. stearothermophilus, penicillinase by B. licheniformis, and invertase by yeast and Neurospora crassa has been examined in detailed manner by Lampen (262).

Specific soil inhibiting bacteria and fungi have been isolated from soils which produce phosphatase (195,200), nucleases and phosphatase (133), and phytase (204). Similarily, the role of hydrolytic enzymes of soil streptomycetes in the decomposition of soil organic matter has been

examined (263) and shown to be of significant magnitude in the organic matter transformations.

Many investigators have tried to find a correlation between bacterial numbers in soil and soil enzymatic activity. In certain cases the activity may be correlated with bacterial proliferation, for example, Daragan-Suschova and Katsnelson (264) were able to correlate activities of several soil enzymes with those of microorganisms. Geller and Dobrotvorskaya (197) suggest that phosphatase accumulates in soil as a result of the activity of microorganisms, but contradictory data do not support this conclusion (203). Kiss and Peterfi (219) concludes that α-glucosidase, β-galactosidase, amylase and inulase are produced in soil by microorganisms, whereas invertase is mainly released into soil by plants. Investigations by Balicka and Trzebinski (265) also did not bear out a clear-cut correlation between enzymatic and microbial activities. Hofmann and coworkers are of the opinion that microorganisms are the exclusive agents supplying soil with free enzymes (266,267). It should be noted that similarily to the generally known behavior of microorganisms _in vitro_, in soil the enzymatic activities may be increased adaptively by addition of substrates, for example, invertase activity (144,214) is increased by addition of sucrose and lichenase by addition of straw (159). A number of investigators (112,143,172,176,211,222,268) have shown that there is a considerably higher activity of many enzymes in the rhizosphere. It is by no means clear whether the increase in activity is due to a specific rhizosphere flora or to the enzyme release by plant roots, or both. However, the increased enzymatic activity in a rhizosphere is not unexpected.

Next to the enzymes mentioned above which have been detected in root exudates, Knudson and Smith (269) have demonstrated amylase secretion by plant roots; Rogers et al (191) have demonstrated that corn and tomato roots are a source of phosphatase in soils and that at least partially roots are a source also for soil nucleases (17).

Any contribution of soil fauna to the enzyme contents in soil has scarcely been studied. Kiss (208) examined the contribution to invertase activity by earthworms, *Lumbricus terrestris*, and showed that the earthworm excreta in grassland and in cultivated fields considerably increased invertase activity, especially in the surface layers of the soil. Activity of ants in soil has a negligible contribution to an increase of invertase. However, a further study by Kozlov (270) supports the conclusions of Kiss that soil animals provide some contribution to the enzyme content of soils.

In evaluating the enzymatic activities in soils a contribution of inorganic catalysts to the apparent results should not be excluded. Some aspects of the hydrogen peroxide decomposition in soils due to inorganic catalysts, were discussed above. It has been known for some time, for example, that ion exchange materials promote ester hydrolysis (271,272) and La, Ce, Th, and other hydroxides promote hydrolysis of glycerophosphates at normal temperature and near neutrality (273). It has been shown that cyclodextrins catalyze the decarboxylation of various acetic acid derivatives (274), and similarily, a dehydrogenase model has been suggested (275).

VI. State of Enzymes in Soil

Enzymes are accumulated in soils and generally they are more resistant to inactivation by various inhibitory agents than similar enzymes studied *in vitro*. Also it has been very difficult to extract active enzymes from soil. Apparently enzymes exist in soil in a certain physical and chemical association with the soil particles that renders the protein molecules more stable and unaccessible to inhibitory and extracting agents.

A point-to-point variation in the concentration of all solutes and gases in a matrix of clays, sand, and humus characterizes the *micro-environment* in soils. At the surfaces of soil particles, as well as at the plant roots and on the surfaces of the cells of microorganisms themselves there is a further variation in the *molecular environment* characterized by gradations in ion concentrations, including the pH, and the reduction-oxidation potential. The enzymatic reactions take place in this molecular environment where the solid phase is characterized by descrete solid organic and inorganic particles, mainly of colloidal size, indispersed with larger size mineral particles. Most of the biologically important chemical reactions take place at the liquid-soil interfaces. Understanding of the effect of physical and chemical behavior of this colloidal matrix on the enzymatic reactions in soil requires examination of colloidal properties of organic and inorganic soil solids, adsorption and exchange of solutes and ions, interactions among colloidal particles, amphoteric behavior of organic colloids, Donnan equilibrium conditions, and others, as has been recently discussed (276).

Because of the charged surfaces, typical to colloidal particles, the soil colloids exhibit strong sorbtive properties.

About one-third of the total nitrogen in soil may be in a protein-derived form but the actual processes of the protein immobilization in soil are not clear. Most of the protein released in soil is rapidly metabolized by microorganisms although much of the metabolically available protein is rapidly adsorbed by clay particles. The adsorption of proteins on montmorillonite was studied by Ensminger and Gieseking (277) and later the processes of protein sorption by clays were examined in detail by McLaren (278). Generally, proteins are adsorbed on clays in a wide pH range and rather stable clay-protein complexes are formed. Proteolytic enzymes may be adsorbed on the clay-protein complexes. The adsorbed enzymes retain their proteolytic activities and hydrolyze adsorbed proteins. The enzymes may be desorbed with a minimal loss in activity (279,280).

Upon adsorption of protein the clays expand as the protein molecules enter the interlayer space of the crystal lattices (281,282). Any protein present in the interlayer space can be utilized by microorganisms as can protein adsorbed on outside surfaces of clay particles. This suggests that extracellular proteolytic enzymes have access to the interlayer space (283). Retarding and stimulatory effects of adsorbents on the metabolic rates of microorganisms have been reviewed (284), showing that data may be hard to evaluate and that several competing factors may be involved at the molecular level.

Studies (283,284) have shown that an adsorbed substrate is metabolized slower than the same in non-adsorbed state; however, monolayers of

denatured lysozyme on kaolinite were hydrolyzed more rapidly by the extracellular proteinases of Pseudomonas and Flavobacterium than denatured lysozyme in solution. In a protein-clay complex paste growth of Bacillus subtilis exhibited a prolonged lag period although the hydrolysis of the substrate protein occurred before the exponential growth phase of organisms started (285). Durand (227) showed that in the presence of bentonite Cu^{++} was considerably less effective as an inhibitor. Stimulation of the activity of urate oxidase was evident when the enzyme was adsorbed on bentonite (185, 286). However, a study on the retardation of the proteolytic activity in presence of clays (287), revealed that the type of clay used for adsorption has a drastic influence on the activity: allophanic clays inhibited the protease activity to a much greater extent than montmorillonite or halloysitic clays.

It is evident that the chemistry of the clay is important in the stimulating or retarding effects it might exert on the activity of the adsorbed enzyme, at present all mechanisms are not known.

Kroll and Kramer (193) showed that addition of montmorillonite to soil had no effect on phosphatase activity. Similarily, there was no effect on invertase by addition of kaolinite to soil, however, when kaolinite was added in the presence of sucrose, considerable increase in invertase activity was observed apparently due to the adsorption of invertase by clay and thus the denaturation of invertase was limited (214,288). The influence of clay minerals on the breakdown of various organic substrates has been studied also by Lynch et al (289,290). Hydrolysis of cellulose dextrin was retarded by attapulgite, but the clay had no effect on the hydrolysis of gelatin. Apparently dextrin entered the interlattice space

and became unavailable to enzymatic degradation, whereas gelatin molecules were either too extended for interlattice layering or else the pH was unfavorable for sorption.

Considerable inactivation of added invertase, urease, and peroxidase was observed by Galstyan (291) and inactivation of other carbohydrazes by Hübner (292).

An interesting observation was made by Krasilnikov and Kotelev (293): they noted that phosphatase from bacterial lysates and from pure phosphatase preparations was adsorbed by corn roots grown under sterile conditions.

Enzymatic activities in three soil aggregate fractions in a rendzina grassland was followed throughout the season (Ambroz [294]). Enzymatic activities (catalase, invertase, amylase, proteinase) were higher in the microaggregates than in macroaggregates throughout the year.

In a study of esterase activity in soil, Haig (35) fractionated a fine sandy loam to obtain information on the localization of enzymatic activity on the soil particles. The clay fraction had the strongest activity toward phenylacetate. Considerable activity was present also in the silt fraction, but very little in sand. Similar fractionation was performed also by Hoffmann (70). He found the highest carbohydrase activity in the silt fraction, that of urease in clay. As there were practically no microbes present in the clay fraction, it was evident that urease, released from lysed cells has been adsorbed and remained active on the clay.

Organic and inorganic soil colloids and the crystalline clay particles usually have an electronegative charge. This charge on the

clay particles is due to the unbalance of the ionic charges in the crystal lattices, and as such it exerts an electrokinetic ("zeta") potential. In an aqueous phase the negative zeta potential causes a cloud of increased concentration of cations to neutralize the charge. This cloud includes the biologically important H^+ ion, thus changing the effective pH near the surfaces of the colloidal soil particle. The consequences of the existance of Δ pH in biological systems at surfaces have been evaluated by McLaren (295) and by McLaren and Babcock (296). This phenomenon may play an important role for enzymatic reactions in soil, as all enzymatic reactions in soils occur at interfaces.

The precise physical state of the extracellular enzymes in soil is not understood, but it is apparent that the enzymes are likely adsorbed on surfaces of the colloidal soil particles and also in some type of a covalently bound form with inorganic or organic macromolecular components. Incidentally, catalytically active enzyme derivatives, covalently bound to organic polyelectrolyte copolymers, have been prepared by Riesel and Katchalski, (297) and by Levin et al (298). In studies with adsorbed chymotrypsin (299) phosphatase (203), or urease (227,286) on clay particles, or with the trypsin-polyelectrolyte copolymer (300), it was evident that because of the ionic double-layer surrounding the clay particles, the observed pH maxima of the respective enzymatic activities were considerably higher than in liquid solutions. Not only the H^+ and OH^- equilibrium around the charged particles is of importance, but these ions may be replaced by other anionic and cationic species that may be present in quite an excess over the H^+/OH^- species; and in these cases correlation with pH might be only coincidental.

When considering the enzymatic reactions in soil one should be aware of the changes the molecular environment in the soil imposes on the enzyme kinetics. The terms used in general enzyme chemistry, like "moles per liter", are meaningless in such structurally restricted systems (296) and equations using mole fractions instead of concentrations have been developed (301). The enzymatic kinetics, expressed in mole fractions are useful in a structured environment and thus the reaction rates on surfaces and in gels may be meaningfully examined.

VII. Extraction of Enzymes

Because of the character of soil particle - enzyme molecule interaction, it has been extremely difficult to extract active enzymes from soil. Some unsuccessful efforts have been reported by Conrad (80) who tried to extract urease, and by Haig (35) whose attempts to extract urease and phosphatase were also unsuccessful. Hübner (292) was unsuccessful in extracting cellulase and pectinase. However, in 1910 Fermi reported (7) that a proteolytically active fraction had been extracted with phenol and Subrahmanyan (9) reported precipitation of the active principle for deamination of glycine. Ukhtomskaya (106) has reported desorption of several enzymes from soil with phosphate solutions.

Antoniani et al (163) were able to precipitate protein with a cathepsin-like activity from soil using ammonium sulfate and sodium tungstate as precipitating agents. Briggs and Segal (14) were able to isolate 12 mg protein with urease activity (75 Sumner units per mg) from 25 kg of soil. They characterized the "soil urease" by ultracentrifugation (molecular weights of the fractions were 217,000,

131,000, and 42,000). The urease appeared to be different from that isolated from other sources. Martin-Smith (125) extracted 2 different uricolytic enzymes from urate enriched soils. The two urate oxidases were extracted with 0.1 M phosphate at pH 7.0, and with 0.1 M "tris" at pH 8.5, respectively.

VIII. Applications

Various tests on soil enzymes have been used to correlate enzymatic activities with soil fertility and with microbial activities in order to establish a "biological index" in soils and to apply enzymatic tests in practical agriculture. In general, such a correlation has not been successful. These negative results are not unexpected because the enzyme activity in soils, as determined *in vitro*, is a manifestation of several biological parameters in soil. The contributing factors to the total enzymatic activity in soils are:

1) free enzymes adsorbed or otherwise bound to soil organic and inorganic fractions,
2) free enzymes released into soil from lysed microorganisms due to the action of bacteriostatic agents,
3) enzymes accessible in dead but not lysed cells,
4) free enzymes released into soil from plant roots, or enzymes on the surfaces of roots,
5) any metabolic activities of live cells and roots present in the soil, and
6) similar contributions, as above, by soil animals.

It is evident that unless greatly improved methods for the separation and examination of the separate activities are found, the total picture of the enzymatic activities in soil will only be partially understood.

The biological activity of the soil is regularily determined by the activities of soil microorganisms. Direct and indirect methods have been used for the determination of biological activity. In the direct method the total numbers of microbes are determined, but the determination of the "true" numbers are limited by the selection of the medium for the cultivation of microbes, or by the factors involved in direct counting under microscope. In the indirect method the number of microbes is not determined. Instead, a) from the changes produced in a soil the total number of microorganisms, or the number of microbes belonging to a certain group, is deduced; b) biologic activity is indicated by the quantity of CO_2 produced (respiration); and, c) the activities of certain enzymes are used as indicators of biological activity. The enzyme activity of the soil is compared with 1) content of microorganisms, 2) soil respiration, 3) other biological activities in soil. A survey of the results in the literature show that a positive correlation of enzyme activity and number of microbes in soils is rather an exception than a rule.

Similar conclusions have been reached for soil enzyme activity and respiration. The sources of CO_2 in soils are respiration of microorganisms, of soil animals, and the respiration of roots of higher plants. Some CO_2 is released in soil due to H_2CO_3 production by chemical reactions. Most CO_2, however, comes from microorganisms and soil respiration varies with seasons and days. Much of the available data are contradictory.

Hofmann, Wolf, and Schmidt (302) found no connection between soil respiration and invertase activity. Seegerer (303) and Hofmann (304) observed the same phenomena. No correlation between invertase activity and soil respiration was observed also by Koepf (305), Drobnik and Seifert (306) and Katsnelson and Ershov (105).

Jackman and Black (68) have added powdered alfalfa to soil at 25% water content. The soil was sterilized, and to it a water extract of a fresh soil was added. During the 5 days of incubation at 28°C, the CO_2 production was measured and after incubation the phytase activity was determined. Under such a treatment a direct correlation was found between CO_2 production and phytase activity. If a pure bacterial culture was added after sterilization, no such correspondence between CO_2 production and phytase activity was observed.

According to Kroll (307) the invertase activity and respiration decreases in a parallel manner with soil depth. Mashtakav _et al_ (116) observed a correlation between respiration, invertase and catalase activity at different soil depths. Seifert (111) added glucose in different quantities to soils and incubated for several days. Both CO_2 production and catalytic activity increased. Turkova and Srogl (308) showed that the correlation between CO_2 production and amylase and invertase activities varied under different plant associations in the same habitat, even on identical soil types. No correlation between activities of carbohydrates, urease and respiration was found by Galstyan (309) in chernozems and dark chestnut soils. The respiration rate was low, whereas carbohydrates and urease showed comparatively high activities; however, catalase activity in these soils was low. In semi-arid soils

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the correlation between respiration and activities of catalase, invertase and urease were more apparent (310). Invertase and catalase activities and respiration rates are affected also by different methods of tillage (311).

Efforts have been made also to find correlation between enzymatic activities and some biochemical cycles in soil. Drobnik and Seifert (306) found no correlation between ammonification and the invertase activity in forest soils. Seifert (111) has added 1% glucose to soil samples, and after adding water, the samples were incubated for 9 days. During incubation catalytic activity and nitrification changed in a parallel manner. Galstyan observed just the opposite (312). Treatment with natural fertilizers increased the nitrate quantity while catalase activity decreased, because of the catalase inhibitory effect of NO_3^-.

There is some correlation between catalytic activity and productivity of the soil. However, this correlation is not so strong that catalytic activity can be used as a measure of productivity, although Hofmann (266, 313) uses the activity of soil enzymes as a measure of the biological activity and productivity. To measure the biological activity he considers the enzymatic method more useful than the determination of the number of microorganisms, or measuring respiration. According to Mashtakav _et al_ (116) the determination of enzymatic activity is equivalent to the biological activity. Kuprevich (314) recognizes the present limited knowledge in soil enzymology but suggests that there is evidence for a direct correlation between enzymatic activity and soil fertility that could be utilized for practical purposes. On the other hand, Scheffer and Twachtmann (144) and Koepf (81,228,305,315) do not believe

in the general use of enzymatic method for the determination of biological activity. Horn (316) considers the enzymatic method questionable, especially in the case of strongly adsorbing soils.

In the production of soil enzymes the presence of specific substrates plays an important role. Therefore, Drobnik and Seifert (306) believe that the enzymatic method is suitable only for qualitative measurements. Generally, it is apparent that no close correlation between enzyme activity, productivity of soils and biological activity has been demonstrated. Although such correlation probably exists, new and improved methods for its demonstration are needed.

At the present time we are unable to state how much of the enzyme activity manifest by soil is due to extracellular enzymes and even whether or not free, extracellular enzyme activity is of agricultural significance. The subject is clearly in its infancy.

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III EXPERIMENTAL

A. Determination of Soil Phosphatase Activity by a Fluorometric Technique.*

Numerous methods have been used for the determination of soil phosphatase activity. Earlier attempts to measure soil phosphatase activity (1, 2) were based on the determination of the extractable inorganic phosphate after long incubations with organic phosphorus substrates. Later, methods using phenylphosphates (3) as substrates were developed, and the phenol extracted from the soil, upon completion of the incubation period, was determined colorimetrically. Recently, measurement of the extractable unreacted substrate at the end of the incubation period was used as index for the phosphatase activity of soils (4). However, none of these methods can be as rapid and sensitive as a fluorometric measurement. In the fluorometric method, fluorogenic substrates, hydrolyzed by the soil enzymes, yield fluorescent compounds which can be measured directly in the soil extract.

This report describes a fluorometric technique for the determination of soil phosphatase activity based on the use of Na-β-naphthylphosphate (NP) as the fluorogenic substrate as well as some additional observations with glycerophosphate (GP).

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*Submitted for publication in Enzymologia.

Materials and Methods

Soils. Fresh surface soil samples from cultivated greenhouse loam and air-dried soil samples from Dublin clay loam, Yolo silt loam and Aiken clay were studied; all of them were screened through a 2 mm. sieve.

Irradiated soil. Air-dried Dublin soil was irradiated with an electron beam (5.36 Megarads), as described by McLaren, et al. (5).

Substrates. Na-β-glycerophosphate from Fisher Scientific Co., Fair Lawn, N.J., Lot No. 783796, and Na-β-naphthylphosphate from Calbiochem, Los Angeles, California, Lots No. 34925 and 42280 were used.

Buffer. Modified universal buffer was prepared as described by Skujins, et al. (4).

Fluorescence excitation and emission spectra.

The excitation and emission spectra of β-naphthol, Na-β-naphthylphosphate (hereafter designated NP), NP hydrolysis products in greenhouse soil extract, NP plus soil extract, and soil extract alone were determined by means of a Spinco-Bowman spectrofluorimeter.

β-Naphthol determination in the soil extract.

Soil samples of about one gram were placed in 12 x 1.5 cm. screw-capped glass vials and two ml. of modified universal buffer (hereafter designated MUB) of the desired pH value were added to each vial. Known amounts of either β-naphthol or NP were added to each vial and the volume was brought to 8 ml. by adding distilled water. The capped vials,

containing the soil mixtures, were then placed radially on a vertical wheel of 10-cm. radius and turned on at 9.3 rpm. Two ml. of 0.5M sodium hydroxide or 2 ml of a more diluted sodium hydroxide solution were added to each vial to stop the reaction, in the case of hydrolysis, and to bring the soil extract to a pH above 11.

The soil suspensions were spun in a refrigerated centrifuge at 27,000 g for 15 minutes. Aliquots of the supernatant fluid were removed and diluted to appropriate volumes. Usually a dilution ratio of 1:100 was used. The amount of β-naphthol present in the supernatant fluid of each sample was determined by fluorometric measurements and compared with a standard curve at a concentration below 5×10^{-6}M.

All fluorometric measurements were taken in a Brice-Phoenix Universal Light Scattering Photometer provided with a monochromatic ultraviolet filter (350-370 mμ band) between the UV-light source and the sample holder and with a blue filter Klett No. 42 between the sample holder and the photocell. The blue filter cuts off the light wavelengths below 400 and above 450 mμ. The fluorescence emitted was measured at 90° with respect to the direction of the exciting light.

β-Naphthol adsorption on soil.

To one-gram soil samples various amounts of β-naphthol were added and after incubation for one hour at pH 7 and 25°C the amount of β-naphthol present in the soil extracts was determined. Controls were run for each soil consisting of the soil sample plus buffer and distilled water to bring the volume up to 8 ml.

Hydrolysis of NP as a function of substrate concentration.

One-gram soil samples were treated with various amounts of NP by adding suitable volumes from a 0.005M NP solution. The amount of β-naphthol released to the soil extract was determined by fluorometric measurements after incubation for one hour at pH 7 and 25°C. Controls were run as described above.

Hydrolysis of NP as a function of pH.

Two ml. of 0.005M NP (10 μ moles) and two ml. of MUB of the desired pH value were added to one-gram soil samples. The MUB pH values ranged between 2 and 12. The amount of β-naphthol present in the soil extract was determined after an incubation period of one hour at 25°C. Controls with soil-water suspensions adjusted to similar pH values and NP solutions alone were also run.

Hydrolysis of Na-β-glycerophosphate as a function of pH.

Two ml. of MUB and one ml. of glycerophosphate (30 μmoles) were added to one-gram Dublin soil samples into 30-ml screw-capped glass vials. Controls were run for each pH value. The volume of each vial was increased to 8 ml. by adding 5 ml. of distilled water. All the treated samples were agitated on the vertical wheel for 6 hours at room temperature. After incubation the samples were centrifuged at 27,000 g for 15 minutes. The inorganic phosphate present in a 5-ml. aliquot of the supernatant liquid of each sample was determined by a modified Martin and Doty (6) procedure.

Results

β-naphthol has maximal and constant fluorescence at pH values above 10 (β-naphthol $pK_a = 9.5$). β-naphthol also shows an almost

linear relationship between its concentration and its emittance of fluorescence at concentration below 5×10^{-6}M.

Excitation and emission spectra.

The excitation spectrum for β-naphthol shows three peaks at 240, 285 and 350 mμ with λ emission at 420 mμ and its emission spectrum shows one peak at 420 mμ with λ excitation at 350 mμ. On the other hand, the excitation spectrum for NP shows three peaks at 230, 280 and 320 mμ with λ emission at 360 mμ and its emission spectrum shows only one peak at 355 mμ with λ excitation at 280 mμ. These results agree with those obtained by Moss et al. (7).

The excitation and emission spectra for NP hydrolysis products obtained after incubation of NP with greenhouse soil for one hour at 25°C are presented in Figure 1. The excitation spectrum shows three peaks at 240, 285, and 350 mμ with λ emission at 420 mμ. These are the characteristic peaks of the β-naphthol excitation spectrum. The emission spectrum shows the distinctive peak of β-naphthol at 420 mμ with λ excitation at 350 mμ. Figure 1 also presents the emission spectrum of greenhouse soil extract alone with λ excitation at 350 mμ. This emission spectrum shows that the contribution of greenhouse soil extract to the fluorescence intensity of NP hydrolysis products is completely negligible at λ emission above 400 mμ.

Figure 2 shows the excitation and emission spectra of Dublin soil extract plus 1.25×10^{-3}M NP with λ emission at 420 and λ excitation at 350 mμ, respectively. It also includes the emission spectrum of Dublin

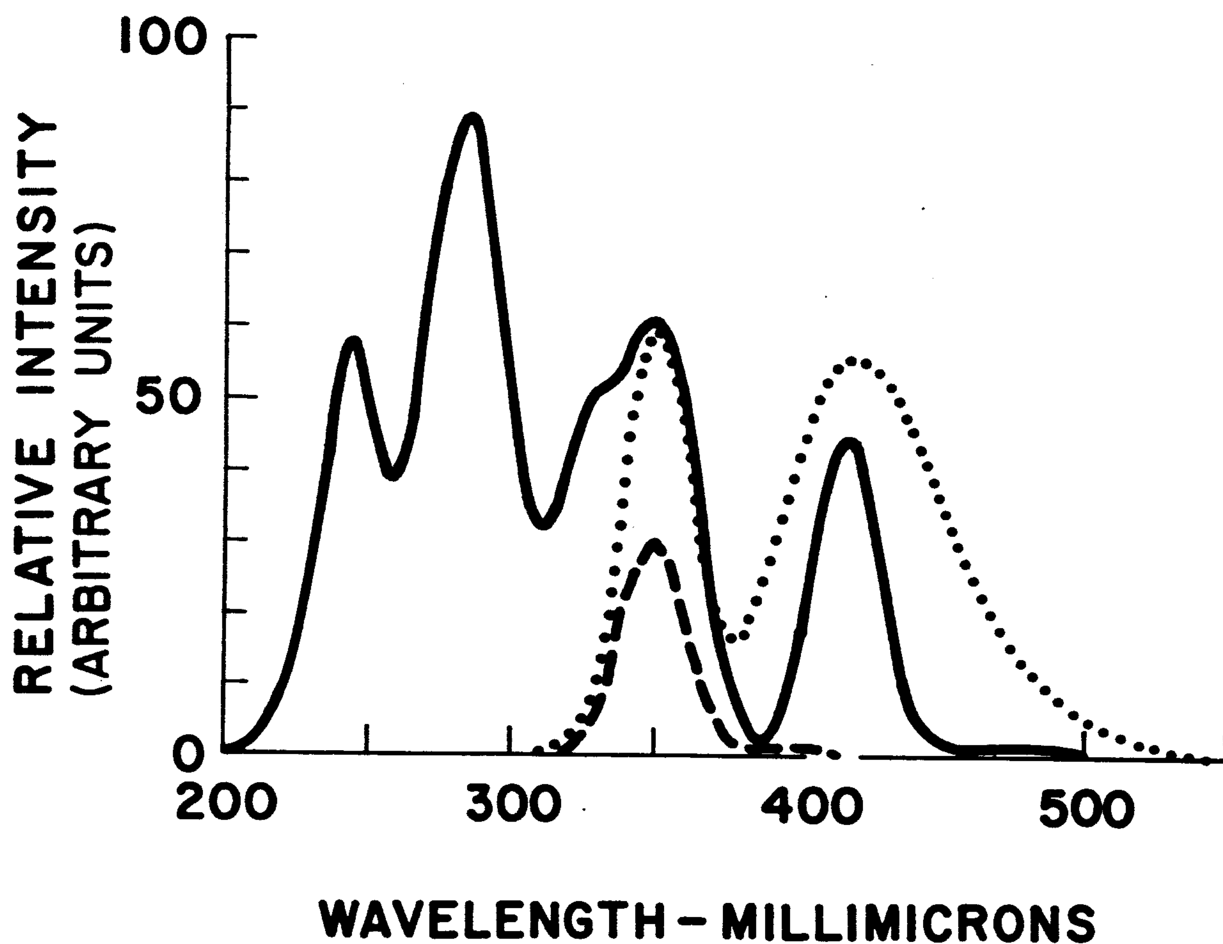

Fig. 1. EXCITATION SPECTRUM OF NP HYDROLYSIS PRODUCTS WITH

$\lambda_{emis.}$ = 420 mμ (———).

EMISSION SPECTRUM OF NP HYDROLYSIS PRODUCTS WITH

$\lambda_{excit.}$ = 350 mμ (•••••).

EMISSION SPECTRUM OF GREENHOUSE SOIL EXTRACT WITH

$\lambda_{excit.}$ = 350 mμ (-----).

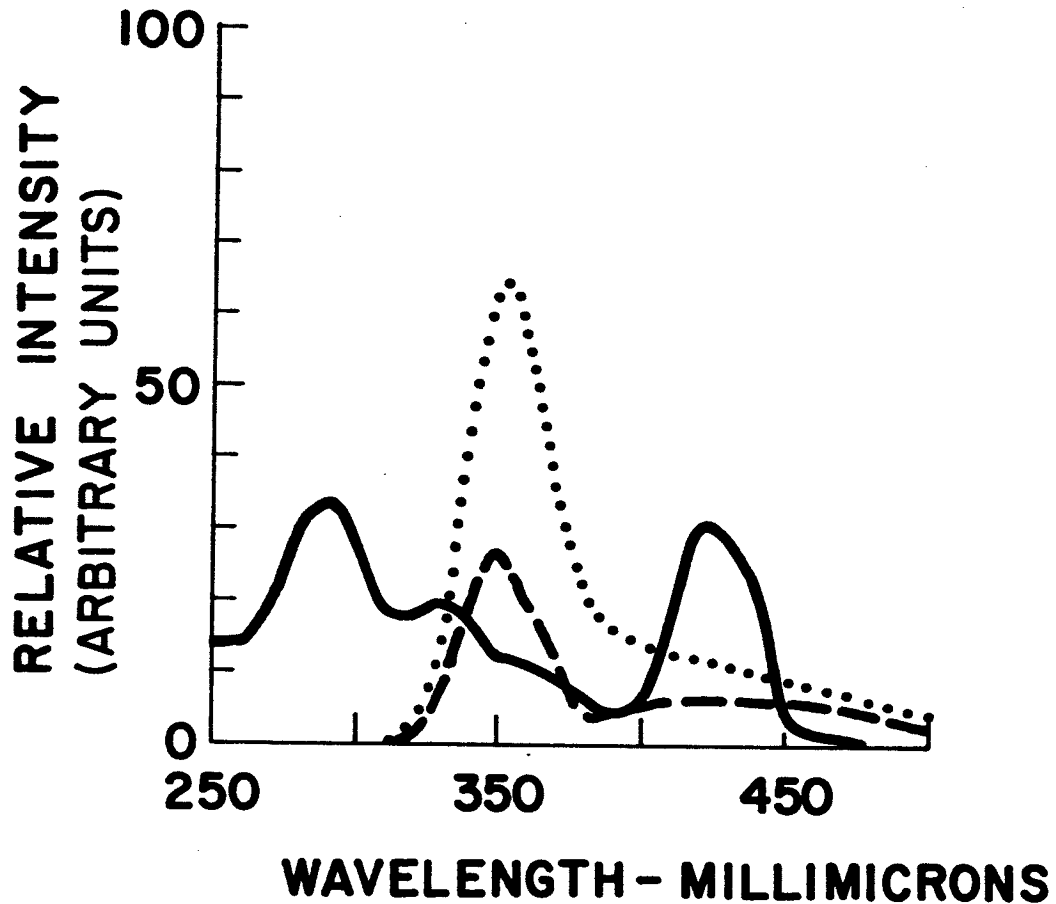

Fig. 2. EXCITATION SPECTRUM OF DUBLIN SOIL EXTRACT PLUS NP WITH

$\lambda_{emis.}$ = 420 mμ (——).

EMISSION SPECTRUM OF DUBLIN SOIL EXTRACT PLUS NP WITH

$\lambda_{excit.}$ = 350 mμ (····).

EMISSION SPECTRUM OF DUBLIN SOIL EXTRACT ALONE WITH

$\lambda_{excit.}$ = 350 mμ (----).

soil extract alone with λ excitation at 350 mμ. These spectra show that the fluorescence intensity contributed by either NP or Dublin soil extract is small when measured at λ excitation around 350 mμ and λ emission at 420 mμ. In fact, in the case of Dublin soil the fluorescence intensity of the soil extract plus NP is less than 15% of the fluorescence intensity of the Np hydrolysis products obtained from the same soil after an incubation period of one hour at 25°C.

Behaviour of β-naphthol added to soil.

When a known amount of β-naphthol is added to one-gram soil samples the recovery is not complete. If a known amount of β-naphthol is added to the extract of either soil alone or soil previously treated with β-naphthol, it is found that the soil extract does not quench the β-naphthol fluorescence. On the other hand, β-naphthol has been shown to be decomposed by a particular soil microorganism only if this microorganism is grown in a medium with β-naphthol as the sole carbon source for several days (8). Thus, the amount of β-naphthol which can not be recovered has to be accounted for as β-naphthol adsorbed to the soil.

The amount of β-naphthol adsorbed depends on the type of soil involved. The adsorption curves for the clay loam soil and the loam soil are shown in Figure 3. In the range of 0 to 4 μmoles of β-naphthol added per gram of Dublin soil the amount adsorbed shows a linear relationship with the amount extracted. Aobve 4 μmoles the curve levels off, and at higher concentrations probably reaches a plateau corresponding to a saturation level. In the case of greenhouse soil, the

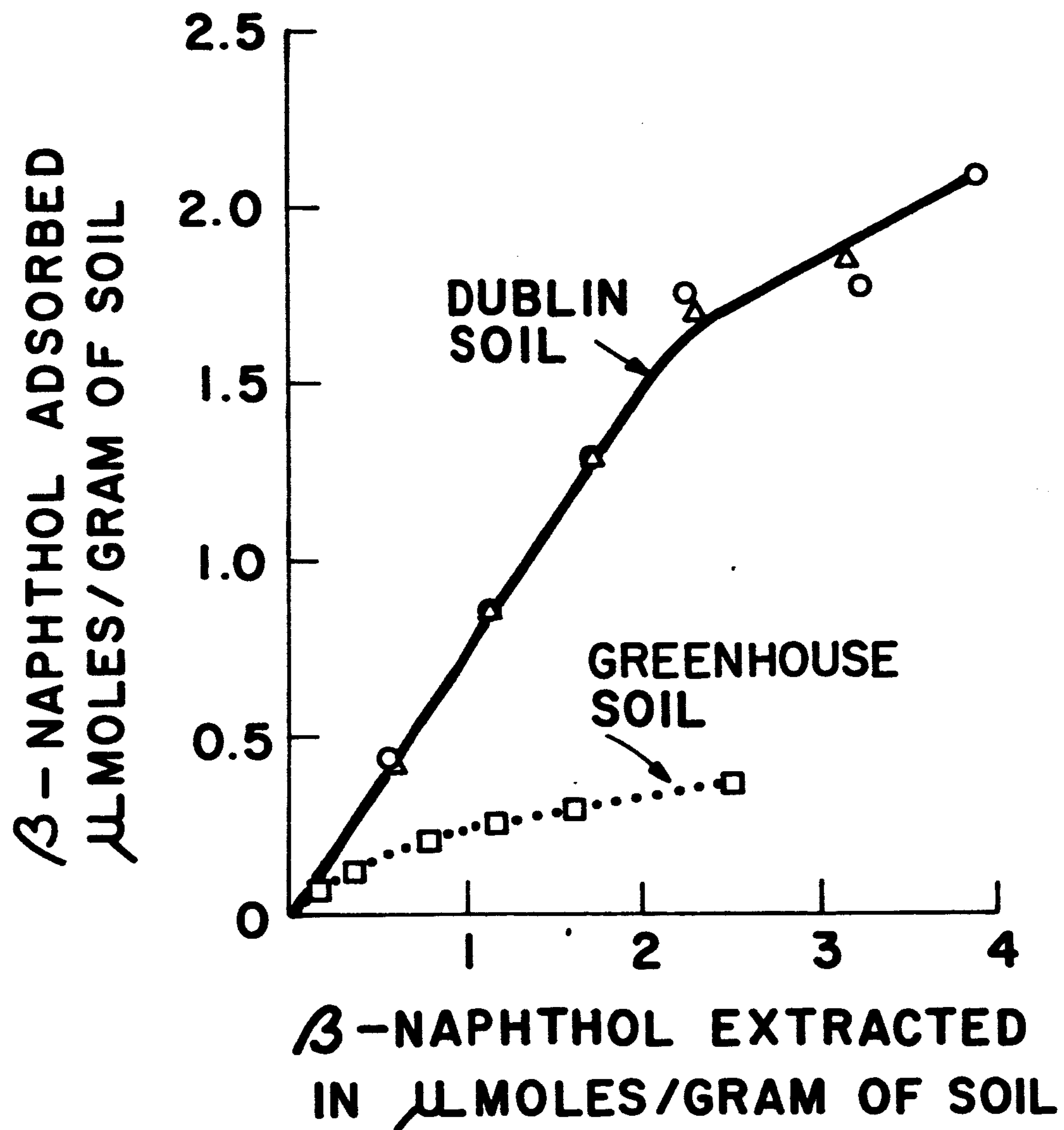

Fig. 3. β-NAPHTHOL ADSORPTION CURVES FOR DUBLIN AND GREENHOUSE SOILS.

linear relationship between the amount of β-naphthol adsorbed and the amount extracted occurs above concentrations of one μmole of β-naphthol added per gram of soil. The clay loam soil retains considerably larger amounts of β-naphthol than the loam soil under the experimental conditions used.

The effect of the dilution of the soil extract on the reproducibility of the fluorometric measurements at different dilution ratios was also investigated. In soils with very low phosphatase activity (e.g. Yolo) it is found that reproducible measurements at different dilution ratios can be obtained only for ratios greater than four parts of distilled water to one part of soil extract. In soils with relatively high phosphatase activity (e.g. Dublin and greenhouse) high dilution ratios are required, and so reproducible measurements are insured when several dilution ratios for the soil extracts are used.

In some soils (e.g. Aiken) the addition of NaOH to the soil mixture after incubation with NP produces a dark colored soil extract in which β —naphthol fluorescence is somehow masked by the background fluorescence. In this type of soil it is advisable that immediately after incubation the soil mixture be spun down in a centrifuge at 4°C (cold treatment). Aliquots of the supernatant fluid then can be diluted and adjusted to a pH value above 11 in order to take the fluorometric measurements. With the greenhouse soil no interference due to masking of β-naphthol fluorescence is found when NaOH is added directly to the soil mixture after the incubation with NP ends (NaOH treatment). The NaOH treatment is found to be 50% more effective in extracting

β-naphthol from the greenhouse soil than the cold treatment described above. On the other hand, when the controls for the cold treatment are compared with those for the NaOH treatment, it is observed that the latter have a low and constant value through the pH range between 2 and 12, whereas those of the cold treatment show higher values which gradually increase from neutral pH towards the acidity and alkalinity sides.

Hydrolysis of NP as a function of substrate concentration.

In both the Dublin and the greenhouse soils the rate of hydrolysis of NP becomes independent of the concentration of the substrate at concentrations above 10^{-3}M. At concentrations below 10^{-3}M the rate of hydrolysis does not increase proportionately with the substrate concentration [see, however, the report on soil phytase by Jackman and Black (9)].

Hydrolysis of NP and glycerophosphate (GP) as a function of pH.

Both curves for the hydrolysis of NP and GP by sterile irradiated Dublin soil as a function of final pH are identical in shape and coincide at pH values below the optimal one for GP hydrolysis (Figure 4). The pH optimum for NP hydrolysis is slightly higher than the corresponding one for GP hydrolysis. In the case of the hydrolysis of GP the incubation period was six hours, whereas for the NP hydrolysis it was only one hour. The curve for NP hydrolysis by unirradiated Dublin soil also exhibits the same shape and pH optimum value shown by the corresponding irradiated Dublin soil curve.

It was noted that NP is very stable in solution in the pH range from 2 to 12.

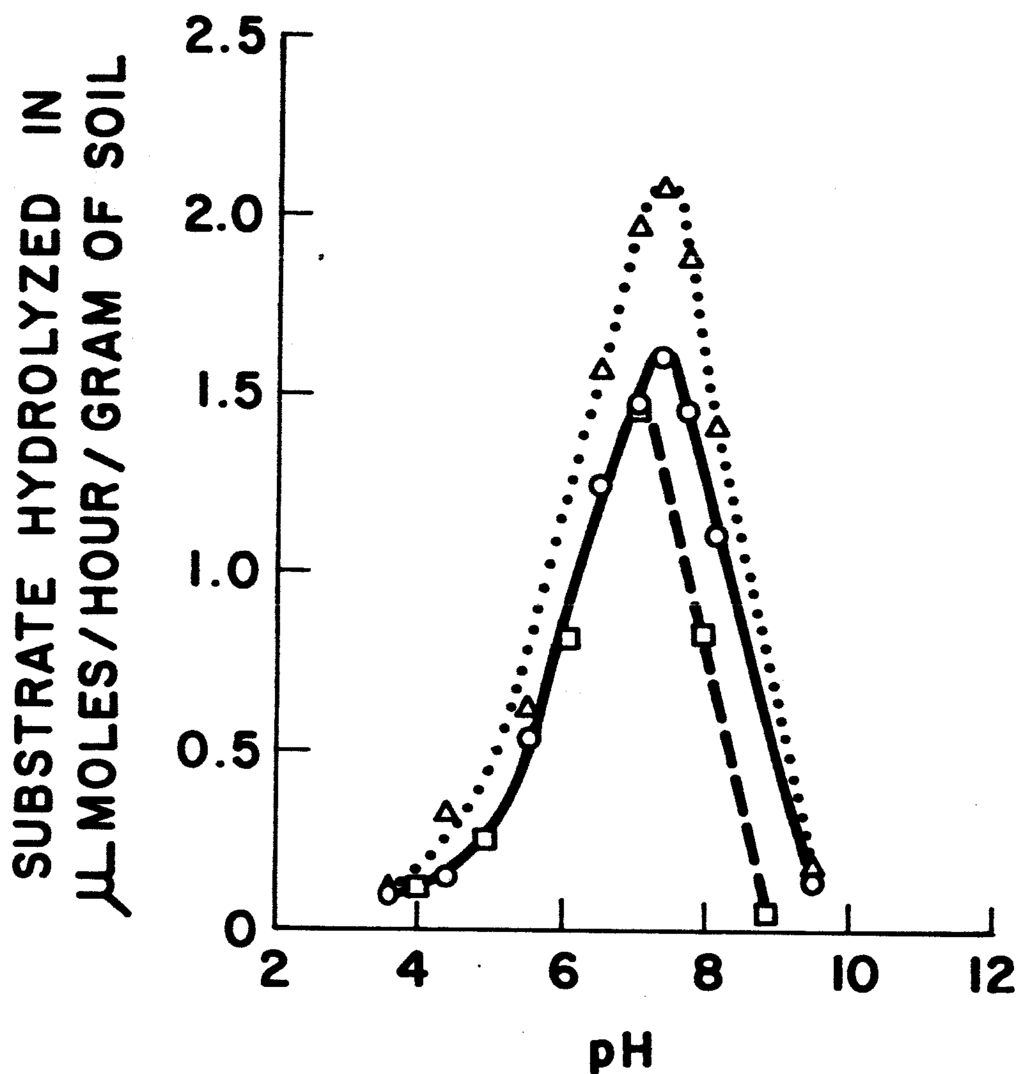

Fig. 4. RELATIVE AMOUNTS OF NP (O———O) AND OF GP (□ --- □) HYDROLYZED BY IRRADIATED DUBLIN SOIL AS A FUNCTION OF pH. RELATIVE AMOUNT OF NP (△·····△) HYDROLYZED BY UNIRRADIATED DUBLIN SOIL AS A FUNCTION OF pH.

Discussion

Na-β-naphthylphosphate (NP) was preferred to the α-isomer as the substrate for the determination of phosphatase activity in soils because one of its hydrolysis products (β-naphthol) shows a three-fold greater fluorescence intensity than the corresponding hydrolysis product of the α-isomer (10). NP has also been shown to be hydrolyzed 1.2 times faster than the α-isomer at maximum velocity (11). On the other hand, β-naphthol is less easily oxidized than the α-isomer under alkaline conditions (12, 13). Moreover, β-naphthol is less toxic than the α-isomer (14). Hammerbacher (15) demonstrated that α-naphthol precipitates proteins whereas β-naphthol does not. More recently, α-naphthol has been shown to be an inhibitory agent of some enzyme systems (16) at concentrations comparable to those resulting in determinations of soil phosphatase activity.

The excitation and emission spectra shown in Figure 1 and 2 indicate that the fluorescence emittance measured in the NP hydrolysis products after incubation of NP with soil is due specifically to β-naphthol when fluorimeter set-up previously described is used. Thus, it may be concluded that the fluorometric technique for the determination of soil phosphatase activity is highly reliable and that no interference is offered by either unhydrolyzed NP or the soil extract in the soils tested. Incidentally, the fluorimeter set-up specifically designed for β-naphthol determinations was found to be as efficient as the Aminco-Bowman spectro-fluorimeter at the β-naphthol concentration levels used.

In obtaining the excitation and emission spectra it was observed that β-naphthol fluorescence decreases on long exposures to UV-light, indicating that some sort of photodecomposition was taking place. This observation agrees with studies by Hercules, et al. (17) who reported that when β-naphthol solutions were exposed directly to a high intensity, low pressure mercury lamp, large changes were observed, but that moderate exposure to UV-radiation did not cause significant changes.

In the determination of soil phosphatase activity by using the fluorometric technique it is necessary to make corrections for the amount of fluorescent hydrolysis product which is retained by the soil under the experimental conditions used. A distorted view of the phosphatase activity of a soil is obtained if no allowance is made for this particular correction.

The facts that the rate of hydrolysis of NP by soil is independent of the substrate concentration at concentrations above 10^{-3}M, and that there is not a linear relationship between the rate of hydrolysis of NP and the substrate concentration at lower concentrations, suggests an enzymatic nature of the reaction. Further evidence in this regard was obtained when it was found that oven-dried (105°C) irradiated and unirradiated greenhouse soil failed to hydrolyze NP.

Hochstein (18) has independently developed a fluorometric assay for soil phosphatase at the mμ mole level using α-naphthylphosphate as substrate. Contrary to the results discussed above using β-naphthylphosphate as substrate, he reports that the assay was complicated by the

native fluorescence of soil and by an apparent quenching of fluorescence by soil. The independence of the rate of hydrolysis above a determined substrate concentration observed in our experiments, however, agrees with his findings. This is also the case with regard to the highest phosphatase activity found by us around neutral pH in most of the soils studied (to be reported in detail in a further publication).

The pH optimum for dublin soil phosphatase activity is about the same whether NP or GP is used as substrate. The striking similarity of the pH optimum curves for soil phosphatase activity in Dublin soil using either NP or GP as substrates (Figure 4), gives additional support for the claim that the determination of soil phosphatase activity by the fluorometric technique has great dependability. Results are usually obtained three hours after sample collection. The outstanding sensitivity and rapidity of this phosphatase assay makes its application especially advantageous either when large numbers of soils with various levels of phosphatase activity are to be studied, or when short-term incubation periods are required in order to exclude the phosphatase activity due to microbial proliferation.

Summary

A fluorometric technique is described for the determination of phosphatase activity in soils based on the measurement of β-naphthol released to the soil extract upon hydrolysis of Na-β-naphthylphosphate. A spectrographic analysis of the fluorometric assay was used to demonstrate that the detection of β-naphthol released to the soil extract is not affected by either unhydrolyzed substrate or the soil extract in the soils tested. Retention by soil of the hydrolysis product being measured must be accounted for when the phosphatase activity of soils is expressed quantitatively. The agreement found for the pH optimum curves of Dublin soil using either Na-β-naphthylphosphate or glycerophosphate as substrate, shows the adequacy of the technique described. The fluorometric technique with its simple and rapid measurements can advantageously replace the long and tedious procedures required in most previous soil phosphatase assays.

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B. Stability of Urea at Elevated Temperatures.

Work is in progress to obtain data on urea stability and to determine the thermodynamic behavior of urea decomposition products at elevated temperatures.

It has been suggested here, that urea might be used as a substrate for the detection of catalysts (i.e., enzymes) causing its eventual decomposition in the Martian soil. Therefore, it is of interest to evaluate its heat stability towards the proposed sterilization procedures and temperatures to which an automated biological laboratory would be subjected.

This report describes the stability of urea when heated in the absence of water for 15 hours and for 35 hours at temperature from 110.0° C - 140.0° C. The amount of NH_3 formed and the amount of biologically available urea remaining was determined quantitatively. Runs were made at both 0.5 mm and 760 mm Hg pressure (starting pressure).

Materials and Methods.

a) Urea, purified by the ion exchange method, as described (First Semiannual Progress Report, March 9, 1965, p. 42) and maintained under vacuum in a dry state.

b) The urease used for the determination of the amount of biologically available urea remaining after heating was either urease 3xNF (Nutritional Biochemical Corporation), 10 mg per tube, or urease tablets (Matheson Colman and Bell) - 25 mg urease/tablet.

c) Resin: Bio-Rad AG 50W-X8 (Dowex 50W-X8), 400 mesh, H^+ form, exchange capacity, 1.7 meq/ml of resin bed. Changed to Na^+ form by washing with 1 N to 0.05 N NaOH followed by excessive washing with 1 N to 0.05 N NaCl.

d) Column: 18 x 9 mm, void volume 1.3 ml. Tube: Pyrex 36290-3C.

e) Method: A 100mg sample of urea was placed in a dry glass tube, approximately 9 mm in diameter, sealed at the desired pressure, and placed in an oil bath, set at a predetermined temperature, for 15 hours or 35 hours.

After heating the outside of the tube was washed with benzene, distilled water and dried. The dried tube was then broken under 10 ml of water, to trap the volatilized ammonia; also the entire solid content was dissolved in that same 10 ml of water. Each sample was adjusted to pH 6.5-7.0 with 1-5 drops of acidic or basic acetate and was then eluted through the ion exchange column, to separate the ammonia formed from the biologically available urea, according to the procedure described in the First Semiannual Progress Report, March 9, 1965, p. 44.

Analysis of the volatilized ammonia:

After the proper dilution the eluted NH_4^+ was nesselerized according to the standard procedure:

Add into a photometer tube in the order shown:

1) 1 ml sample,
2) 10 ml NaOH, 0.02 M.,
3) 1 ml gum acacia, 0.2%
4) 1 ml Nessler's reagent.

After 20 minutes read on the "Spectronic 20" at 420 mμ.

Analysis of the residual urea:

To the eluted urea 10 mg of urease 3xNF was added and the sample was incubated with continued stirring, for 1 hour. Next, the sample was centrifuged at 27,000 G and 0° C for 15 minutes. One ml of the supernatant solution was placed in the column following the same procedure as described above. The ammonia formed by the hydrolysis of urea by urease, was collected. After the proper dilution this sample was then nesselerized as before.

Results

The results are presented graphically in Fig. 5 and Fig. 6.

The main objective of this investigation was to determine, by enzyme assay, the amount of biologically available urea that remained after 100 mg of urea was heated for 15 hours and 35 hours at 110-140° C in the absence of water.

The residual urea was analyzed by subjecting it to urease action. Any urease inhibiting substances present would decrease the "biologically available" amount of residual urea, thus giving the desired results.

There was no significant difference in the amount of the residual urea between the samples heated at standard air pressure and those heated at 0.5 mm Hg. It is apparent that at 110° C there was approximately 98% of the urea left. This amount decreased almost linearly with regular increases in temperature to 125°C. From 125°C to 140°C the results indicate

FIGURE 5

RESIDUAL UREA

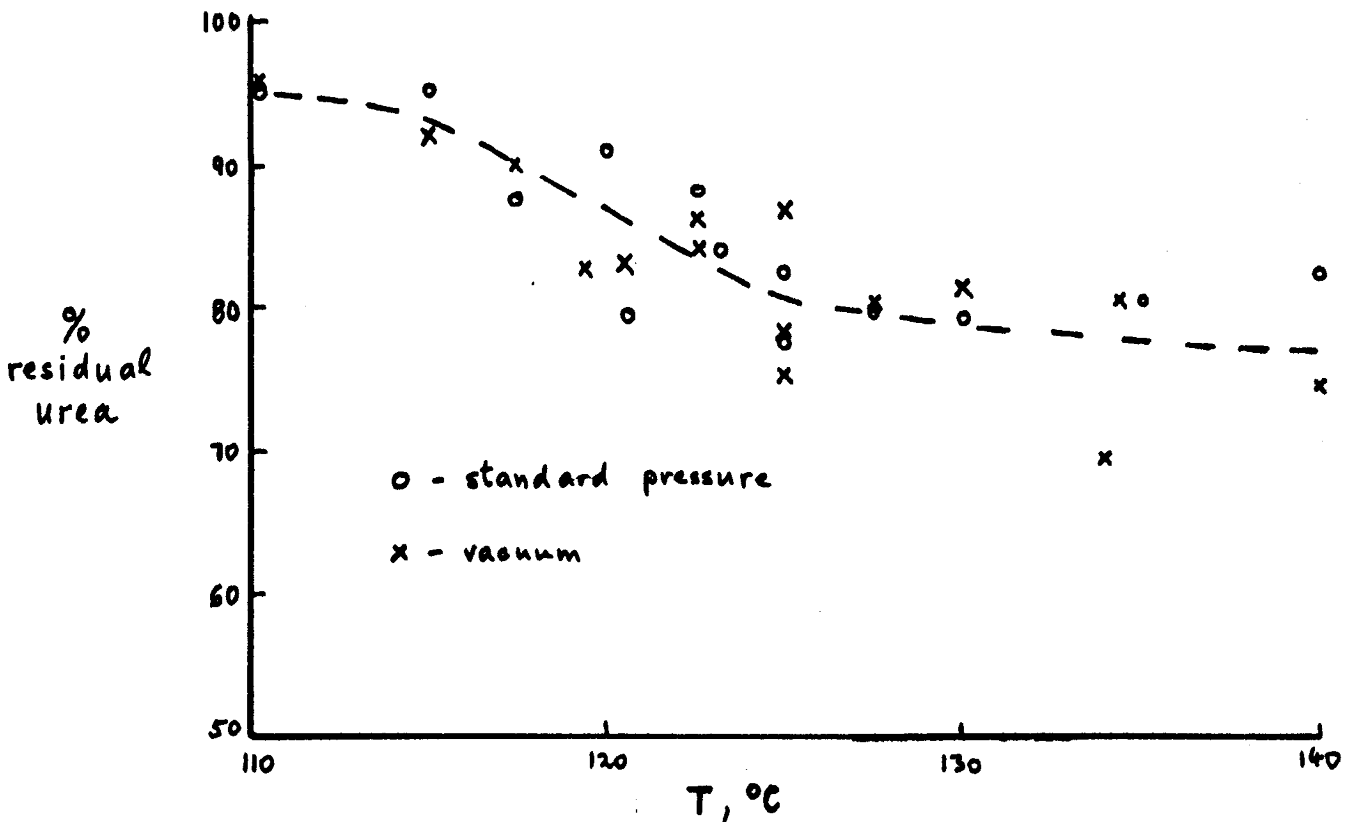
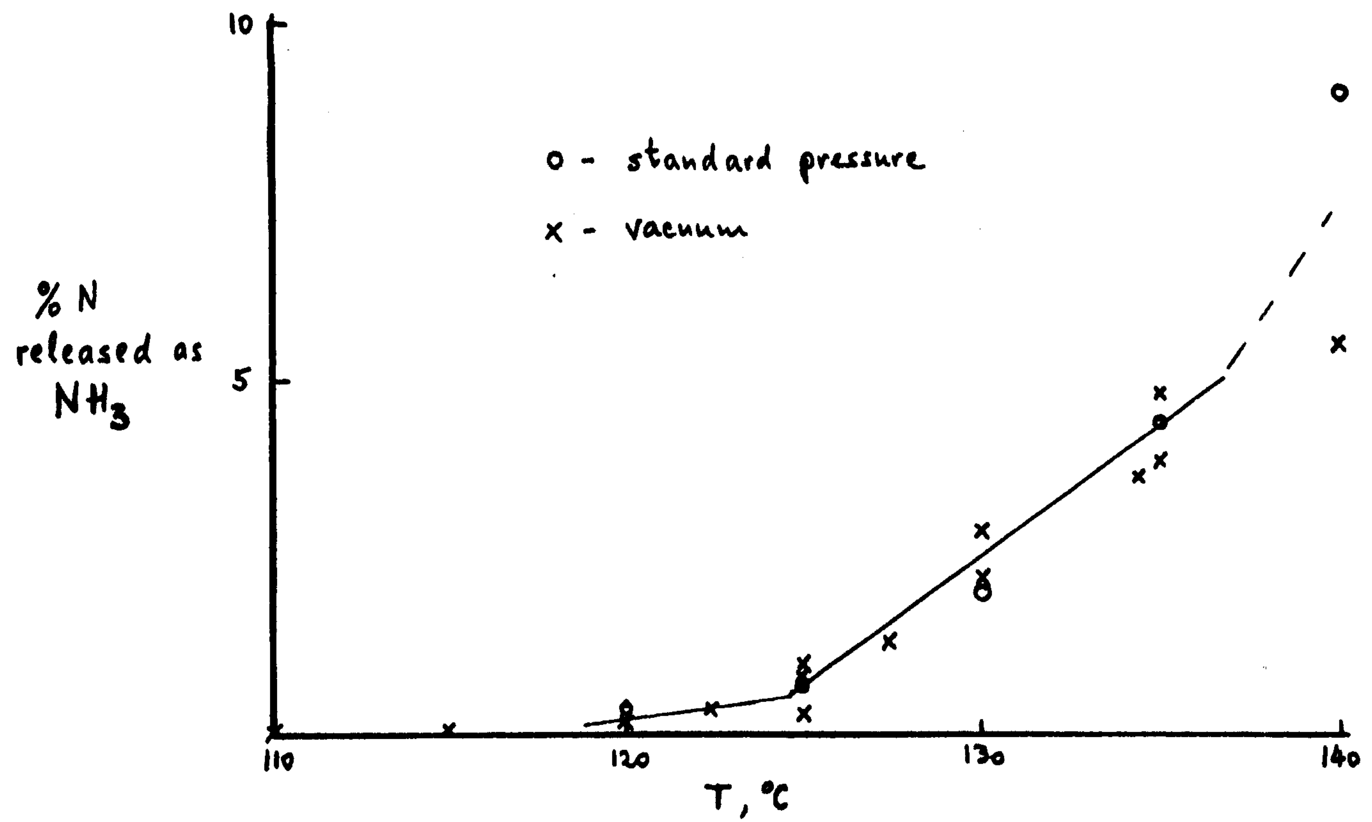


FIGURE 6

AMMONIA RELEASE



that the amount of biologically available urea remaining is about 80%. The differences between the 15 hours and 35 hours heated samples were small - there was a slightly higher release of ammonia.

The per cent N as volatilized NH_4^+ does not seem to correlate with the amount of residual urea, as it is less than 1% from 110-125°C. From 125°C to 140° C it increased gradually to approximately 7%. Evidently there was also a soluble product(s) formed which contained 1% to 20% of the total N. Again, since only the amount of biologically available urea was of interest, this product was not identified; however, chromatographic evidence suggests that most of this product is biuret.

C. Determination of Urease in Soils - Influence of Microbial Proliferation.

The determination of the urease activity is one means by which the presence of life may be detected in Martian soil. Urease is specific to a single substrate, urea, which it hydrolyses to ammonia and carbon dioxide. If, therefore, urea is added to a soil, the activity of urease present in it may be determined quantitatively by measuring the amount of ammonia evolved.

The Conway method (2) for the determination of ammonia has been widely used with subsequent modifications (1,7). It has also been used for some time in this laboratory (6) with minor changes. Recently, a comparison of methods for determining urease activity in this laboratory (Second Semiannual Progress Report, July 20, 1965) has revealed the

need for a re-assessment of some aspects of the Conway procedure.

Two aspects of the Conway procedure are presently being investigated. The first of these concerns the possible multiplication of microorganisms in the soil sample during the incubation period. Stevenson (8) demonstrated that the increase in bacterial numbers after re-moistening air-dried soil starts after six hours, whereas Griffiths and Birch (5) found an increase in the number of coccoid forms of more than 50 per cent after only three hours although rod-like forms showed no net multiplication before 12 hours following re-moistening. Accordingly, the change in number of microorganisms with duration of incubation time was examined using three different soils.

The other part of the Conway procedure currently under investigation concerns the testing of all the variables that might lead to the evolution of ammonia from sources other than the urea added or for causes other than the urease originally present in the soil sample. The methods are being developed currently. This aspect involves also examination of a possible loss of ammonia during incubation by cation fixation or other mechanisms.

Methods and Materials

Properties of the soils used are given in Table I.

Change of microbial numbers in soil samples with duration of the incubation period was examined by the dilution plate colony count method. Incubation time was taken from the moment of addition of water to the air-dried soil and ended with mixing of a sample with water in the first

Table I

Properties of the Soils Used

Name	Characteristics	Air-dry storage length	pH	Wt. loss on ignition	Organic C content	Clay	Silt	Sand
				%	%	%	%	%
Yolo	silt loam	8 years	6.8-7.0	9.3	1.27	12	21	68
Dublin	adobe clay loam; top 7"	10 years	5.6-5.7	14.5	2.74	29	25	46
Hilgard No. 7	Kern R. Delta soil; top 12"	70 years	6.8	8.8	----	11	--	--
Agricultural (Oxford Tract)	cultivated loam (used for soil extract)	6 months	6.7	---	2.83	20	33	47

dilution step. Distilled water was added at the rate of 0.4 millilitres per gram of air-dried soil for all samples and soil types except for one waterlogged test wherein 1.0 millilitre was added per gram. Samples were placed in petri dishes with the lids on to prevent evaporation during incubation which took place at room temperature.

At the end of each incubation period, 1.4 grams of moist (≐ 1.0 grams of air-dried) soil were diluted with sterile tap water and shaken vigorously by hand: for one minute at the first dilution (1:100), for 30 seconds at each subsequent dilution, and for shorter intervals regularly during plating (1 millilitre aliquots of the final dilutions were used). Two dilutions were plated out: 10^{-4} and 10^{-5}. Warm agar

medium, stored at 50-55 °C., was then poured over the samples and swirled until thoroughly mixed. The plates were incubated at room temperature, and counted after five days. Five replicates were used.

The agar medium used for all tests was a soil-extract agar prepared after Fred and Waksman (4). The soil extract was prepared as follows: 1000 ml of tap water were added to 1000 g of air-dried, sieved (3mm) Agricultural soil (see Table I) and autoclaved for 20 minutes. The extract solution was filtered with a filter candle, after half a gram of calcium carbonate had been added, and stored in the refrigerator until required. The initial pH of the soil-extract agar mixture was 8.2-8.5 and was therefore adjusted to pH 7.0 prior to autoclaving of the agar medium. The glucose(5% solution) was autoclaved separately (3) and stored at the same (50-55 °C.) temperature as the agar. They were mixed immediately before pouring the first plates in each series.

Finally, one soil was amended with urea, in the same amount that is added in the Conway analysis, to determine the effect of urea on microbial numbers. This series was moistened with 0.3 ml of distilled water per gram of air-dried soil. The Hilgard No. 7 soil sample was conserved by using a half instead of one gram per series.

Urease activity: the activity of urease in soil is being measured by determining the amount of ammonia evolved in the presence of an excess of the substrate urea. A modification of the Conway procedure is being used as follows: - (a) Incubation: to the centre well of a

Conway porcelain microdiffusion unit is added 2 ml. of 0.02 N H_2SO_4; to the outer well is added 1 g. of sample, 1 ml. of urea aqueous solution (10 mg./ml.), and 1 ml. of water or 1 ml. of 0.05 M K-phosphate buffer (pH 7.0). The unit is sealed with a glass lid and petroleum jelly and incubated for 4 hours at 25°C. (b) Diffusion: at the end of the incubation period the unit is opened by sliding back the glass lid, 1 ml. of 10% KOH (w/v) is added to the outer well, and the lid is re-sealed. Diffusion from the outer to the centre well is allowed to take place at room temperature for periods ranging from 3 to 20 hours. (c) Nesslerization: at the end of the diffusion period the contents of the centre well are removed to a test tube with 2 washings of 2 ml. of 0.02 N H_2SO_4. The amount of ammonia absorbed is then determined by Nesslerization by adding to each test tube 1 ml. of 0.2% gum acacia (w/v), 1 ml. of Nessler's reagent (prepared by mixing stock Nessler's reagent 1:5 with 10% NaOH), and making up to 10 ml. by adding 2 ml. of distilled water. After 10 minutes the tubes are read in a Klett-Summerson photometer using a No. 42 blue filter and a single colorimeter tube to avoid tube calibration problems.

Before reliance can be placed on data thus obtained it is necessary to determine the errors contributed at different stages. Accordingly, the following "blanks" are being run to assess any such errors: - To the outer well of a Conway unit is added (a) 2 ml. water + 1 ml. urea; and KOH is added at incubation time (b) 1 ml. each of water, buffer, and urea; KOH at incubation time (c) 1 g. soil + 2 ml. water; KOH at incubation and at zero times (d) 1 g. soil + 1 ml. water + 1 ml. urea;

KOH at incubation and at zero times (e) 1 g. soil + 1 ml. buffer; KOH at incubation and at zero times (f) 1 g. soil + 1 ml. water + 1 ml. $(NH_4)_2SO_4$(2 mg./ml.). In addition, a series of diffusion times from 3 to 20 hours, and incubation times from 1 to 4 hours is being tried.

Results and Discussion.

The average colony count for each set of five replicates is presented in Figures 7-10. The highest values were obtained with the Yolo soil which yielded a maximum of about 7.6×10^6 per gram of air-dried soil at one hour's incubation time. Dublin soil yielded a maximum of 2.4×10^6, and Hilgard No. 7 yielded 1.9×10^6 microorganisms per gram. These values for Yolo and Dublin soils are higher and in different proportion to each other to those obtained previously (Second Semiannual Progress Report, July 20, 1965). This is primarily attributed to the relatively infertile soil that was used to prepare the soil extract previously, although there were other differences in technique also. It should be noted that the Hilgard soil stored for 70 years gave a count of the same order of magnitude as the Dublin soil stored for only 10 years (Figs. 8 and 10).

The difference in numbers obtained between the first and second trials with Yolo soil (Fig. 7) and between the first and second trials with Dublin soil (Fig. 8) may be attributed entirely to differences in shaking technique.

Figure 7: the relatively sharp rise in numbers in all four graphs during the first one to two hours is believed to be due to

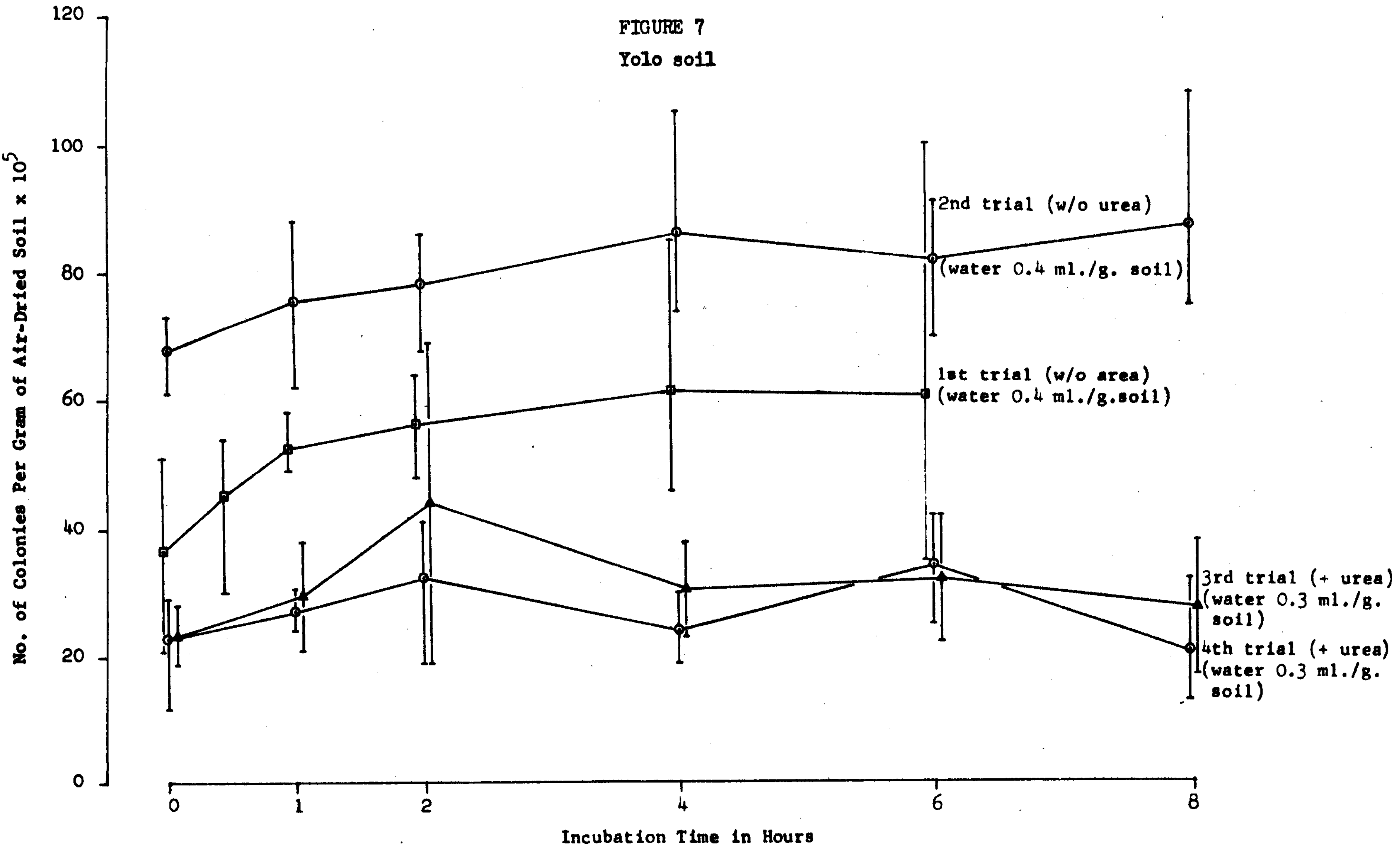


FIGURE 7
Yolo soil
2nd trial (w/o urea)
(water 0.4 ml./g. soil)
1st trial (w/o area)
(water 0.4 ml./g.soil)
3rd trial (+ urea)
(water 0.3 ml./g.
soil)
4th trial (+ urea)
(water 0.3 ml./g.
soil)
No. of Colonies Per Gram of Air-Dried Soil x 10^5
120
100
80
60
40
20
0
0
1
2
4
6
8
Incubation Time in Hours

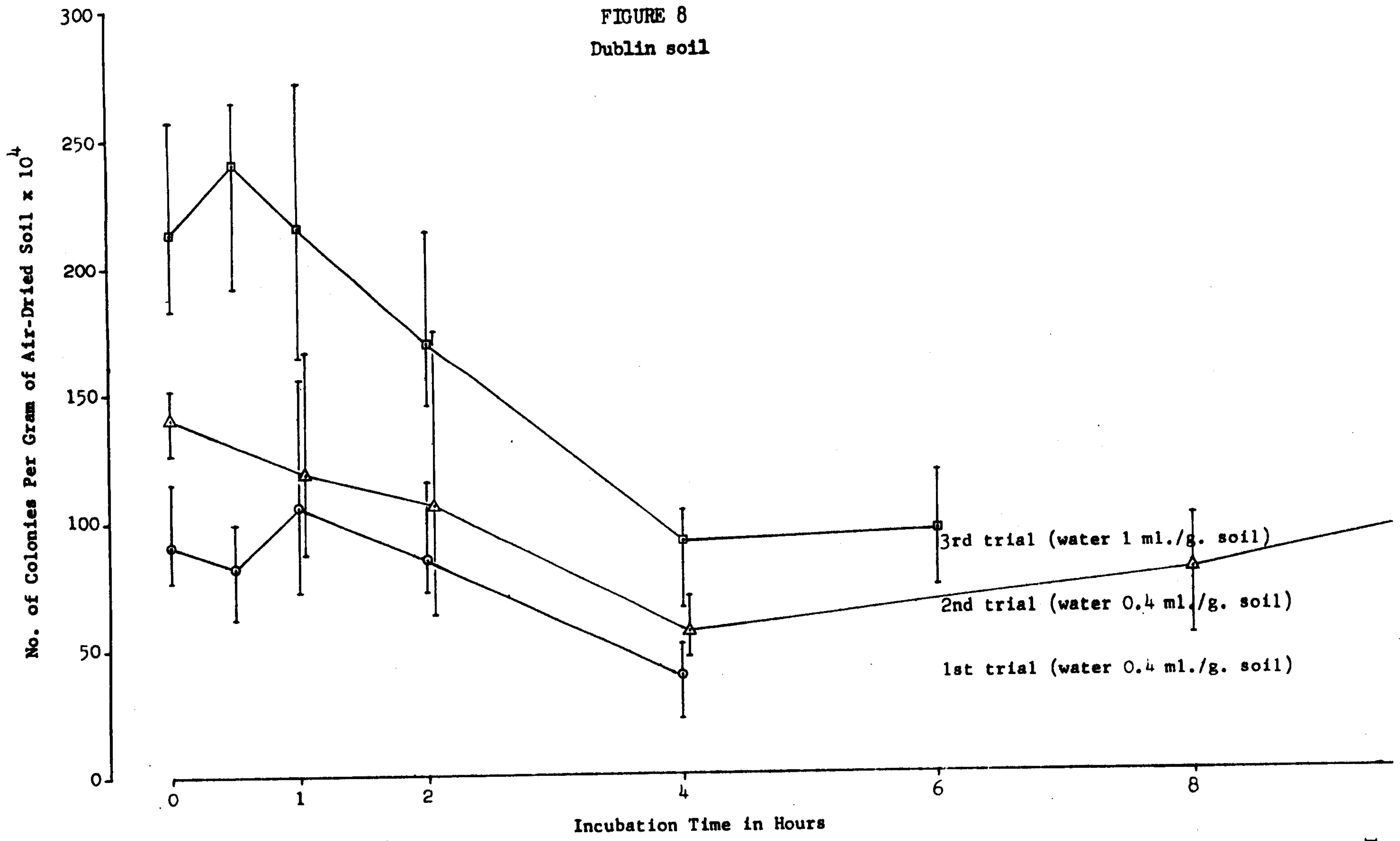
separation of microbial cells which became stuck together during the original air-drying. The subsequent levelling off (in the case of the first two trials) and slight decrease (the third and fourth trials) indicate that no multiplication of cells occurred since conditions remained virtually constant. The comparison of the first and second trials, in which only water was added, and the third and fourth trials, in which urea was added, clearly indicates that the urea depressed the growth of microorganisms from air-dried soil under the conditions of this study.

Figures 8 and 9: Fig. 9 is the full plot of the data from the second trial with Dublin soil. This graph is shown in part in Fig. 8 for comparison with the other two trials. The initial decline in numbers in all three trials lasted for about 4 hours and then levelled off. Fig. 9 shows that an upward trend begins between 4 and 8 hours and increases at an increasing rate, although not until 16 hours have elapsed does the colony count surpass the initial one. It is therefore unlikely that before this time there is any significant increase in the amount of urease present in the soil.

The third, or "waterlogged", trial shows the same basic trend as the others, but yielded a significantly higher colony count.

Figure 10: the single trial with Hilgard No. 7 soil yielded viable cell counts that show the same general trend and are of the same order of magnitude as the Dublin soil trials. The stable, levelling off, period is somewhat longer, extending from about 4 to at least 8 hours.

FIGURE 8
Dublin soil



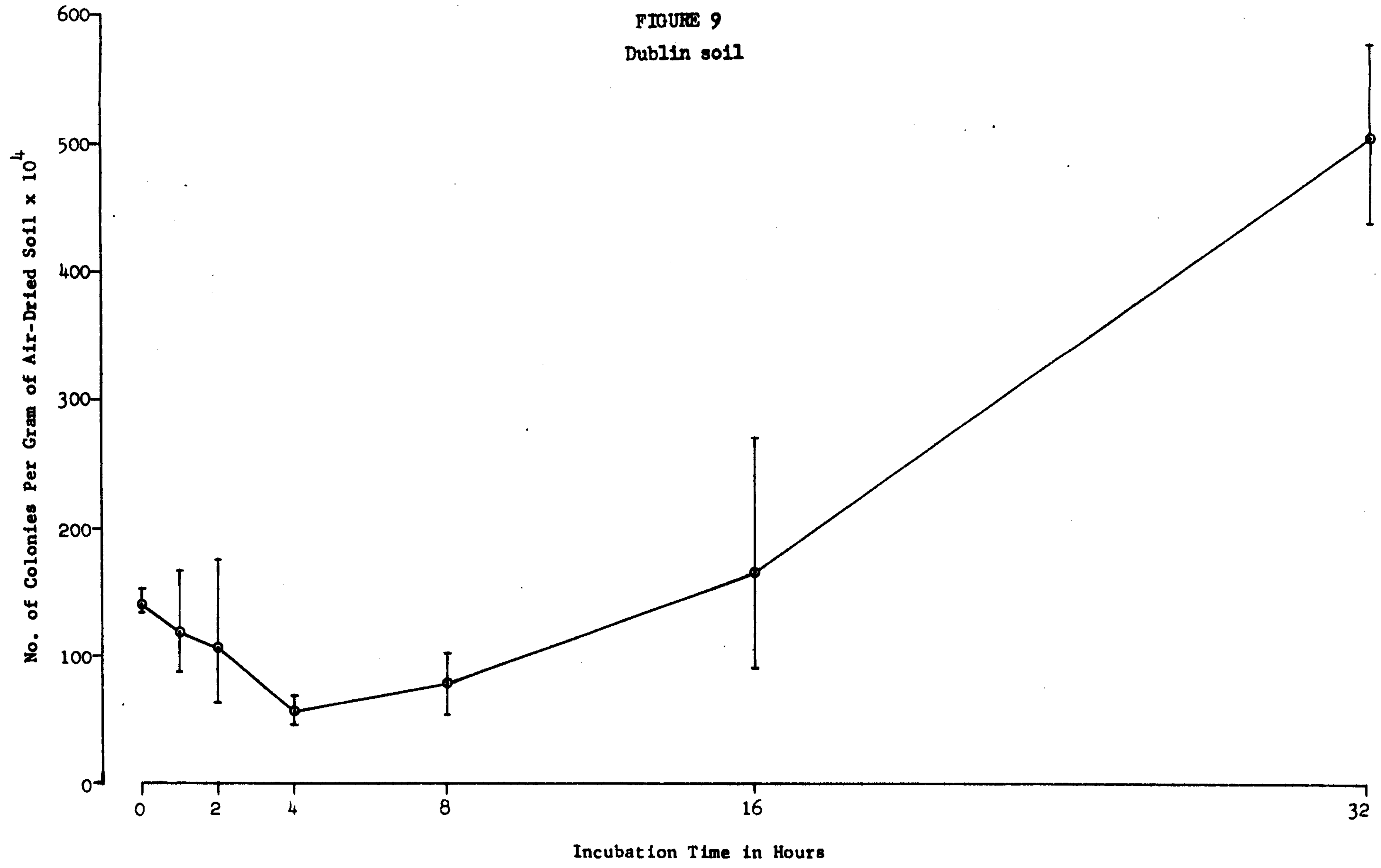
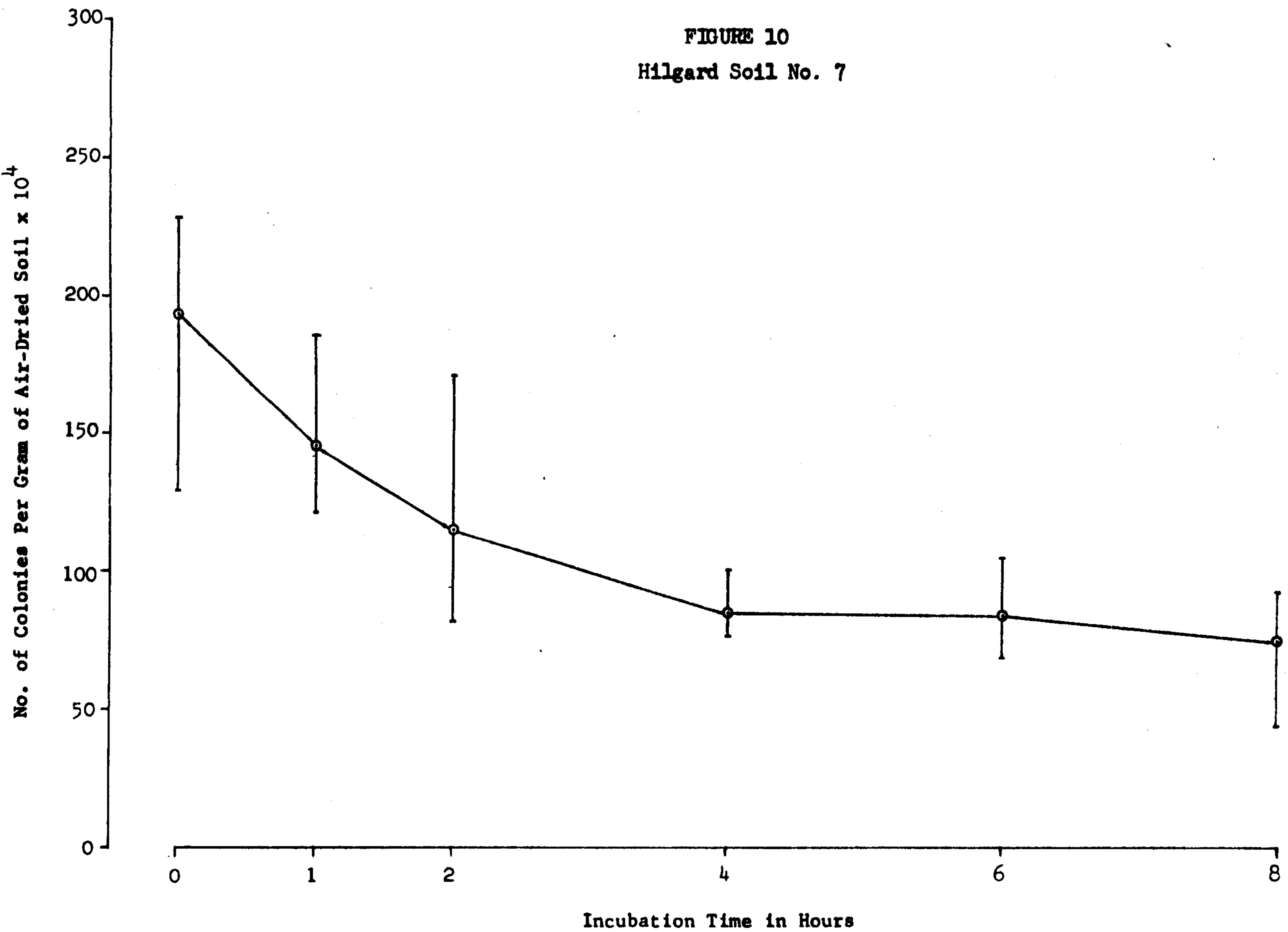


FIGURE 9
Dublin soil
No. of Colonies Per Gram of Air-Dried Soil x 10^4
600
500
400
300
200
100
0
0
2
4
8
16
32
Incubation Time in Hours

FIGURE 10
Hilgard Soil No. 7



The same amount of water was added to each soil type at the beginning of the incubation period to facilitate ready replication. Since the three soils do not possess the same water-holding capacities, the effective moisture content is different for each soil. Any large difference in water-holding capacity as, for example, might be expected between the Yolo and Dublin soils might also complicate the comparison of trends. However, although Dublin soil has a significantly higher capacity than Yolo soil, it was the Dublin soil that gave a trend essentially the same as that expected and obtained under waterlogged conditions and the low water-holding Yolo soil that gave the trend expected in aerobic conditions in moistened soil. Furthermore, the Hilgard No. 7 soil also behaved as if waterlogged. Under waterlogged conditions it is the restriction imposed on gaseous exchange which results in the suppression of aerobic and stimulation of anaerobic microorganisms. It is apparent that in the given textured soils anaerobic conditions are induced at lower moisture contents relative to water-holding capacity than in coarse soils due to the smaller spaces between the soil particles. It should be noted that the waterlogged conditions approximate most nearly the incubation conditions of the Conway procedure.

The addition of 10 mg. urea/g. air-dried Yolo soil significantly lowered the colony count as compared with unamended soil. Work is in progress to evaluate these results.

Conclusions.

Dilution plate colony counts with three soils clearly indicate that there is no significant increase in the number of viable microorganisms

over an incubation period of at least 6 and perhaps as much as 16 hours under moisture conditions of approximately field capacity or waterlogging. Consequently, it can be expected that there is no error introduced into the Conway procedure by microbial multiplication during the 4-hour incubation period used.

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D. Microbiological Characterization of Soils.

The microbiological characteristics of the stored Hilgard soils were determined by Roy E. Cameron, Jet Propulsion Laboratory, Pasadena, California.

This information supplements Table II, Characteristics of Soils, Second Semiannual Progress Report, July 20, 1965, pg. 41.

Media: Aerobes and actinomycetes: trypticase soy agar.

Facultative anaerobes: fluid thioglycollate medium.

Anaerobes: trypticase soy agar; incubation in CO_2 atmosphere.

Fungi: Rose Bengal agar.

Algae: Pochon's salt medium with soil extract.

The results are presented in Table II.

Table II. Microbiological Characteristics of Hilgard Soils.

Soil	Aerobes: Bacteria + Actinomycetes x 10^3	Facultative Anaerobes[1),3)] Growth in dilutions 10^{-4}	10^{-5}	10^{-6}	10^{-7}	Anaerobes x 10^3	Fungi	Algae[2),3)] Growth in dilutions 1:10	10^{-2}	10^{-3}	10^{-4}
Hilgard 1	830	+	+	+	–	33	0	+	+	–	–
2	59	+	+	+	–	5.55	0	–	–	–	–
3	38	+	+	+	–	4.40	0	–	–	–	–
4	11	+	–	–	–	0.02	0	–	–	–	–
5	530	+	+	–	–	8.84	0	+	–	–	–
6	30	+	–	–	–	0.880	0	–	–	–	–
7	5000	+	+	+	+	240	0	+	+	–	–
8	1850	+	+	+	–	24.5	0	–	–	–	–
9	395	+	+	–	+	0.305	0	–	–	–	–
10	160	+	+	–	–	1.18	0	–	–	–	–
11	362	+	+	+	+	10.9	0	+	+	+	–
12	160	+	+	+	+	5.55	0	–	–	–	–

Numbers per gram of soil.

1) Includes microaerophillic bacteria

2) Includes microscopic examination for growth

3) For dilution cultures + = growth; – = no growth

E. Adsorption and Reactions of Chitinase on Chitin.

The significance of adsorption phenomenon involved in enzyme reactions at surfaces has been discussed by McLaren (1).

Chitin-chitinase and chitin-lysozyme systems provide with an opportunity to study *in vitro* the adsorption of a soluble enzyme on the surface of insoluble substrates and the characteristics of its catalytic breakdown. Although many reports have been published on the hydrolysis of chitin (2-26, 29, 31) only scant data are available regarding adsorption of enzymes on chitin (7,27).

Chitinase has been investigated by Skujins et al. (31) previously, and an extensive research has been done by McLaren (37) on the adsorption of proteins and enzymes, including lysozyme, on clays.

In this report we describe the initial results in our studies on the adsorption and reactions of chitinase and lysozyme on chitin.

Materials and Methods.

Substrate. Technical crustacean chitin, Kylan PC (Moretex Chemical Products, Spartanburg, South Carolina) was used for preparation of the dispersed chitin, according to the following method:

10 g. of Kylan PC was shaken with 200 ml of conc. HCl in an Erlenmeyer screw cap flask. It was kept at +4°C for 6 hours, and it was shaken occasionally. It was filtered through glass wool and the filtrate was poured slowly into 4 liter size beaker containing 3 liters of 50% (v./v.) aqueous ethanol while stirring it vigorously.

The beaker with the finely dispersed chitin was stored in refrigerator. When the chitin settled the clear supernatant was syphoned off and replaced with distilled water, and mixed again. The washing was repeated 18 times during the following 5 days.

Microorganisms and media. Two different *Streptomyces* sp. strains were used for chitinase production.

The first strain, designated as 3-C is the same which was used in investigations by Skujins et al. (31). The second strain - 2-B - was isolated from a soil sample of U.C. Berkeley Campus grounds.

About 120 g. of surface soil was mixed with 20 g. of chitin and was kept moist in a crystallization dish, in dark, at room temperature. After two weeks about 2 g. of soil was suspended in ca 30 ml. of water and 1:1000 to 1:100,000 dilutions were inoculated on surface of agar plates containing dispersed chitin as the single source of C and N, as used by Reynolds (2) and as recommended by Lingappa and Lockwood (32):

Dispersed chitin	2.5 g.		
K_2HPO_4	0.7 g.	$ZnSO_4$	0.001 g.
KH_2PO_4	0.3 g.	Agar	20 g.
$MgSO_4$	0.5 g.	Water	1000 ml.
$FeSO_4$	0.01 g.	pH	7.0

Twelve different strains of *Streptomyces* were isolated. Strain number 2-B produced the widest clear zone around the colonies and was chosen for a cultivation in liquid medium.

The basal liquid medium was prepared according to Skujins et al. (31):

K_2HPO_4	0.8 g.	$CaCl_2 \cdot 2\ H_2O$	0.01 g.
KH_2PO_4	0.2 g.	$ZnSO_4 \cdot 7\ H_2O$	0.001 g.
$(NH_4)_2SO_4$	0.5 g.	Water	1000 ml.
$MgSO_4 \cdot 7\ H_2O$	0.2 g.	pH 7.0	
$FeCl_3 \cdot 6\ H_2O$	0.01 g.		

Erlenmeyer flasks of 500 ml. size were used. Each flask contained 1.5 g. of commercial technical crustacean chitin and 325 ml. of basal liquid medium. They were plugged with cotton and covered with paper cups. The flasks were autoclaved at 15 lb. for 20 minutes, cooled, and each inoculated with the whole growth of a 2-B slant, washed off in 5-10 ml. of basal liquid medium. Flasks were placed on a rotary shaker and incubated at 28-31°C.

Crude chitinase preparation and purification. In cultures of streptomycete 2-B the maximum extracellular chitinase activity was reached after 4 days of growth. The 4-day old streptomycete No. 2-B cultures were filtered through No. 42 Watman filter paper using a Buchner funnel. The proteins were precipitated with $(NH_4)_2SO_4$ (special enzyme grade Mann Research Laboratories, New York) at 85% saturation and left overnight in refrigerator. The precipitate was collected in a Buchner funnel using No. 42 Watman filter paper and Celite suspension. Then the filter paper with Celite pad and precipitate was removed, crushed and washed out in 50 ml. of cold 0.01 M Na phosphate buffer pH 7.0. The dissolved precipitate was filtered off

through No. 42 filter paper.

Depending on amount of buffer used for washing the yield is about 50 ml. of crude chitinase, usually of a dark brown color. Crude chitinase preparations were kept in a frozen state in small plastic bottles until being used.

Purification of crude chitinase was done by elution from DEAE-cellulose column with 0.01M Na phosphate buffer, pH 8.4. As shown by Skujins et al. (31) the procedure separated chitinase of many proteins and pigmented substances. However, some colored substances in crude chitinase obtained from cultures of streptomycete 2-B could not be removed by elution from DEAE-cellulose column. Therefore, it was passed in addition through a column of sephadex G-50. As eluent 0.01 M Na phosphate buffer pH 7.0 was used.

The diethylaminoethyl cellulose (DEAE-cellulose) (Cellex-D, Bio-Rad Laboratories, Richmond, California) exchange capacity 0.73 meq./gm. was prepared as follows:

1. Suspended in 1 N HCl, followed by a wash with distilled water,
2. suspended in 0.2N NaOH; washed,
3. suspended in 0.2M Na_2HPO_4, washed,
4. suspended in 0.01M, pH 8.4 Na-phosphate, stirred and adjusted to pH 8.4 with H_3PO_4 or NaOH. Sufficient time was allowed for phosphate and cellulose to equilibrate. Sephadex G-50 fine mesh was suspended in 0.01M Na-phosphate buffer pH 7.0. Six hours were allowed for swelling before it was packed in the column.

Assay procedure for determination of chitinase activity.

Definition of chitinase activity unit:

One chitinase unit is the amount of enzyme required to catalyze the release of one micromole of N-acetyl-hexosamine, expressed as N-acetylglucosamine, from its substrate. The poly-N-acetylglucosamine (dispersed chitin), per minute at 37° and pH 5.5.

N-acetylglucosamine was determined with the p-dimethyl-aminobenzaldehyde reagent according to the method of Reissig et al. (34).

Chemicals and reagents used:

A. N-acetyl-d-glucosamine, (A grade Calbiochem).

B. Na-phosphate-acetate buffer, pH 5.5, 0.05M in Na, was used for chitin-chitinase and for chitin-lysozyme incubations.

C. Potassium-borate buffer (K-tetraborate Rg.) (33):

$K_2B_4O_7 \cdot 4H_2O$, 0.8M in borate, pH 10.3.

Dissolved in water: 12.35 g. H_3BO_4

4.50 g. KOH

Volume made up to 500 ml. and pH adjusted to 10.3 with KOH.

D. DMAB reagent: 1 part of stock solution diluted with 9 parts of glacial acetic acid before use.

Stock solution: 10 g. p-dimethylaminobenzaldehyde (Eastman Kodak, purified) dissolved in 100 ml. acetic acid containing 12.5% (V/V) 10 N HCl; stored in refrigerator.

General Procedure:

Add to a screw cap tube:

1.0 ml. of dispersed chitin in water (5.0 mg dry weight)

3.0 ml. Na phosphate-acetate buffer pH 5.5, 0.05M

1.0 ml. enzyme in 0.01M Na phosphate pH 7.0

Tubes are mounted on the "Ferris wheel" and set in a water bath at 37° for a predetermined time - usually for 30 minutes, if not stated otherwise. After incubation the tubes are placed in an ice bath for 3 minutes and subsequently centrifuged.

N-acetylglucosamine determination. Add into "Spectronic 20" tubes:

1.0 ml. supernatant

0.2 ml. tetraborate reagent, mix and set into a boiling water bath for exactly 3 minutes and 15 seconds, after which time the tubes are placed in an ice bath. After 5 minutes 5.0 ml. DMAB reagent is added to each tube and they are incubated in a water bath at 37° for 20 minutes. After covering in an ice bath, the A_{585} readings are made.

Lysozyme - 2 x crystallized (Worthington Biochemical Corp., Freehold, New Jersey) — dissolved in 0.05 M Na phosphate buffer pH 7.0.

$E_{280}^{mg/ml}$ lysozyme = 2.64 (38).

<u>General procedure for adsorption of enzyme proteins on chitin.</u>

Usually to 0.2 or 0.5 ml (= 1.0 or 2.5 mg) of chitin suspension in a buffer, the enzyme solution was added to bring the total volume to 4.0 or 5.0 ml.

Tubes were mounted on a Ferris wheel and incubated in a water bath at 25° for a predetermined time. After turning the chitin was sedimented by centrifugation at 1000 G. for 5 minutes. The concentration of the non-adsorbed protein in the supernatant was determined by A_{280} readings in a Beckman DU spectrophotometer.

Buffers:

1) Na-phosphate-acetate pH 5.5, 0.05M in Na,
2) Universal buffer (35):

$Na_2HPO_4 \cdot 7\ H_2O$	13.405	g
Citric acid	7.00	g
H_3BO_3	3.14	g
1.0 N NaOH	243.0	ml

Dissolve in 1 liter. Dilute 10 times and titrate with 0.1N HCl to the desired pH.

RESULTS

Adsorption of chitinase on chitin.

An attempt was made to establish the characteristics of the rate of chitinase adsorption on chitin. The reaction times above 1 minute failed to show any significant differences in the amount of the adsorbed enzyme protein. It is evident that the adsorption of chitinase on chitin at pH 5.5 and 25° takes place in less than a minute.

It can be seen from further data (Fig. 11 and Fig. 12) that 0.25 mg/ml chitin has adsorbed the maximum amount of protein A_{280} = 0.070,*

*As $E_{280}^{mg/ml}$ for our purified chitinase has not been established as yet, all quantitative data are given in absorbance at 280 mμ.

FIGURE 11

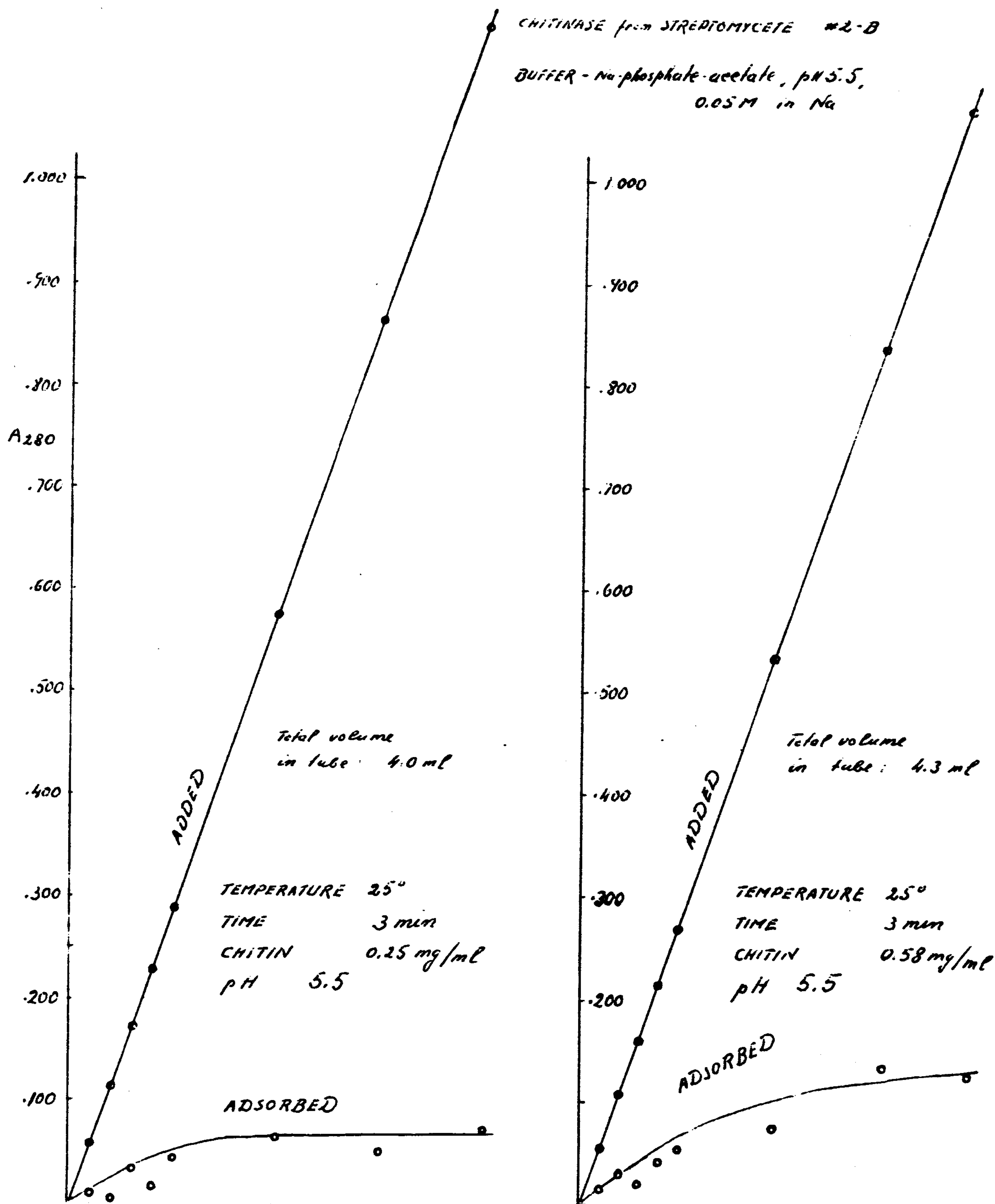
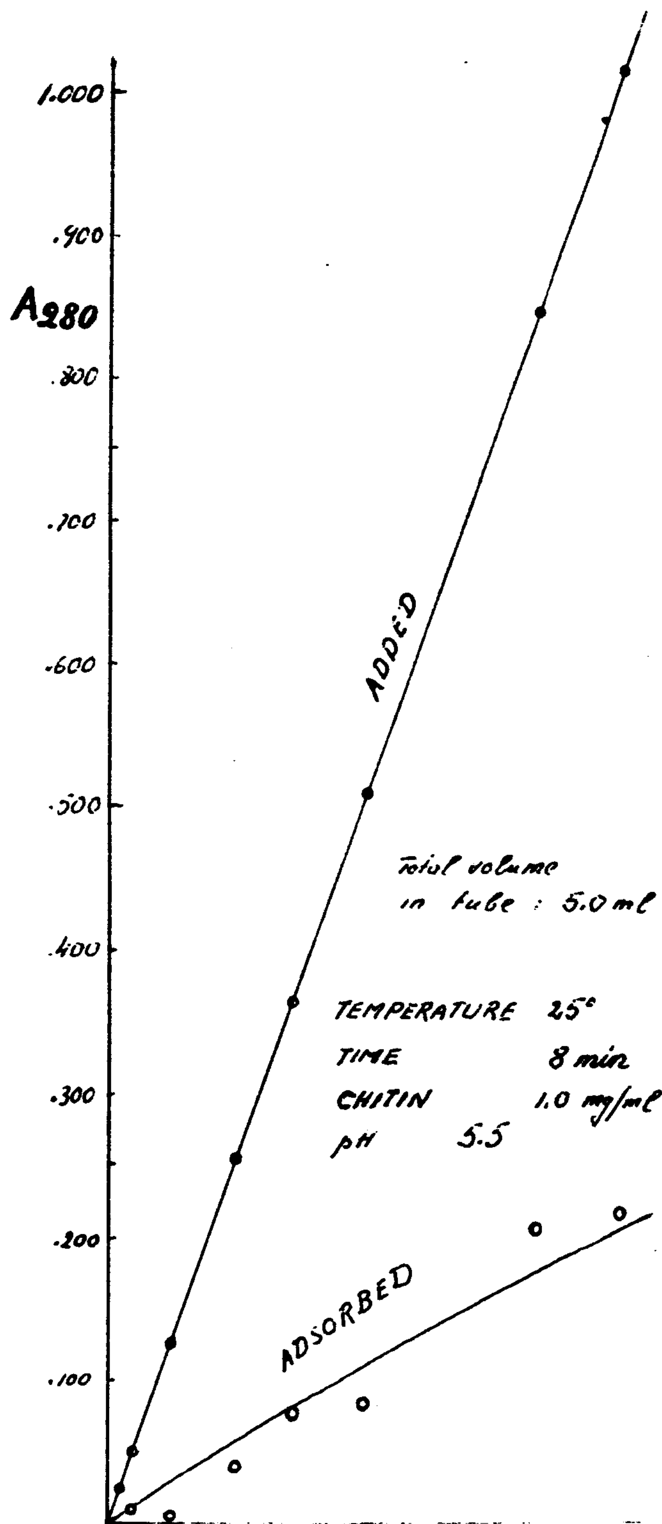


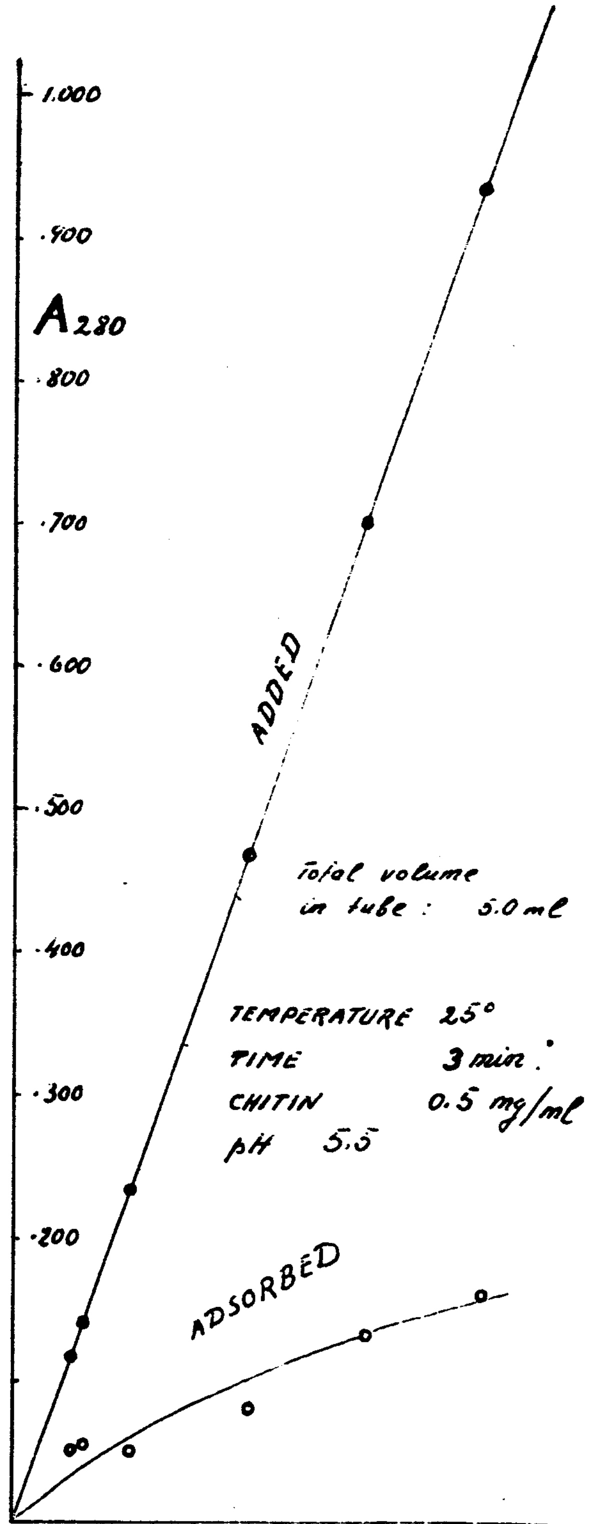
FIGURE 12

ADSORPTION of CHITINASE on CHITIN

CHITINASE - from STREPTOMYCETE #2-B

Na phosphate-acetate buffer pH 5.5, 0.05 M in Na





and that the system is at equilibrium after 3 minutes.

Adsorption of lysozyme on chitin.

Effect of pH. Amount of lysozyme adsorbed on chitin is increasing from pH 3 to pH 9 at different enzyme concentrations and buffer systems (Fig. 13 and Fig. 14).

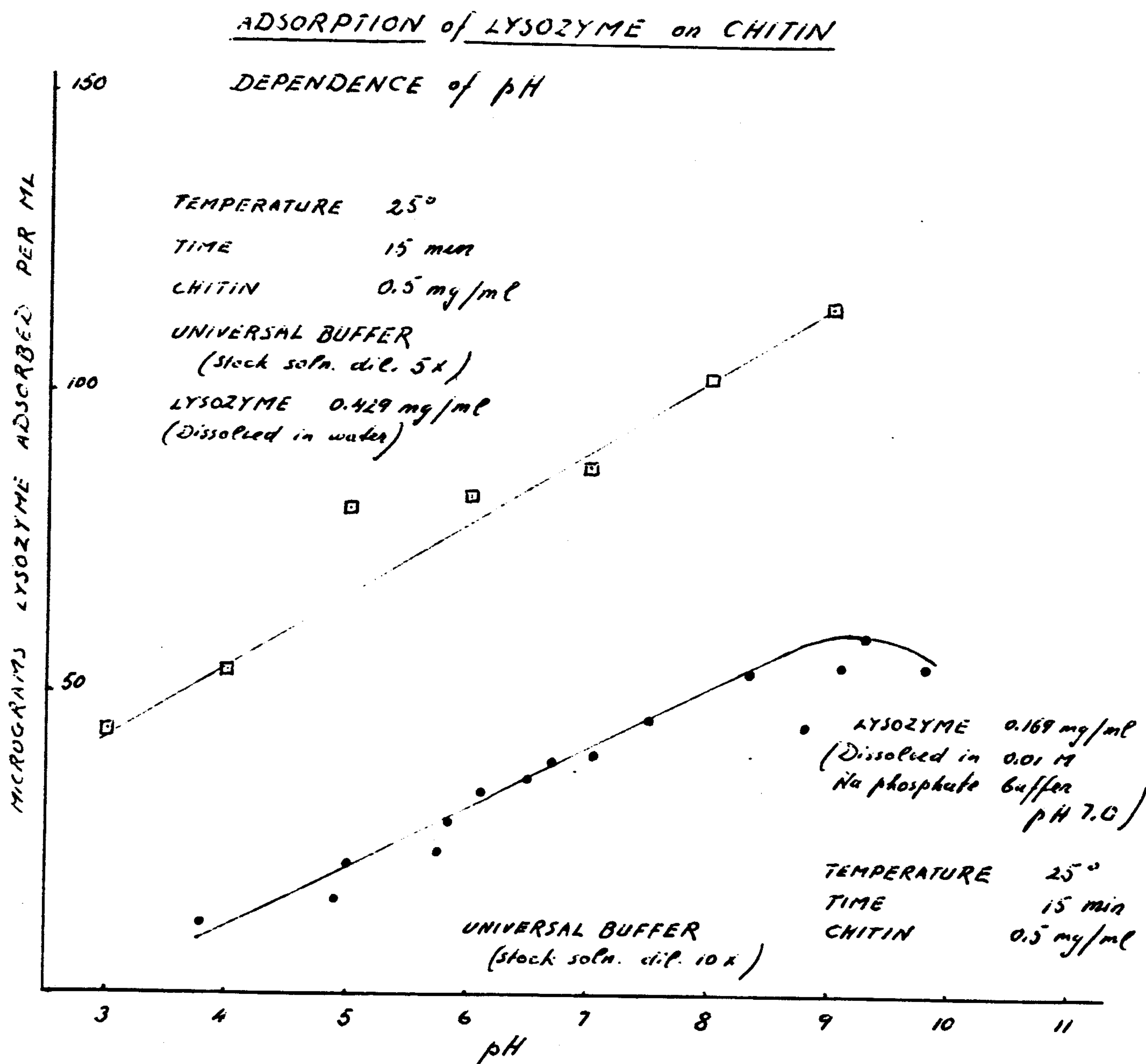
By using an enzyme concentration of 0.16 mg/ml - with a normally (10X) diluted stock universal buffer and with a 5X diluted stock universal buffer the same pattern of adsorption was obtained but the amount of adsorbed lysozyme protein is about twice as much in the first case as in the second.

When different amounts of enzyme were incubated parallely in universal buffer (10 x diluted stock solution) pH 5.5, and in 0.05M Na phosphate-acetate buffer pH 5.5, there were proportionally more lysozyme adsorbed on chitin in presence of the universal buffer than in the presence of phosphate-acetate buffer.

Adsorption of N-acetylglucosamine on chitin.

Determination of chitinase activity with DMAB method would show a lesser enzymatic activity if N-acetylglucosamine would become partially adsorbed on chitin. Therefore, to establish a standard curve for the N-acetylglucosamine assay with this method the determinations of absorbance were performed in the presence and in the absence of chitin. The results showed that some adsorption of N-acetylglucosamine on chitin evidently took place (Fig. 15).

FIGURE 13



$E_{280}^{mg/ml} = 2.64$

FIGURE 14

Adsorption of lysozyme on chitin

Dependence of pH

TEMPERATURE 25°

TIME 10 min

CHITIN 0.5 mg/ml

UNIVERSAL BUFFER
(Stock soln. dil. 5x)

LYSOZYME 162 mg/ml

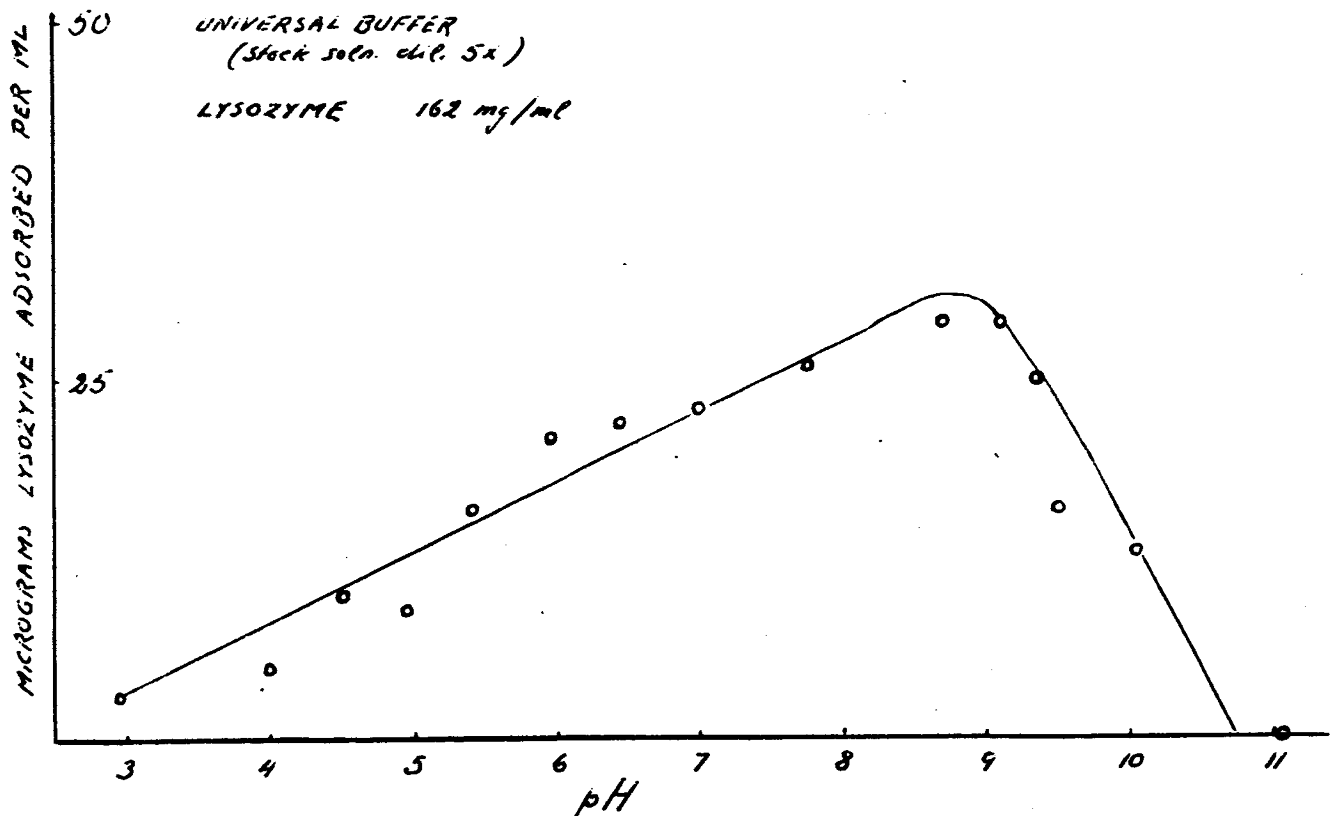
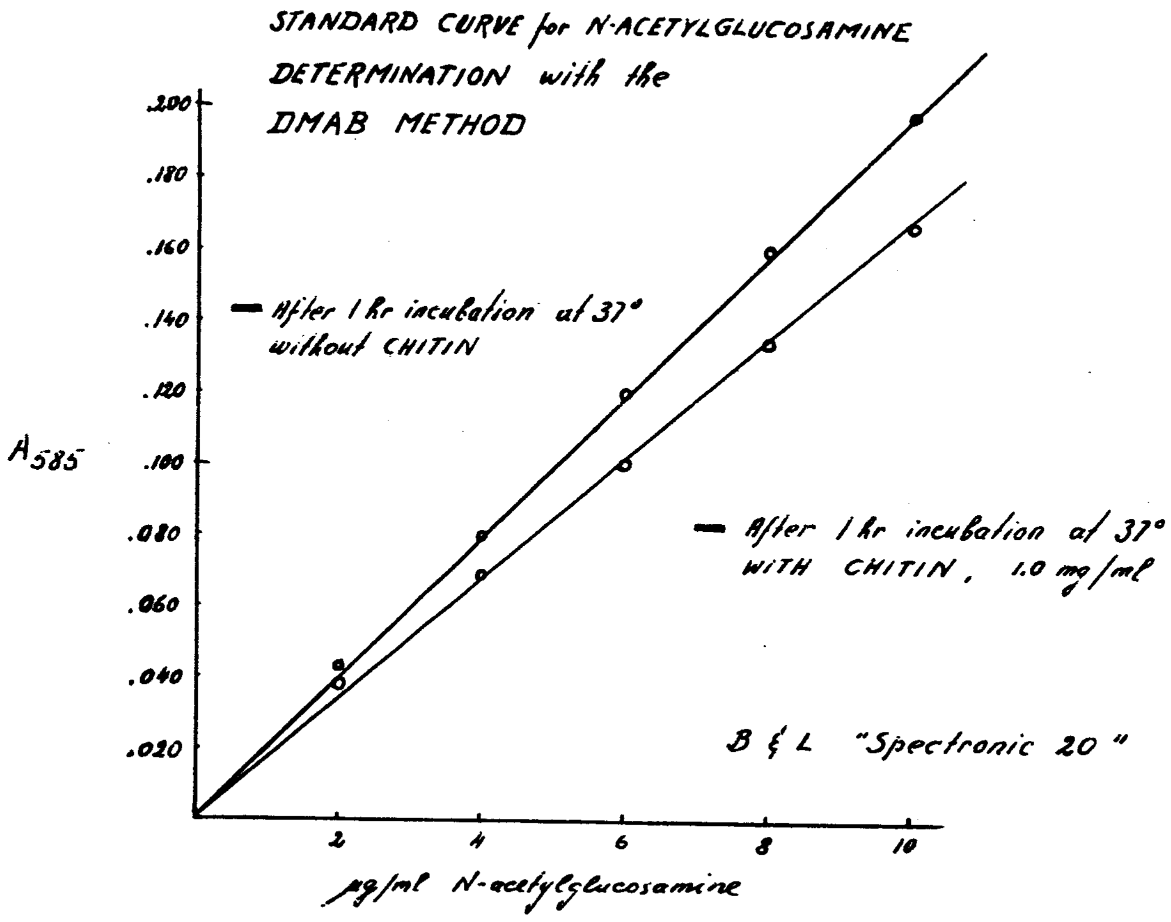
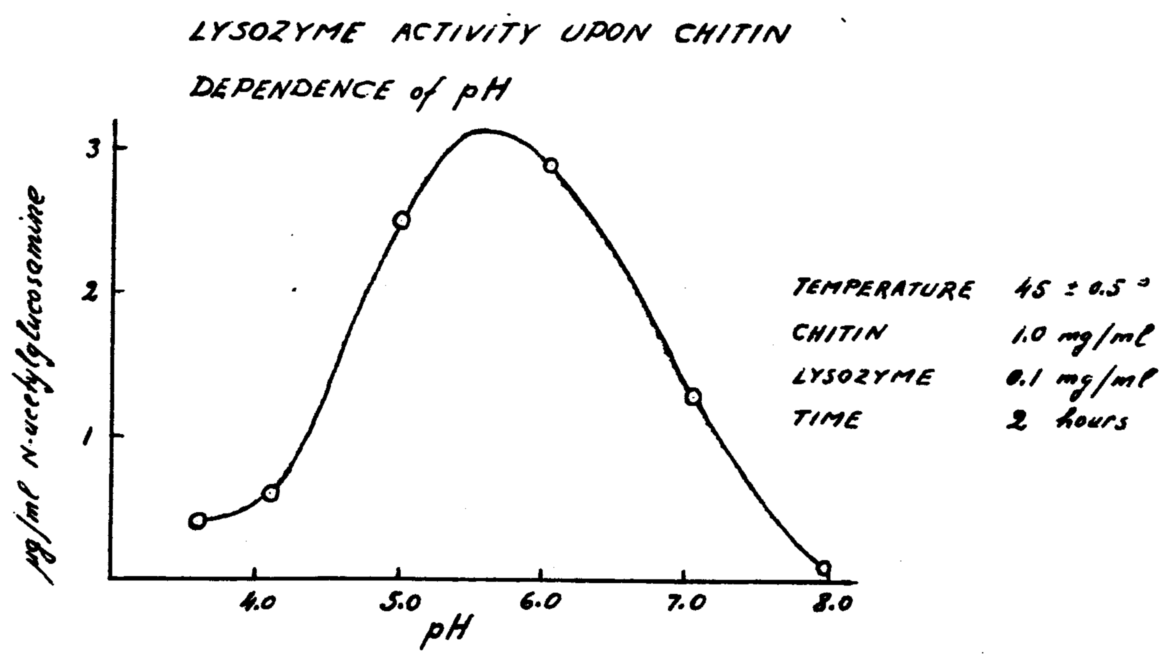


FIGURE 15





Activity of lysozyme upon chitin.

Preliminary experiments confirm that hydrolysis of chitin by lysozyme is a comparatively slow reaction and it is a pH, time, and concentration dependent process (Figs. 15 and 16).

Discussion

The maximum concentration of extra-cellular chitinase in cultures of streptomycetes was reached on sixth day by Reynolds (2), and on fifth day by Skujins et al. (31). Also in cultures of *Aspergillus niger* the highest level of extra-cellular chitinase was reached on fifth day by Otakara (12-18).

The cultures of streptomycete No. 2-B were the richest in extra-cellular chitinase after four days of incubation. After the fifth day there is a sharp decrease in the concentration of chitinase although the streptomycetes continue to increase in weight.

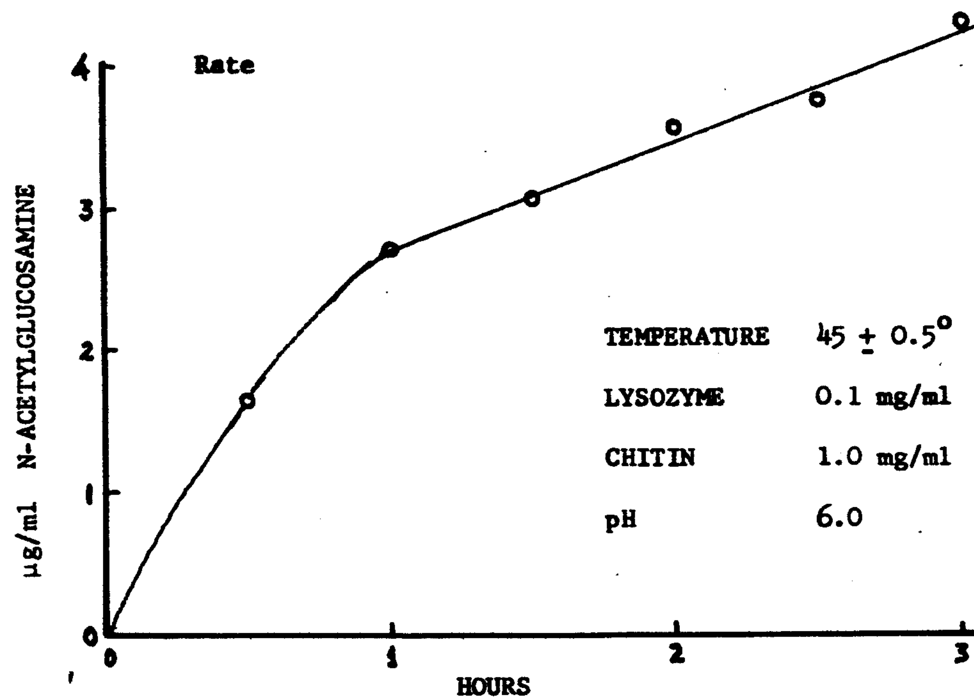
Purified chitinases

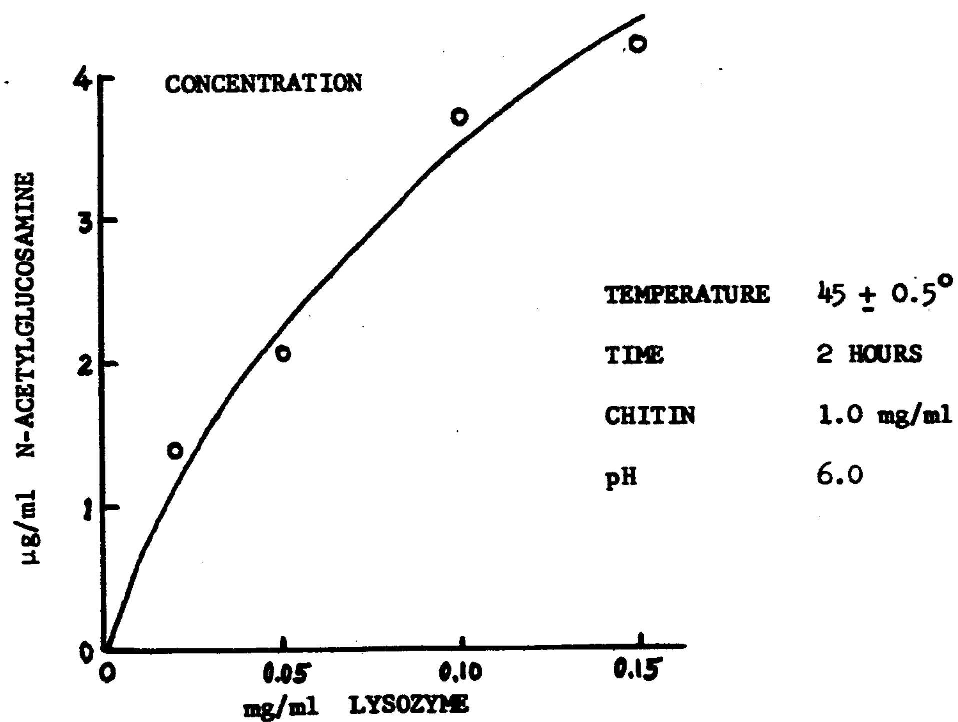
Berger and Reynolds (8) separated the *Streptomyces grisens* chitinase into two components by a zone electrophoresis on starch beds of pH 6.3. One of the fractions was pure but the other was mixed with chitobiase.

Jeuniaux (3,6,7) obtained a purified chitinase fraction from *Streptomyces antibioticus* by adsorbing it on chitin, eluting with buffers and subsequent fractionation with ammonium sulphate. The molecular weight of the pure fraction was estimated about 30,000, with $E_{280}^{mg/ml} = 1.24$, and 95% of its proteins were chitinolytic. However, he was able to separate this fraction of chitinase further by electrophoresis at pH 8.2 into 3 separate components, which all contributed

FIGURE 16

LYSOZYME ACTIVITY ON CHITIN





to the chitinolytic activity.

DEAE - cellulose columns have been used for chitinase purification with good results by Skujins et al (31) and by Powning and Irzykiewitz (19). The later studied plant chitinase and separated it into two components.

The chitinase system of Aspergillus niger has been extensively investigated by Otakara (12-18). He suggests a participation of two different enzymes in the decomposition of glycol-chitin and chitin. Lunt and Kent (11) investigated chitinases obtained from Carcinus maenas. In their opinion depolymerization of the chitin chains could be caused by one and the release of N-acetyl-glucosamine by the second enzyme.

The purified chitinase from streptomycete No. 2-B show two distinct peaks of activity when fractionated from Sephadex column. Further investigations of the properties and characteristics of these enzymes are in progress.

Adsorption of enzymes on chitin

The affinity of chitin to adsorb certain proteins is a well known phenomenon. Nozu (27) suggests the use of chitin as a specific adsorbent of lysozyme. Jeuniaux (3,6,7) is using the adsorption of chitinase on chitin at pH 5.2 as the first step in the purification of extra-cellular microbial chitinase.

Wenzel et al. (36) have reported that N-acetylglucosamine inhibits the activity of lysozyme upon chitin. Investigating the cause Johnson and Phillips (28) found that N-acetylglucosamine and two other inhibitors are binding specifically to one and the same site on the lysozyme molecule. It is evident that not only the adsorption of enzyme on

substrate but also the adsorption of breakdown products on enzyme and probably on substrate as well should be considered.

Activity of lysozyme upon chitin

Hydrolysis of chitin by lysozyme was observed by Berger and Weiser (22). Hamagushi and Funatsu (29) reported that the hydrolysis of glycol-chitin by lysozyme is more rapid than that of native chitin. Later the activity of lysozyme upon glycol-chitin was investigated by Hamagushi et al. (23) and by Hayashi et al. (24,25).

Jeuniaux (7) reported that the rate of hydrolysis of chitin by lysozyme is about 300 times slower than that of chitinase.

Investigators	Substrate mg/ml	Lysozyme mg/ml	Buffer	pH	t°	Time in Hours	H-acetlyglucosamine released μg/ml per hr.
Berger and Weiser (22)	Purified chitin 0.5	0.100	0.01 M Sorensens phosphate	7.0	37	12-40	~0.8
Hamagushi and others (23)	Glycol-chitin 2.6	24 μM if M.W. of Lys. is 14500	$\frac{M}{15}$ phosphate	5.5	33	8-48	~1.0 (glucosamine HCL)
Hayashi et al. (24)	Glycol-chitin 0.5-5.0	0.01-0.60	pH 3.6-4.6 0.1 M acetate; pH 5.6-8.0 0.1 M phosphate	1-8 optimal 4-5	0-100 optimal 50	1-9	
	5.0	0.5		4-5	50	4	~100
Jeuniaux (7)	Colloidal chitin 1.25	0.625	0.1 M phosphate-citrate	5.2	37		0.2

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