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SPACE SCIENCES LABORATORY

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

By: J. J. Skujins and
A. D. McLaren (Principal Investigator)

Tenth Semi-Annual Progress Report on
NASA Grant NGL 05-003-079

Department of Soils and Plant Nutrition

Period: 1 January 1969 to 30 June 1969

1 July 1969

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Berkeley, California 94720

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I. PREFACE

Our objective is twofold. First, we are developing procedures for detection and assay of enzymes in soil suitable for presumptive tests of life in planetary soils. This requires use of enzyme-substrates that are stable to heat, moisture and storage. We have shown previously that urea, as a substrate for urease, meets these requirements very satisfactory. Second, we are exploring the behavior of enzymes in non-classical systems. These include soluble enzyme action at surfaces in gels (insoluble substrate) and in other heterogenous, structurally restricted systems analogous to those found in cells and in soils, including systems of low water activity.

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 substrate by adsorbed enzyme (hydrolysis of chitin by chitinase) is being continued and extended to clay surfaces in order to investigate some of the factors influencing reactions at interfaces analogons to those in soils.

Emphasis has been placed on the detection of urease activity because of the probable primordial origin of urea as an organic substance, because of its stability as an enzyme substrate, and because of the ubiquity of soil urease in the terrestrial environment.

Urease in soils has shown a high resistance to high energy electron-beam irradiation, urease activity has been recovered in over

9000 years old permafrost soil samples, and the hydrolysis of urea by urease in media of low water availability is detectable below 50% relative humidity (at physiological temperatures) and measurable hydrolysis of urea occurs in soils at 70% relative humidity and above. These observations, among others, indicate that there exists in soil an extracellular and enzymologically active moiety of urease.

PERSONNEL

The participants in the currently reported phase of this project included Professor A. D. McLaren, Dr. J. J. Skujins, Mr. A. H. Pukite, Mr. W. H. Brams, and Miss Renee Zin-May Sung.

II EXPERIMENTAL

A. STUDY OF ORGANIC-MATTER-EXHAUSTED SOILS

This report describes the continuation of work initiated on a study of degradability of soil organic matter and the properties of soil organic matter resistant to the activities of microorganisms (1, 2, 3). In particular, the present work is a continuation of a study to determine the retention of enzymatic activities in soil subjected to various treatments of partial or total sterilization.

Materials and Methods

Wet heat treatment: 20 g soil was placed in a screw-capped vial, moistened with glass-distilled water to its water holding capacity, and the wet soil was pressed against the walls of the vial. The vial was tightly capped and placed in a stirred water bath at 80°C for three hours. The soil was then removed, spread in a thin layer in a Petri dish and dried overnight.

Flask culture: 100 g Dublin soil and 100 ml water were placed in a 2000 ml filter flask and inoculated with 20 ml of a suspension of fresh garden soil. The weight of the soil in the inoculum was about 5 grams. The flask was sealed with a rubber stopper to which was attached a plastic test tube containing 10 ml of 5N NaOH. The flask was attached to the respirometer. During incubation (21°C) the flask was swirled vigorously every few days to resuspend the soil but care was taken not to splash any of the NaOH into the soil suspension.

Urease and phosphatase activities were determined as described before (ref. 1, pp 89-92).

Results and Discussion

The oxygen uptake by inoculated, incubated and treated soil samples is shown in Table I. The effect of various treatments on microbial numbers and on enzyme activity is shown in Table II.

Table I. Oxygen uptake by soil samples.

Sample and time interval		O_2 uptake ml/g (dry weight) of soil
Untreated Dublin	0-18 days	0.078
	18-25	0.079
	25-33	0.058
	33-41	0.058
	41-51	0.078
	51-69	--
	69-98	0.080
Methyl bromide treated Dublin	0-18	0.078
Soil dried, treated again with methyl bromide, then re-inoculated and incubated	0-15	0.075
Dublin soil incubated with excess water - "Flask culture"	0-24	0.083

Table II. Effect of treatments on microbial number and on phosphatase and urease activity in Dublin soil

Treatment	Microorganisms per gram soil	Phosphatase β-naphthol μm/g/hr	Urease CO_2 μm/g/hr
none	8.0×10^6	4.7	0.90
wet and dried 4 times	5.9×10^6	4.2	0.27
wet and dried 5 times	6.2×10^6	4.0	0.31
wet and dried 6 times	6.8×10^6	4.0	0.22
heated dry, 80°C, 3 hr.	3.4×10^6	2.0	0.18
heated wet, 80°C, 3 hr.	7.0×10^4	1.8	0.11
methyl bromide treated (Ds)	60	4.3	0.44
Ds inoculated, incubated 18 days, then dried (Dsi)	4.0×10^8	7.5	0.83
Dsi treated with methyl bromide (Dsis)	165	5.5	0.79
Dsis inoculated, incubated 15 days, then dried (Dsisi)	2.5×10^8	4.0	0.08
Untreated Dublin, inoculated and incubated 7 days	2.1×10^8	5.2	0.28
18 days	1.5×10^8	6.9	0.51
25 days	2.1×10^8	5.8	0.31
33 days	2.9×10^8	5.8	0.42
41 days	2.0×10^8	7.1	0.35
51 days	2.0×10^8	5.8	0.38
69 days	2.2×10^8	6.0	0.29
98 days	2.1×10^8	6.1	0.32
Flask culture	6.8×10^7	3.8	0.23

The rate at which oxygen is consumed in an incubating soil sample is a measure of the rate at which the soil organic matter is being broken down and assimilated by the soil micro-organisms. This, in turn, is a measure of the ease with which the soil organic matter can be degraded. The untreated Dublin soil showed a rate of oxygen consumption of about 0.08 ml/g/day for the first 25 days (Table I). This decreased to a rate of about 0.06 ml/g/day during the next 16 days but then the rate returned to its initial value and stayed constant for the remaining period of time, about two months. This constancy in the rate of oxygen consumption indicates a constancy in the "degradability" of the soil organic matter. However, the value of total oxygen uptake for the 98 day period of incubation indicates that only about 7.4% of the total organic matter had been oxidized, and so the property of constant susceptibility to degradation holds only for this fraction (3). The rate of oxygen consumption was similar to that observed by other workers (4) for other soils of the same organic matter content.

The low rate of oxygen consumption by incubating soils may be due to the fact that the organic matter is unavailable to the soil micro-organisms, perhaps by being bound to the clay particles. To release the organic matter and thus enhance its breakdown, a sample of soil was incubated with excess water ("Flask culture"). The rate of oxygen uptake of this culture was only slightly higher than that in which the soil was incubated in the form of moist crumbs, 0.083 vs 0.078. Therefore,

the limitation in the rate of oxygen uptake does not seem to be due to the unavailability of the soil organic matter as shown by H.F. Birch (4).

The rate of oxygen consumption for inoculated and incubated methyl bromide treated soil (Dsi) is similar to that of untreated soil: 0.078 ml/g/day. This soil, treated again with methyl bromide and then reinoculated and incubated showed a rate of 0.075 ml/g/day. This indicates that the methyl bromide treatment neither reduces the susceptibility of the soil organic matter to degradation (e.g., by methylating reactive groups) nor provides an additional food source to the microorganisms (e.g., in the form of methyl groups). Upon treatment of the soils with methyl bromide, the pH drops by about 0.2 units. However, it returns to normal (pH 5.7) after inoculation and incubation. Thus the treatments with methyl bromide seem not to significantly alter the physical and chemical properties of the soil samples.

The effects of wetting and drying Dublin soil to a total of six times are shown in Table II. The number of microorganisms remains about the same as in untreated soil indicating no multiplication or killing has taken place. However, with each treatment the phosphatase activity decreases, reaching a value of 86% of the initial value after six treatments. Similarly, the urease activity decreases, reaching a value of 24% of the initial value after six treatments. The urease activity is more easily destroyed by the treatments, and this sensitivity puts a limit on the number of treatments involving wetting and drying to which Dublin soil can be subjected without reducing the activity below an easily detectable level.

The effect of heating soil in a wet state rather than in a dry state is one of increased destruction, as shown in Table II. Compared to soil heated in a dry state, microbial counts are reduced about 50-fold. Phosphatase activity is 90%, and urease activity is 61% of dry-heated soil. Again, urease activity is more sensitive to denaturing effects than phosphatase activity. The fact that organisms survive and enzyme activities remain after these treatments attests to the remarkable stability of these entities.

The effects of treating soils with methyl bromide, and then inoculating and incubating them are shown in Table II. Methyl bromide gas causes almost complete sterilization, as shown by the value of 60 microorganisms per gram of soil after treatment. Upon inoculation and incubation, the numbers of microorganisms rise to about the same values as for untreated soils, indicating that methyl bromide or its residues causes neither stimulation nor inhibition of microbial growth. Methyl bromide treatment of the inoculated and incubated soil (Dsi) again causes almost complete sterilization (165 organisms/g) indicating that the treatment does not result in the selection of resistant organisms. Reinoculation and incubation lead to the usual numbers of microorganisms, showing as before the methyl bromide treatment does not influence the growth of the organisms. Also it appears that nutrients are not exhaused since the microbial numbers reach the same levels as usual.

These treatments do not cause marked changes in the phosphatase or urease activities. In general the methyl bromide treatments destroy some activity. Soil treated once with methyl bromide and then inoculated

an incubated shows an increase in activities. However, when this soil is sterilized and again inoculated and incubated (soil Dsisi), there is a decrease in activities with an almost complete loss in urease activity. Since enzymes are produced by microorganisms, large increases in the number of microorganisms should produce an increase in the enzyme activities of a soil sample. Since this was not observed, it may be that sufficient phosphatase and urease activity are present in the soil to prevent the induction of such enzymes in the multiplying organisms, in which case the enzymatic activity would remain constant or even fall somewhat if some of the enzymes free in the soil were degraded. In view of the increases in activities after the first incubation, the decreases after the second incubation are hard to explain. Additional cycles of sterilization followed by inoculation and incubation are necessary to establish if there is any trend in the changes with these treatments.

Untreated Dublin soil, inoculated and incubated continuously for 98 days, shows a constancy in microbial numbers and in phosphatase and urease activities (Table II). This constancy could be explained by the situation described above, with the enzymes free in the soil being degraded very slowly, or it may be that the synthesis of new enzymes balances the degradation of the old. One must carefully distinguish between the stability of the activity and the stability of the enzymes; there may be enzyme turnover despite constant apparent activity.

Dublin soil incubated with excess water had about the same number of microorganisms and the same enzymatic activity as soil incubated in the form of crumbs. The treatment does not seem to increase the breakdown of the soil organic matter.

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B. UREASE REACTION RATES IN MEDIA OF LOW WATER ACTIVITY

The chemistry of the hydrolysis of urea by urease has been reviewed extensively in our previous reports (1, 2).

Although the maximum rate of the urease activity on urea is achieved in dilute aqueous solutions, our investigations showed that the hydrolysis of urea by urease took place also in concentrated urea solutions at a measurable rate (1, 2). Further investigations have shown that the reaction takes place also in a "dry" state, when intimately mixed enzyme and substrate powder is exposed to descrete amounts of water vapor ("relative humidities") in air (3, 4, 5, 8) and in soil (6, 7).

In order to explore further the kinetics of enzymatic reactions specifically the hydrolysis of urea by urease at low moisture levels, we have continued a study of the characteristics of this reaction, especially its dependence on temperature at controlled atmospheric humidities with a pure enzyme - substrate mixture.

Materials and Methods

The methods and procedures used for the preparation of dry C^{14}-urea-urease mixture, the assay of urease activity at various atmospheric humidities,and the apparature used has been described in our previous progress report (8) and elsewhere (5, 7).

Results and Discussion

The results of urease activity on urea in an intimately mixed (lyaphilized) powder, exposed to water vapor at various relative humidity levels at descrete temperatures between 2° and 70°C were presented in Figures 1 to 15, ref. 8.

Examination of Figures 1 to 15 show that there is a certain "break", i.e., a considerable increase in the activity between 65 to 85% relative humidity, depending on the temperature. It appears that two separate phenomena at the two relative humidity ranges take place which direct the urease activity at these conditions. An example at 45°C is shown in Figure 1.

Water vapor is adsorbed on urea at relative humidities above 60 to 90%, and urea crystals deliquesce, depending on the temperature, as shown in Figure 2, whereas at relative humidities below 75% no water vapor is adsorbed on urea at room temperature (cf. Fig. 2, ref. 5).

It is evident that the rate of hydrolysis of urea by urease at relative humidities where urea deliquesces is directly influenced and dependent on the water vapor adsorption and a subsequent dissolution of substrate; for the most part urease is suspended in a concentrated urea solution where the reaction takes place. On the other hand, urease activity at relative humidities where urea crystals do not adsorb water are dependent only on the water available by adsorption on protein, i.e., on the water activity, $\underline{a}$.

The "gaps" between the two activity ranges, as shown on Figures 1 to 15, ref. 8, are compared with the water vapor sorption (i.e., deliquescence) of urea with respect to temperature in Figure 2. For clarity an example is shown at 45°C range: the points A and B of Figure 1 are identical with those in Figure 2. Other aspects of these results have been discussed in our previous report (8).

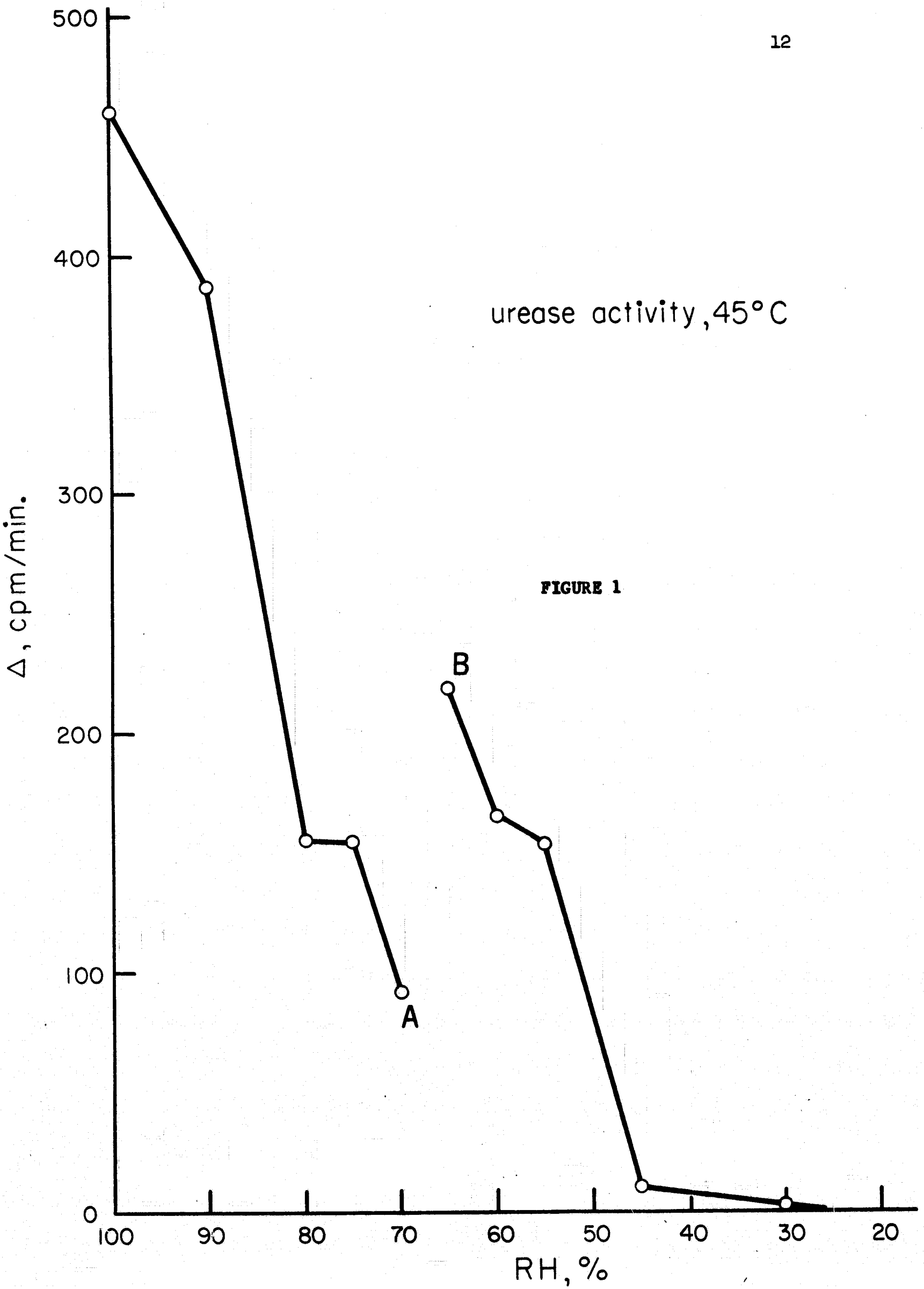
urease activity, 45° C
Δ, cpm/min.
500
400
300
200
100
0
100
90
80
70
60
50
40
30
20
RH, %
A
B

FIGURE 1

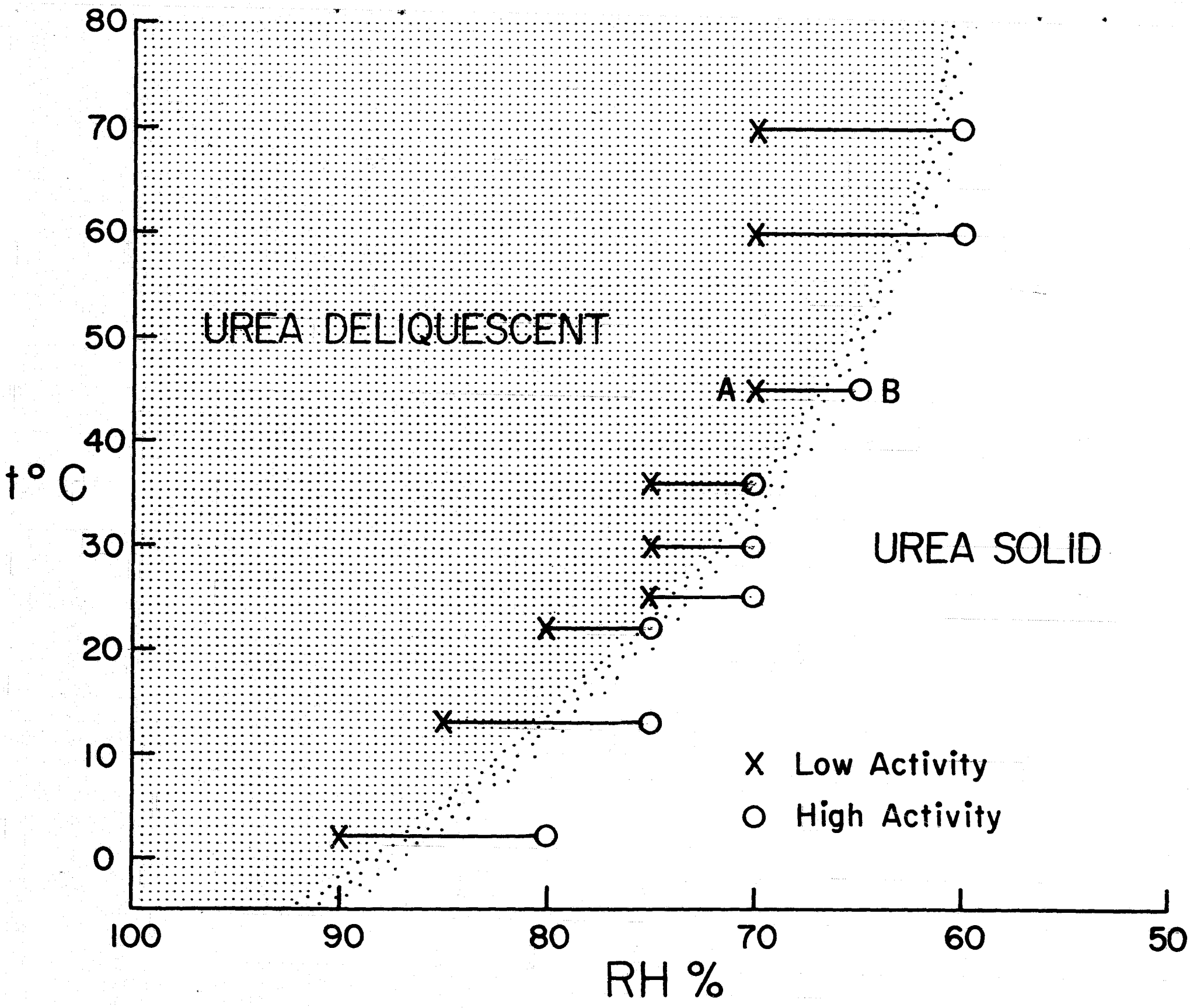

FIGURE 2. Urease activity in presence of deliquesced and solid urea.

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5. Skujins, J. J. and McLaren, A. D. Enzyme reaction rates at limited water activities. Science, 158, 1569-1570 (1967).
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D. ADSORPTION AND REACTIONS OF CHITINASE ON CHITIN AND CLAYS

The currently reported phase of the investigation of chitinase activity in adsorbed state on chitin and in clays is a continuation of the previously described project under the same title (5, 12, 17) on enzyme kinetics in structurally restricted systems.

Chitinase has a vital role in germination of Thielaviopsis basicola spores (1) and is probably involved in many other biological processes in soil (23). As an exoenzyme it has to remain active outside the organisms for some period of time. It is likely that similar to other enzymes in soil it is partly protected from immediate degradation by being adsorbed on soil constituents (2, 8, 10, 11, 18, 19). A considerable amount of data regarding the extent and nature of binding of enzyme proteins by soil minerals is already available (4, 6-9, 20, 22). The information presented here might be of value to facilitate successful extraction of enzyme protein from soil.

Some of the possible chitinase extraction procedures have been considered. Due to the little-known nature of chitinase as a protein, however, it seemed appropriate to carry out some adsorption and activity experiments with chitinase and soil minerals. Kaolinite was chosen as one of the most common clay minerals in soils.

Materials and methods

Most of the materials and methods have been described in previous reports (5, 12-17).

Kaolinite: Peerless No. 2, electrodialyzed, titrated to pH 8 with KOH Base exchange capacity: 2.28 milliequivalents per 100 grams of kaolinite (4).

Absorbancy measurements at 280 μu were corrected for light scattering material present when universal buffer (U.B.) and kaolinite were used (4).

Adsorption of chitinase on kaolinite: Each test tube contained 640 mg kaolinite and 600 μg chitinase in 5.0 ml of U.B., diluted 1:17. The pH values indicated are those measured on the supernatant after turning on the "Ferris wheel" for 2 hours at 25°.

Results and Discussion

The adsorption of chitinase on kaolinite at several ambient pH values is shown in Figure 3. The highest amount of chitinase adsorbed is in the range between pH 2.2 and pH 4.8. Adsorption falls sharply in the range from pH 5.0 to pH 5.5 and is practically nonexistent at pH 7.0. No adsorption peak is evident; at pH 5.5 only about 15% of the maximum amount is adsorbed although the isoelectric point of chitinase is at pH 6.8 (15). The amount of chitinase adsorbed per unit of kaolinite depends also on the chitinase kaolinite ratio (Figures 5, 6).

Once adsorbed on kaolinite chitinase does not desorb at the particular pH when the concentration of nonadsorbed chitinase in mixture is decreased and becomes zero. Chitinase adsorbed on kaolinite at pH 4.5, however, desorbs if the supernatants are replaced with UB buffer of

increasing pH's values; the desorption is complete at pH 7.0, although desorption does not reach the equivalent protein level of the initial adsorption at the particular pH.

Amounts of chitinase bound to kaolinite is increasing with increasing enzyme concentration. In 0.03 M Na acetate, pH 4.5, with 12.8 mg kaolinite per ml, the saturation is reached at chitinase concentration of about 600 µg per ml.

FIGURE 3

ADSORPTION of CHITINASE on KAOLINITE as a FUNCTION of pH

KAOLINITE : 10.7 mg per ml.
UNIVERSAL BUFFER / 17 , pH 2 to pH 10.5
CHITINASE : 100 µg per ml.

2 hours at 25°

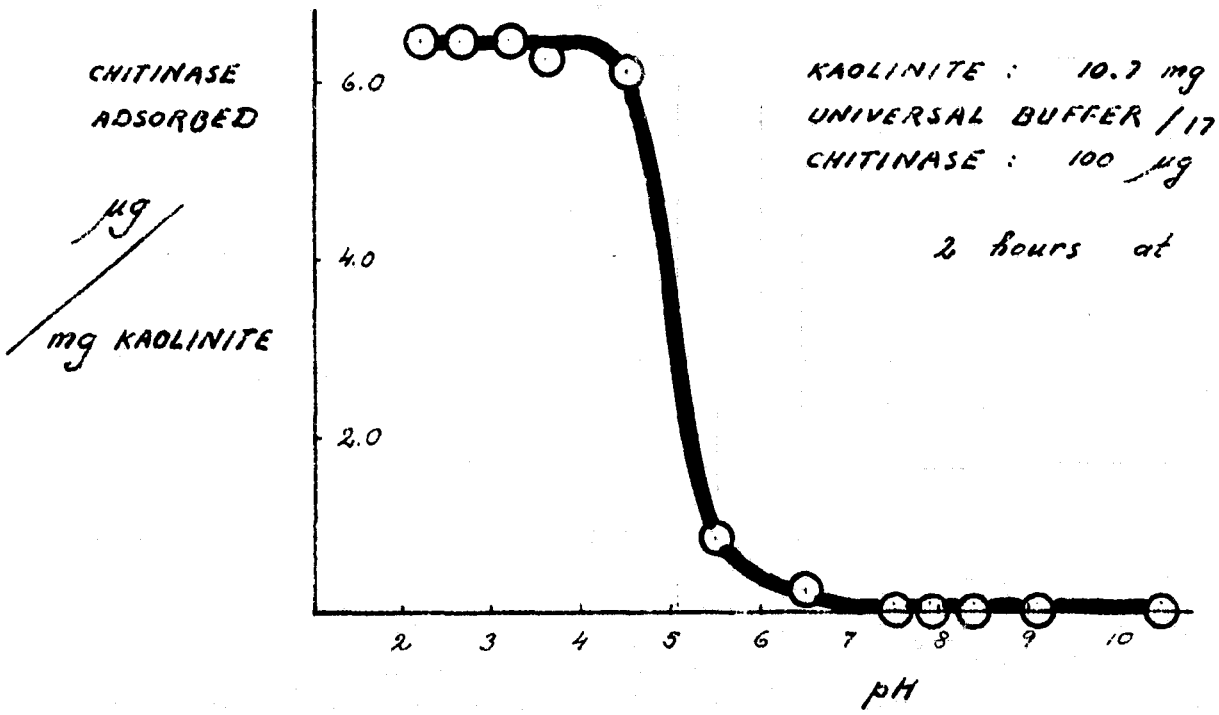


FIGURE 4

DESORPTION of CHITINASE from KAOLINITE — an EFFECT of HIGHER pH

CHITINASE ADSORBED on KAOLINITE at pH 4.5 and 25° AFTER CENTRIFUGATION SUPERNATANT REPLACED with the SAME BUFFER of VARIOUS pH

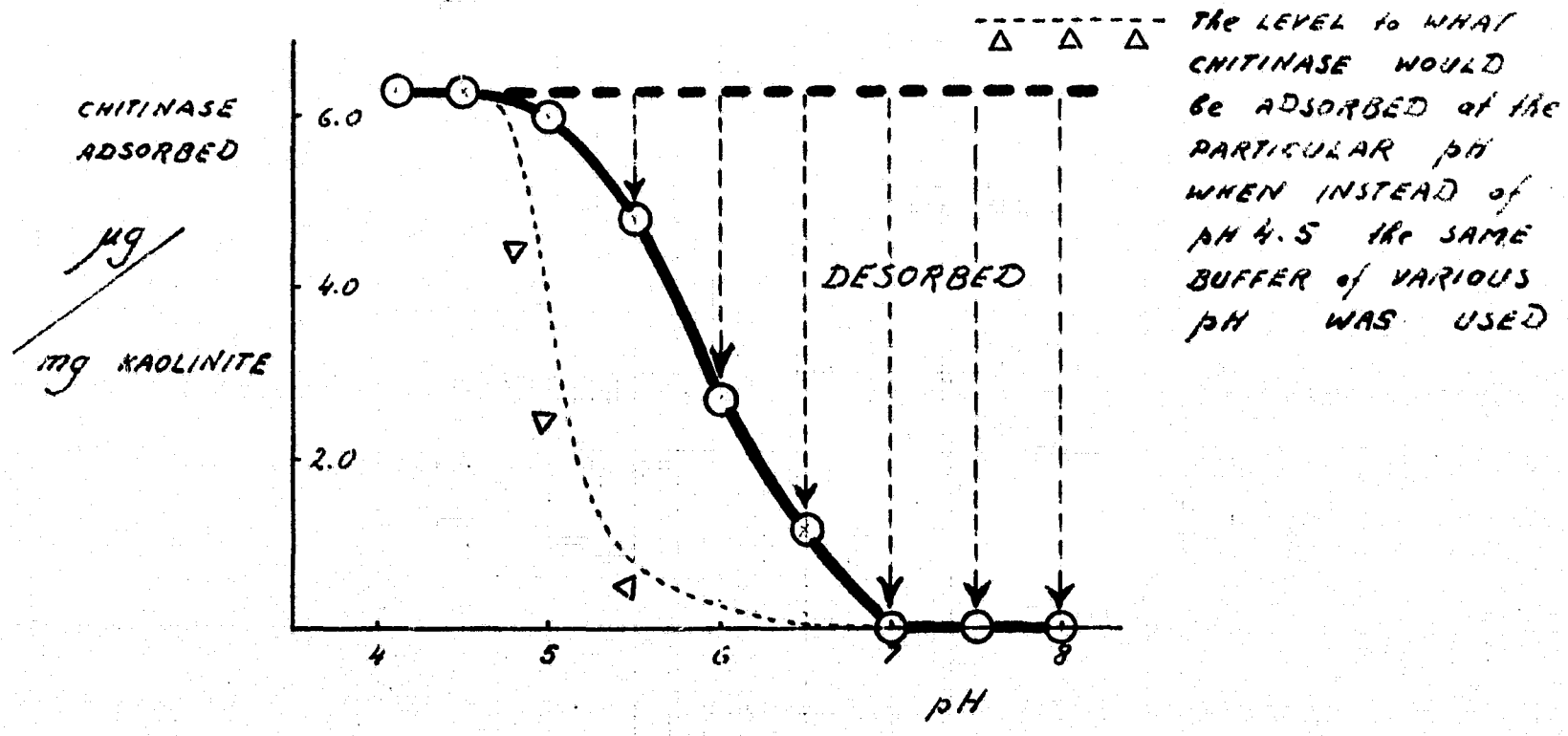


FIGURE 5

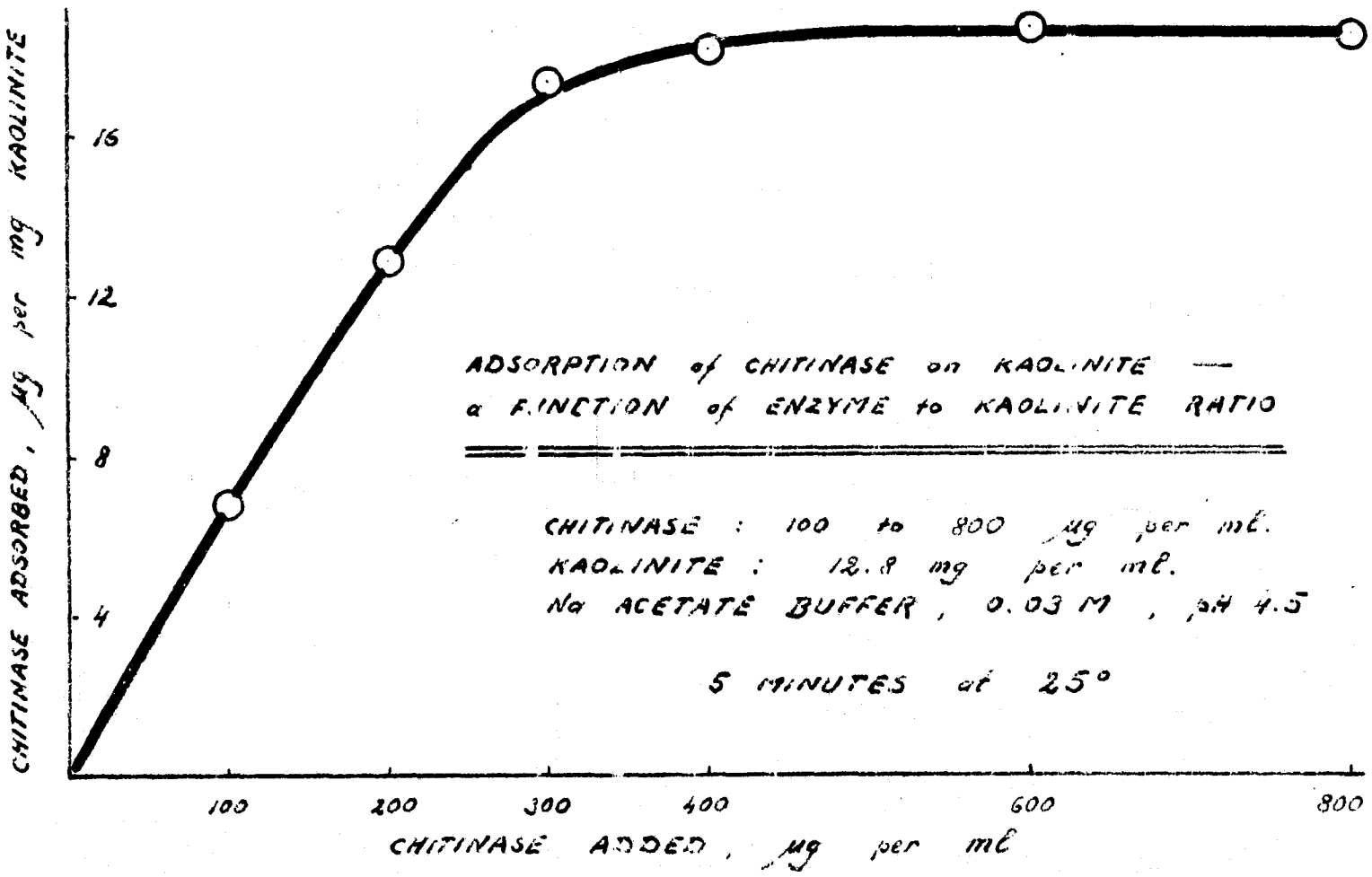
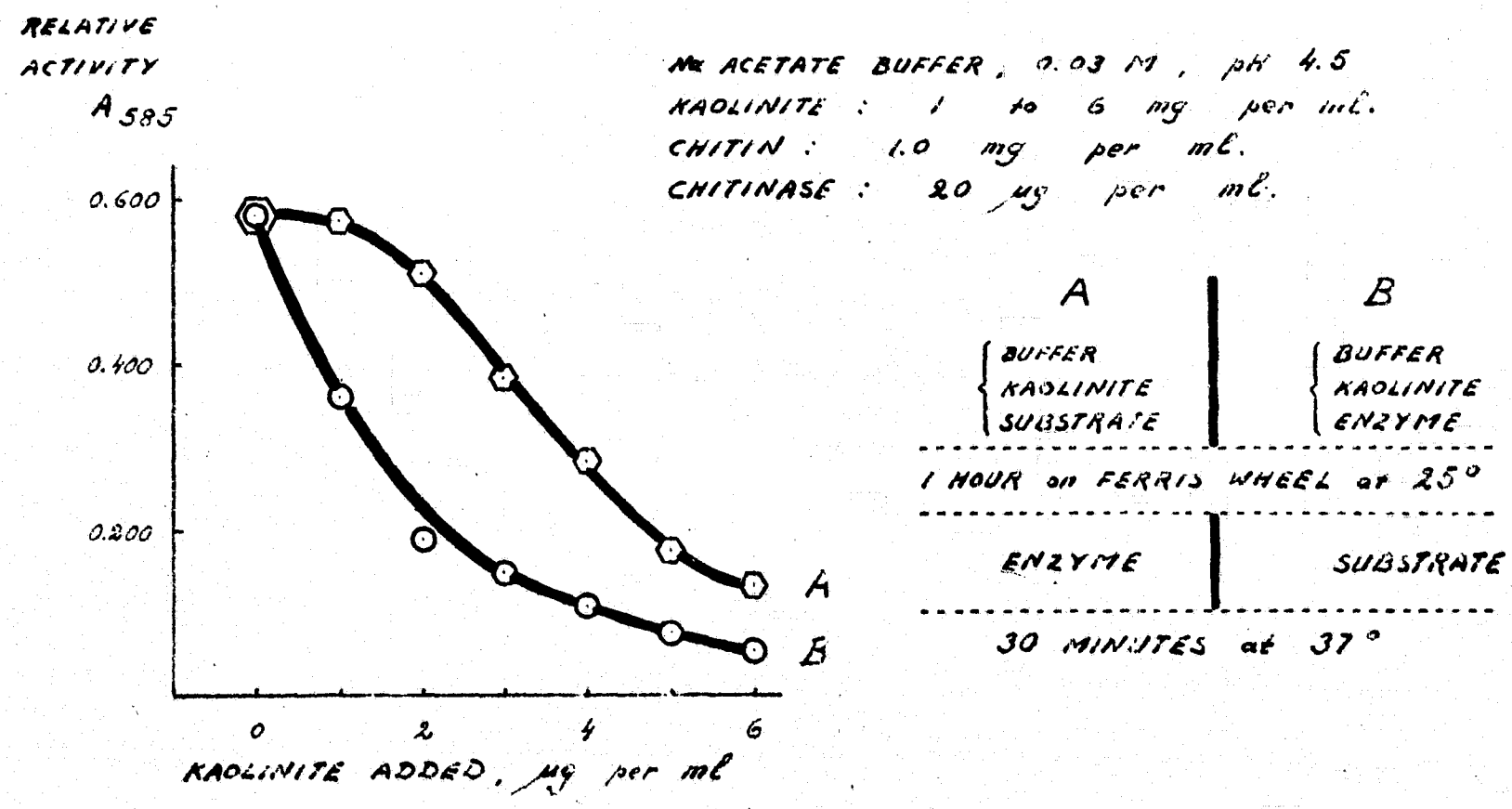


FIGURE 6

ACTIVITY of CHITINASE in the PRESENCE of KAOLINITE



The maximum adsorbed is 18.5 μg chitinase per mg of kaolinite (Figure 5). Rate of binding of chitinase by kaolinite at pH 4.5 is rapid and the adsorption is complete in about 5 minutes (Figure 5).

Activity of the nonadsorbed chitinase in supernatant is decreasing very rapidly at first: about 50% of the activity is lost during the first minute, about 75% are lost in 30 minutes, and only about 12% of activity remains after 4 hours of incubation with kaolinite at pH 4.5 and 25° (Figure 7).

In the presence of kaolinite chitinase activity depends on the enzyme to kaolinite ratio and upon the sequence in which the individual components of the reaction mixture are added (Figure 8).

Upon addition of chitin to kaolinite a chitin-kaolinite complex is formed which minimizes the inactivation of chitinase added later. When chitinase is added to kaolinite before the chitin, a far more severe loss of activity occurs (Figure 6).

As expected the activity reaches an intermediate value if chitinase is added to one half of kaolinite first, and to the other half of kaolinite chitin added first, and mixed (Figure 9).

Adsorbed on kaolinite at lower pH chitinase shows less activity than the one adsorbed at higher pH value (Figure 10). The highest pH at which chitinase may be adsorbed on kaolinite to maximum amount is at pH 4.5. When tested for activity at different pH values such adsorbed chitinase is showing an activity maximum at pH 5.7 instead of pH 4.7, as in the absence of kaolinite (16). The same pH 5.7 is also the maximal activity pH for the nonadsorbed fraction of chitinase (in supernatant). As a

consequence the optimal pH for chitinase activity is pH 5.7 when kaolinite is simply added to the reaction mixture, regardless of the amount of enzyme adsorbed or nonadsorbed (Figure 11). This phenomemon may be an artifact, caused by colloidal clay particles in the supernatant which have not been centrifuged out at the particular g values used.

FIGURE 7

ADSORPTION of CHITINASE on KAOLINITE and the ACTIVITY of NONADSORBED CHITINASE THEREAFTER as a FUNCTION of TIME

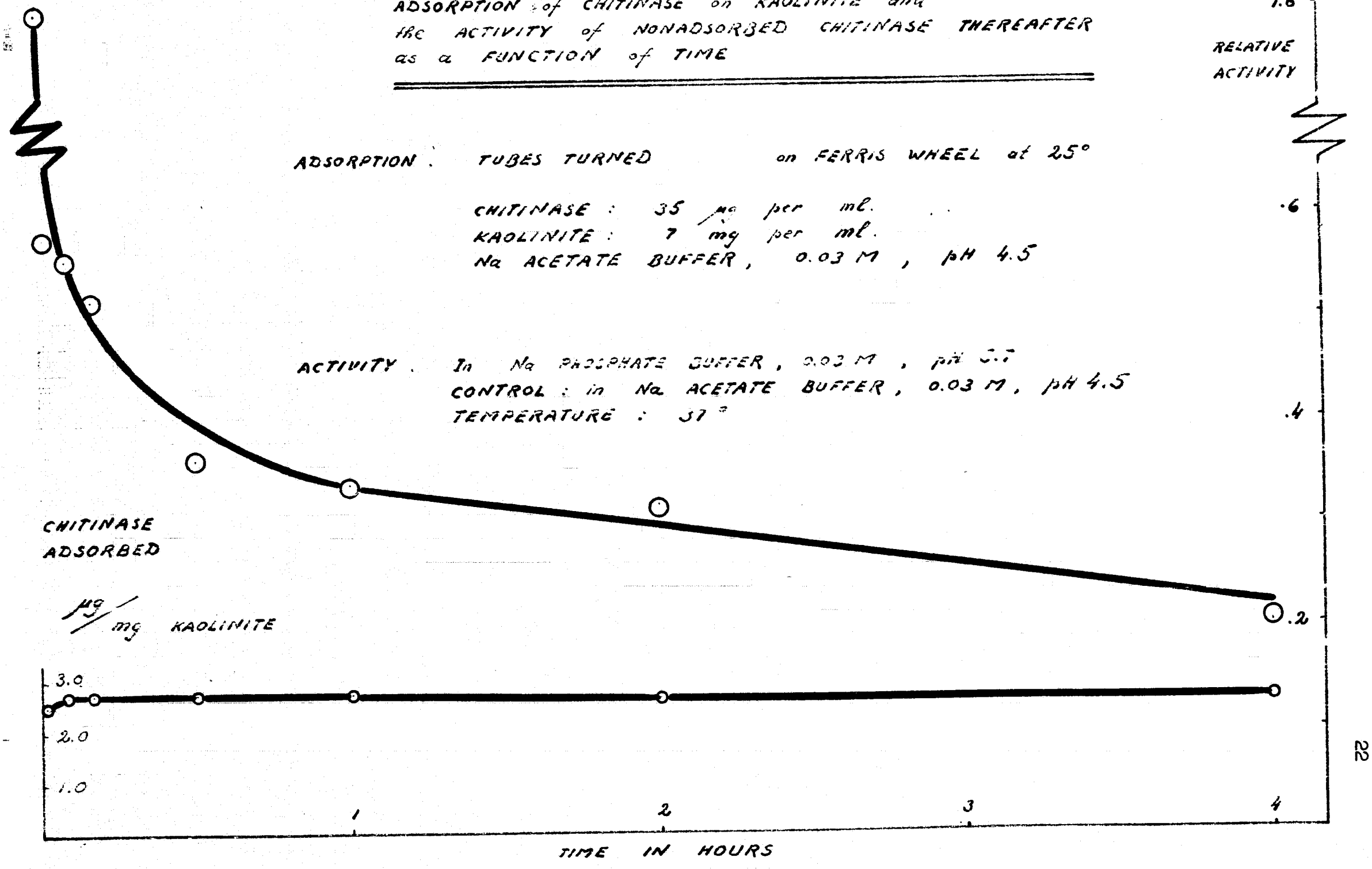


FIGURE 8

ACTIVITY of CHITINASE
in the PRESENCE of KAOLINITE

Na ACETATE BUFFER, 0.03 M, pH 4.5
KAOLINITE : 2.0 mg per mℓ ; 7.0 mg per mℓ.
CHITIN : 1.0 mg per mℓ.
CHITINASE : 20 µg per mℓ.

ADSORPTION — 2 HOURS on FERRIS WHEEL at 25°
HYDROLYSIS — at 37°, 5 to 60 MINUTES

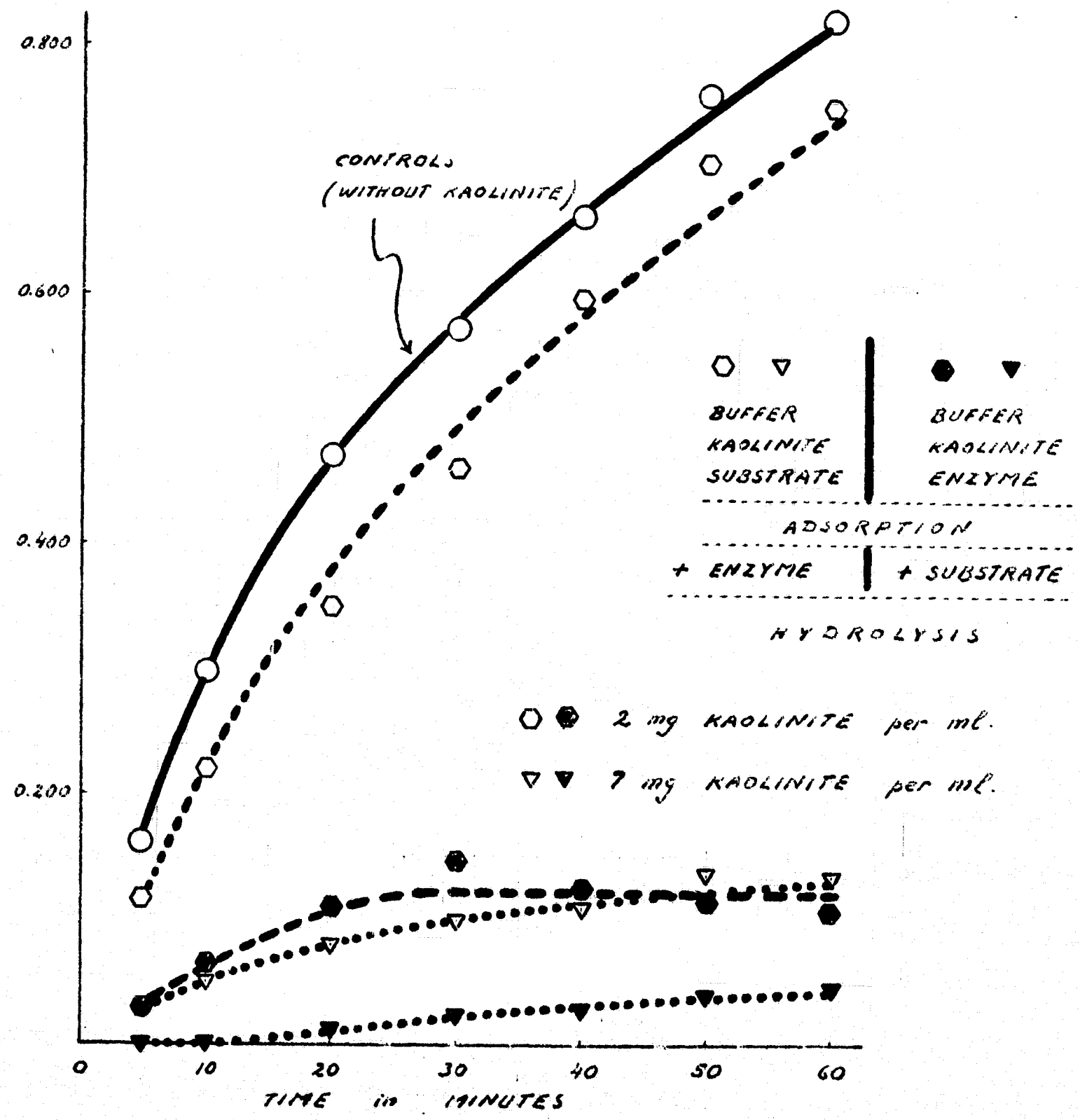
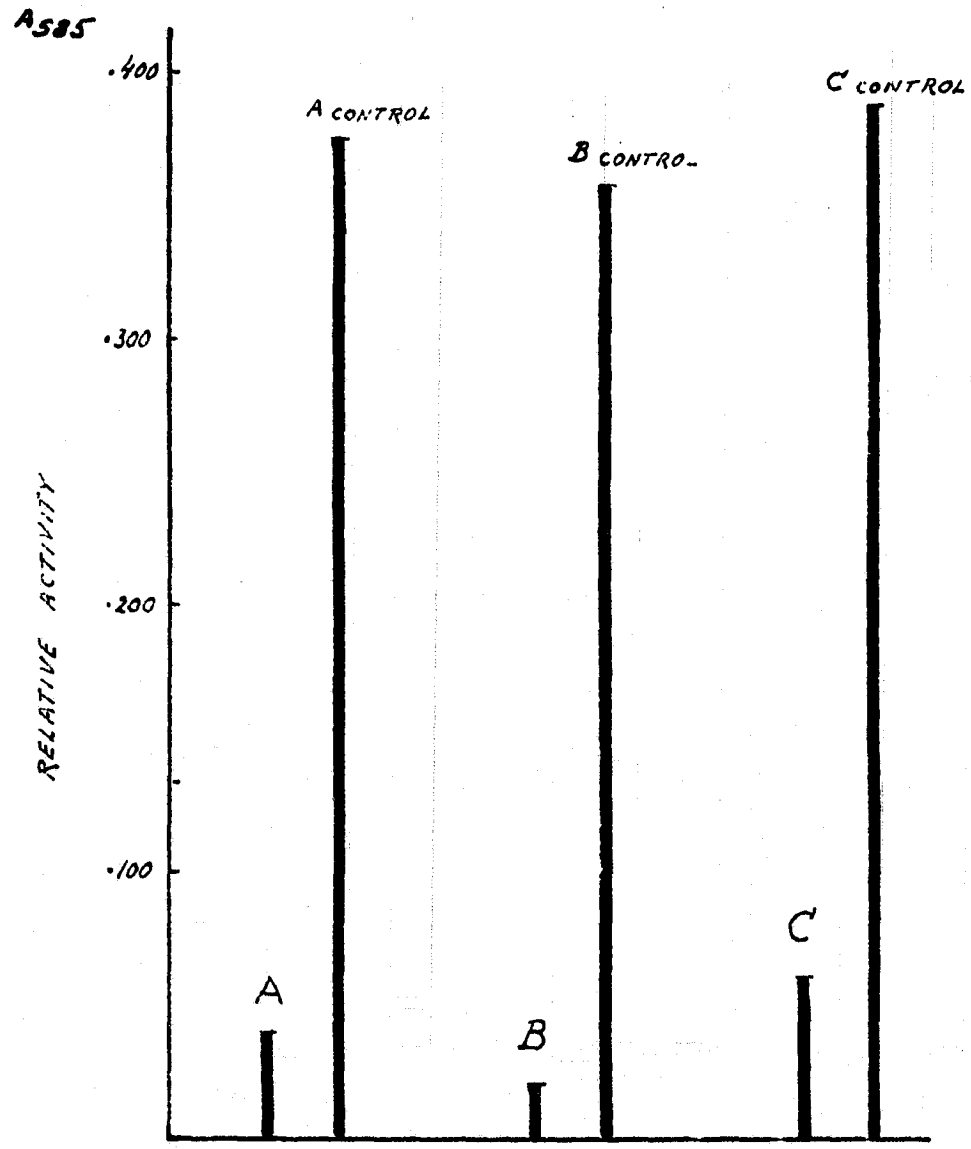


FIGURE 9

CHITINASE ACTIVITY IN THE PRESENCE OF KAOLINITE



TIME 30 min.
TEMPERATURE 37°

REACTION MIXTURE :
CHITINASE 14 µg per ml.
Na ACETATE BUFFER 0.03M, pH 4.5
KAOLINITE 7 mg. per ml. *)
CHITIN 1 mg. per ml.

*) NO KAOLINITE IN CONTROLS - KAOLINITE SUSPENSION REPLACED WITH WATER

A. (1)	(2)	B.	C.
CHITINASE	CHITIN	CHITINASE	CHITIN
KAOLINITE	KAOLINITE	KAOLINITE	KAOLINITE
BUFFER	BUFFER	BUFFER	BUFFER

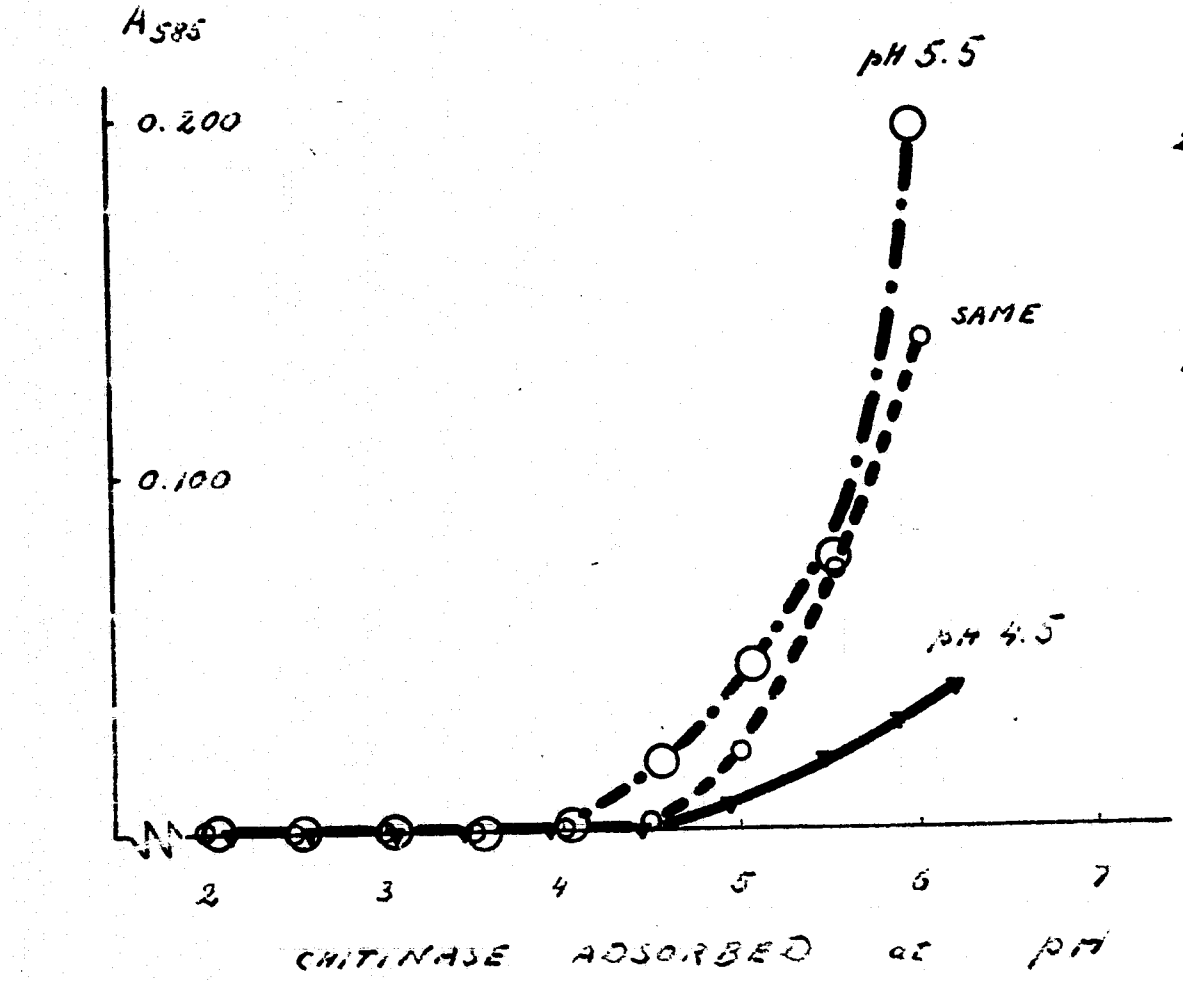
TURNED on FERRIS WHEEL at 25° for 10 min.

A: + CONTENT of tube (2) ADDED to CONTENT of tube (1)
B: + CHITIN
C: + CHITINASE

TURNED on FERRIS WHEEL at 37° for 30 min

FIGURE 10

ACTIVITY of CHITINASE BOUND to KAOLINITE at DIFFERENT pH



ADSORBED in 3 SIMILAR SETS of TUBES —
UNIVERSAL BUFFER $\frac{1}{11}$, pH 2 to 8
KAOLINITE : 12.8 mg per ml.
CHITINASE : 100 µg per ml.

2 HOURS on FERRIS WHEEL at 25°

ACTIVITY — counted for 20 µg of CHITINASE per MILLILITER

KAOLINITE PELLET with ALL the CHITINASE ADSORBED in ½ of the PARTICULAR pH
UNIVERSAL BUFFER : pH 4.5
pH 5.5
pH 2 - pH 6.5 (SAME)

30 MINUTES on FERRIS WHEEL at 37°

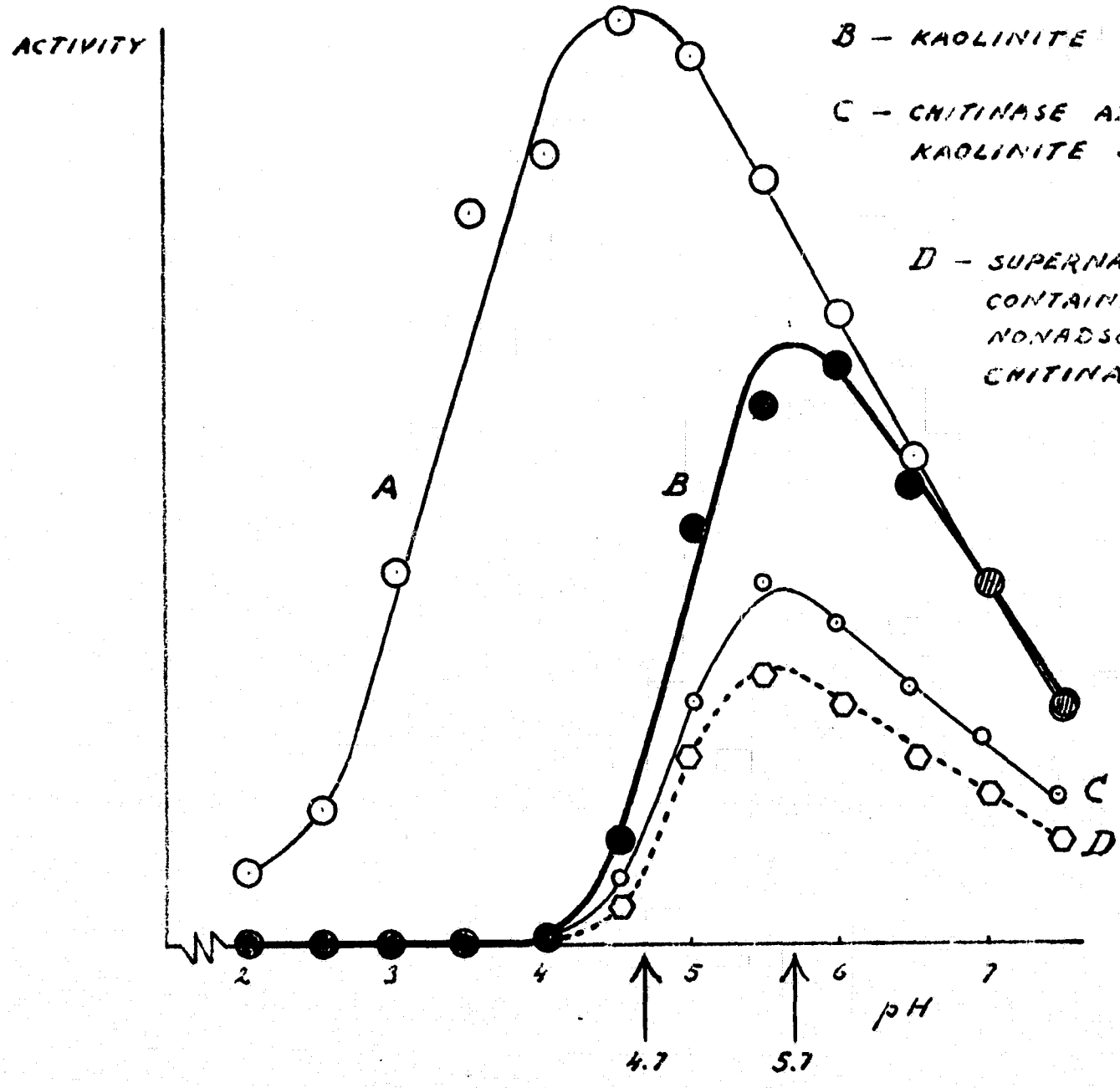
FIGURE 11

ACTIVITY of CHITINASE at VARIOUS pH
in the PRESENCE and ABSENCE of KAOLINITE

UNIVERSAL BUFFER/11
CHITIN : 1.0 mg per mℓ.
KAOLINITE : 5 mg per mℓ, if PRESENT
CHITINASE : 20 γ per mℓ.

ADSORPTION — 2 HOURS on FERRIS WHEEL at 25°, in pH 4.5 BUFFER
HYDROLYSIS — 30 MIN. " " " " 37°

ACTIVITY SCALE (on ORDINATE) is NOT the SAME for ALL CURVES



A — CONTROL (WITHOUT KAOLINITE)

B — KAOLINITE PRESENT

C — CHITINASE ADSORBED on KAOLINITE at pH 4.5

D — SUPERNATANT, CONTAINING the NONADSORBED CHITINASE

Several other explanations, however, for this behavior are possible. First, the enzyme most likely consists of two isoenzymes. In absence of kaolinite one isoenzyme may have the optimal activity below pH 4.7 and the other isoenzyme at pH 5.7, together showing the highest activity at pH 4.7 as an effect of addition. In the presence of kaolinite the first isoenzyme is adsorbed and because of this the pH optimum is shifted to pH 5.7 - to the same value which is the optimum for activity of the other nonadsorbed isoenzyme. (This may be verified by isolating the respective adsorbed and non-adsorbed isoenzyme moieties).

Another possibility is that calcium atoms in the chitinase molecule are strongly attracted to kaolinite and that some of them are dissociated from chitinase, thus causing reduction of activity and also causing a change in pH for the optimal activity. Such phenomenon had been observed with riboflavin containing coenzyme of the old yellow enzyme when it was adsorbed on kaolin "Frankonite" (21).

The role of calcium in the activity of chitinase (15, 16, 17) is supported also by an unsuccessful attempt to obtain calcium free enzyme protein by prolonged dialysis against 0.01 M Na_4EDTA. After 8 changes in 96 hours of dialysis in cold, and subsequent two changes of glass distilled water in the next 24 hours, the chitinase protein still contained 0.3% Ca, which is about 2 Ca atoms per molecule of chitinase, M.W. 29,000.

Tested for activity with similarly dialysed chitin suspension as a substrate, with 0.05 ml of 0.04 Na acetate solution added and containing 16 µg of Ca per tube, the activity was 50% higher than that in

control tests without such an addition. It should be noted that for these experiments all glassware was washed for 24 hrs in 0.01 M Na_4 EDTA solution, with a subsequent 4 changes of glass distilled water in 2 days.

All adsorption experiments require a large amount of enzyme. An average yield of a batch of culture gives 20 mg chitinase. Because of this reason only a limited number of experiments were performed and some were not repeated a sufficient number of times for strict quantitative evaluation. Experiments to determine the effects of concentration and chemical composition of buffers were omitted. Additional work on adsorption of various temperatures, pH, and rates appears desirable.

Elution with ethylamine HCl was not tested. The elution experiment by changing pH (Figure 4) should be repeated in a modified manner: supernatants should not be discarded and replaced, but their pH values should be changed by titration to retain the enzyme protein in the supernatant.

The properties of desorbed chitinase should be investigated. It is not known if chitinase exposed to kaolinite in a buffer solution above pH 7 remains unaffected. It seems that altering of chitinase protein by kaolinite could be prevented if the adsorbing surface of kaolinite would be saturated with inorganic or organic cations. May be that the positive results of enzyme extraction from soil with Na pyrophosphate (17) are associated with the order of replacing capacity of cations: H^+, Ca^{++}, Mg^{++}, K^+, NH_4^+, and Na^+ in the extraction procedure. Regarding the extraction of chitinase from soil it should be noted that the most likely damage to chitinase protein during the extraction may be the loss of its Ca^{++} by adsorption on clay minerals.

The following general outline may be presented regarding the required conditions for the extraction of chitinase protein from soil:

(1) The need for a cold room is an option, not a necessity. The work with cooled solutions at room temperature should give the same results qualitatively.

(2) Chitinase tolerates a wide range of pH values in its environment (pH 2 to 9), but wherever possible pH 7 should be maintained to avoid possible denaturation of enzyme protein.

(3) Adsorbing surface of clay minerals in soil should be saturated with cations which would displace Ca^{++}. Such conditions should be maintained where any of the soil minerals are present.

(4) $(NH_4)_2SO_4$ and Ca-acetate precipitations may be used routinely. Alcohol should be used only at $-18^{\circ}C$, and all work must be done in the cold room (3, 15).

(5) For the separation of precipitates, centrifuges should be used instead of filter paper and other filter aids. The loss of enzyme protein adsorbed on these surfaces are often of significant magnitude (15).

(6) The separation of chitinase enzyme from other soil proteins are not expected to create difficulties. The usual method worked well in previous experiments (16).

(7) There are only small quantities of adsorbed proteins in the soil. Initially kilogram quantities of soil should be used.

Summary

The amount of chitinase adsorbed to kaolinite depends on the concentration and on the ratio of enzyme to kaolinite. Chitinase is adsorbed only below its isoelectric point, pH 6.8. Maximum adsorption is reached below pH 4.6.

Adsorption results in reduction of chitinase activity, the severity of it depending on the amount of kaolinite present, on pH, and on the length of exposure time. Once the chitinase has been in contact with kaolinite in a medium below pH 6.8, regardless of the conditions of sorption, the activity is significantly weaker and the optimal pH for its activity has shifted from pH 4.7 to pH 5.7.

The presence of chitinase isoenzymes and the possible role of Ca^{++} in chitinase molecule are discussed. The possibility of chitinase extraction from the soil is discussed and a short outline of suggested working conditions is given.

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D. THE SURFACE MORPHOLOGY OF SPORES OF STREPTOMYCES STRAINS 2B AND 3C

In conjunction with the studies on toxonomy of Streptomyces strains 2B and 3C (7) used in our chitinase work we have examined the surface morphology of their hyphae and spores by transmitting electron micrography and by scanning surface electron micrography.

Materials and Methods

Refracting (transmitting) electron micrography. Preparation of samples: Formvar-covered copper grids were gently pressed to the growth of sporulating aerial mycelium. The samples were taken from old growth on agar-containing medium plates. No coatings were used for these observations.

The samples were observed with an RCA model EM U3 electron microscope, located in Hilgard Hall, University of California, Berkeley and photographed at the following magnifications: strain 2B - 17,120x and 33,680x; strain 3C - 11,820x, 22,940x, and 33,680x.

Stereoscan scanning electron micrography. Preparation of samples: Spores (mycelium) was taken from 2 weeks and from few days old cultures, where sporulation was just appearing. Spores and mycelium were attached by pressing cultures to metal stubs coated with a very thin layer of silver paint. The samples were coated with a gold-palladium allay to a 200-600 Å thickness.

Samples were observed and photographed with a Stereoscan scanning electron microscope operated by the Electronics Research Laboratory, Cory Hall, University of California, Berkeley. The apparatus was

operated at 20 Kv and 20° slant. Photographs were made at 5,400x to 56,000x magnifications as shown in Figures where applicable.

Results and Discussion

Surface morphology of streptomycete spores is currently regarded as a useful characteristic for the identification of streptomycete species. Usually four basic types of spore surfaces are distinguished: smooth, warty, spiny, and hairy (1).

The transmitting electron micrographs of Streptomyces strain 2B are showing somewhat apart standing spores with a warty surface, and it appears there is a break in spore wall across the middle (Figures 12, 13).

Spores of strain 3C are clearly smooth-walled as shown in Figure 14 and Figure 15. The less dense areas are caused by the depressions made by partial collapse of spore walls under vacuum (Figure 15).

Under the scanning electron microscope viewing the differences among the strains, i.e., between the "smooth" and "warty" spores somewhat disappeared, apparently caused by the necessary and rather thick metal coating of the specimens. The typical collapsed appearance of the Strain 2B spores, however, was evident (Figures 16 and 17). The overall view of both mycelia was rather similar (Figures 18 and 19).

On the whole spores of strain 2B are more closely together as they appeared at first under transmitting electron microscopy. The narrow connection between two spores were seen only in few places. The spores of Strain 3C for the most part have no spaces between them, but occasional

connecting spacing is evident (Figure 20). The spore surface is warty and the depressions in spore walls are giving a chain-like appearance to the strings of spores.

Spores at the end of sporophores are often elongated, more rounded and with a less warty surface. The elongating mycelial tips show practically no collapse (Figure 21). There is a possibility that in some cases spore surface could change while the spores are growing and maturing. Such cases have been reported (2, 3, 5). Rancourt and Lechevalier (4) showed that in the course of spore development the spines were on a superficial layer which did not adhere strongly to the surface of mature spores. Very likely this is also the case here where spores from cultures of differentages were examined. However, without more detailed study it is not possible to assert whether the shedding of a somewhat superficially warty spore layer is taking place here.

Tresner et al. (6) investigated the spore surfaces of about 600 Streptomyces strains. They found that Streptomyces having spore masses in white, or yellow, to cream or buff shades had smooth-walled spores. With two exceptions smooth spore walls were also in the pinkish-cinnamon to pinkish-tan-spored streptomycete group.

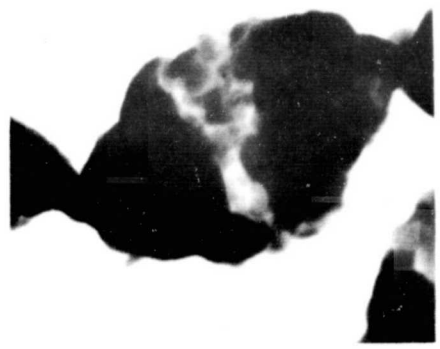
Streptomyces strain 3C has distinctly white colored spores. Its smooth spore walls are in agreement with the observations of Tresner et al. Strain 2B on the other hand show buff colored spores in older cultures and on glycerol-starch-glutamate agar the spores are of lavender color. Having a warty spore surface the 2B strain is another exception in a usually smooth-walled spore group of Streptomyces, as found by Tresner et al.

Figure 12

Electronmicrograph of Streptomyces Strain 2B.

Magnification 17,120x

Figure 13



Electronmicrograph of Streptomyces Strain 2B.

Magnification 33,680x

Figure 14

Electronmicrograph of Streptomyces Strain 3C.

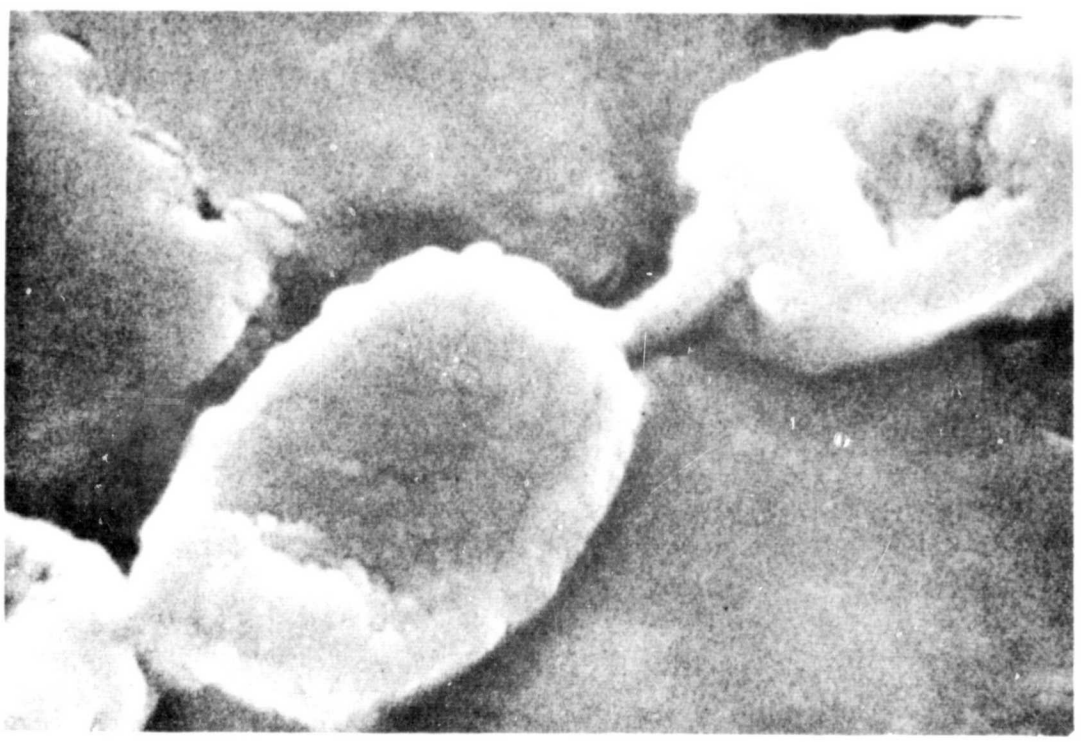
Magnification 11,820x

Figure 15

Electronmicrograph of Streptomyces Strain 3C.

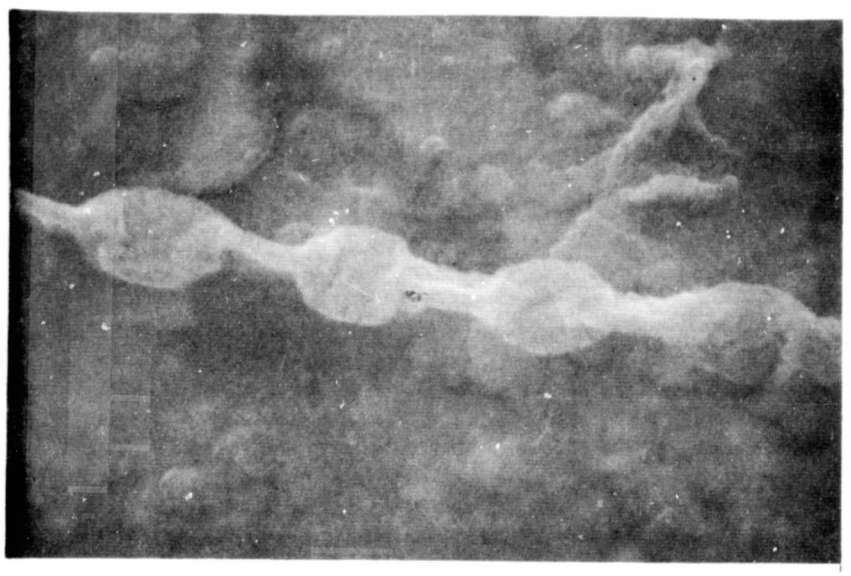
Magnification 22,940x

Figure 16



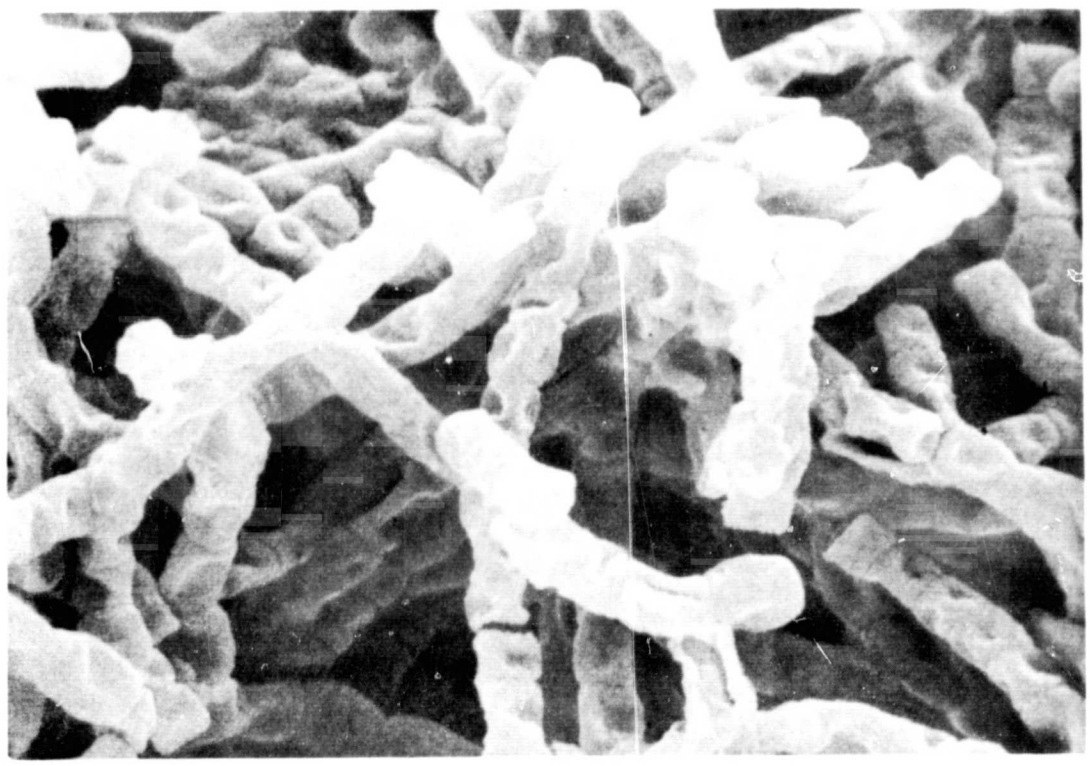
Magnification 22,000x; print enlargement 1.6x

Figure 17



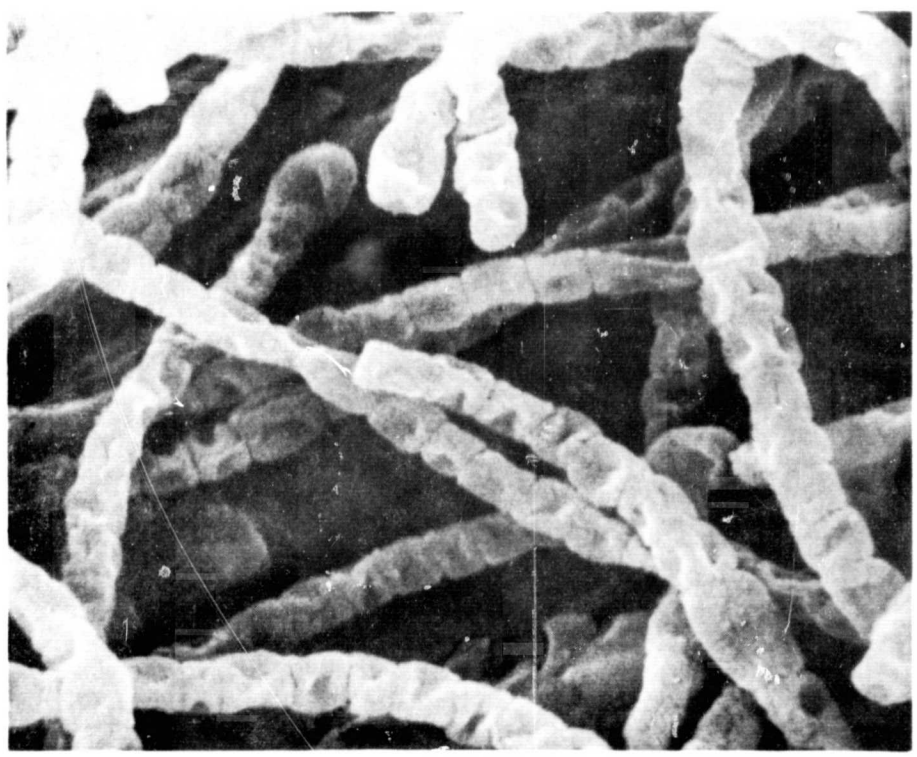
Magnification 11,200x

Figure 18



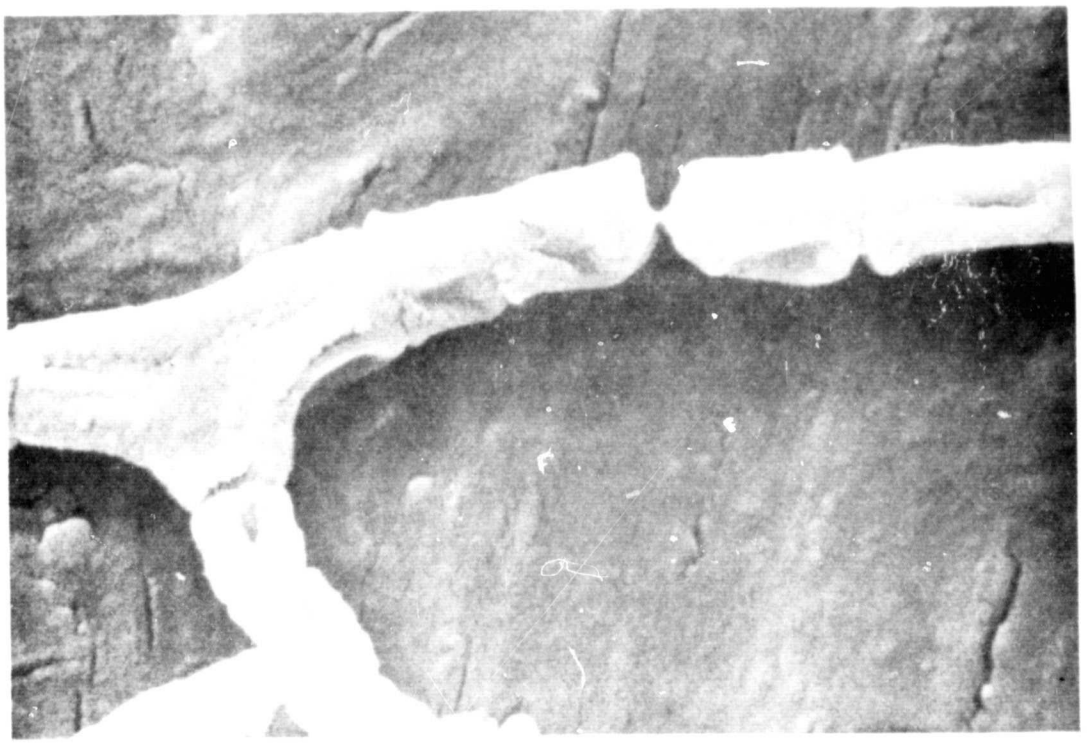
Stereoscan micrograph of Strain 2B. Magnification 5,400x

Figure 19



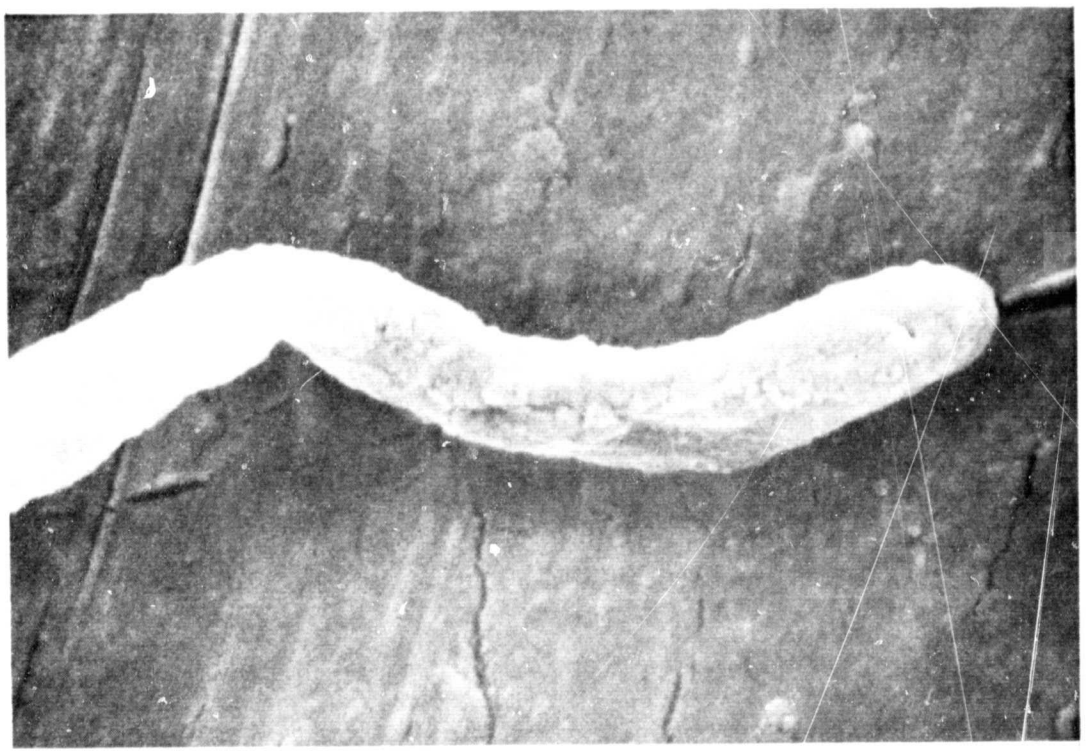
Stereoscan micrograph of Strain 3C. Magnification 5,500x

Figure 20



Stereoscan electronmicrograph of Strain 3C.
Magnification 11,000x; photo enlargement 1.6x

Figure 21



Stereoscan electronmicrograph of Strain 3C.
Magnification 11,000x; photo enlargement 1.6x

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