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The Seventh Semiannual Progress Report on

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

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I. PREFACE

Our objective is twofold. We wish to work out procedures for detection and assay of enzymes in soil suitable for presumptive tests of life in planetary soils. Incidentally, this calls for use of enzyme-substrates that are stable to heat, moisture and storage. Second, we are exploring the behavior of enzymes in non-classical (soluble enzyme & insoluble substrate) systems. These include enzyme action at surfaces in gels and in other heterogeneous, structurally restricted systems analogous to those found in cells and in soils.

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 in order to investigate some of the factors influencing reactions at interfaces. 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.

The hydrolysis of urea by urease in media of low water availability is detectable at 60% relative humidity and measurable hydrolysis of urea occurs in soils at 80% relative humidity and above. Methods are being developed further for their possible use for the detection of such a catalyst in the Martian environment.

Theoretical aspects of the kinetics of enzyme reactions in heterogenous systems and of microorganisms in flow columns are reviewed and discussed.
PERSONNEL

The participants in the currently reported phase of this project included Professor A.D. McLaren, Dr. J.J. Skujins, Mr. A.H. Pukite, Miss C. Wolf, Mr. W.H. Brams, and Miss L. Kersten.
SURVEY OF ENZYME ACTION IN HETEROGENEOUS SYSTEMS

Part I, in this report, includes sections I, II, and III in the following outline.

Part II, to appear in our next progress report, will include a bibliography.
OUTLINE

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I. INTRODUCTION

Some enzyme reactions in nature, both within cells and tissues and without, take place in homogeneous aqueous solutions. In most instances, however, enzyme reactions take place at interfaces within gels and coacervates or in some other heterogeneous milieu (Holter); it is surprising to note how rarely this subject has been treated in textbooks or reviews. Digestion in the gut of animals or at the surfaces of plant roots are natural situations with heterogeneous catalysis. Here a number of such situations will be discussed and some biological examples for which such considerations need to be extended in depth will be described.

Recently interest in the action of enzymes on solid supports and in insoluble gels has emerged, and the results have relevance to soil and food science and preparative and analytical chemistry as well as to cell biology. Others have reviewed various phases of this very broad subject (Augenstein, Alexander, Fraser, Katchalski, Robert, Zittle, McLaren, McLaren, McLaren and Peterson), and in this review we have tried to present a coherent picture of some of the kinetic aspects of the subject together with contrasts with classical solution studies. The scope of the field can be seen in Figure 1 as a vast area for research. Homogeneous aqueous systems take their place as a special case in this scheme. Among all these possibilities water plays a role, and we may ask such questions as at how low a relative humidity can reactions take place; can reactions take place in frozen systems, etc.?
Figure 1. Some combinations of enzyme and substrate giving reactions in heterogeneous systems. Examples are indicated on the tie lines. The classical, homogeneous solution system marked by *, is included for perspective.
Further, we must consider in the case of insoluble enzymes what is the local, surface pH or redox potential at the solid-liquid interface and how do such micro-environmental conditions modify the kinetics deduced for homogeneous systems. Contrariwise, how can one describe the action of an enzyme on an insoluble substrate having a solubility so low that in effect no solution reactions are observed?

Certain topics have recently been reviewed adequately and will only be alluded to in the following. These include enzyme reactions at oil-water and air-water interfaces (James and Augenstein) and the kinetics of translocation through natural membranes (Mitchell, 1967).

II. ELEMENTARY KINETIC AND THERMODYNAMIC FEATURES

A. Some rate equations

Let us consider the usual reaction scheme for the action of an enzyme $E$ on a substrate $S$. $E\cdot S$ is the intermediate, an enzyme-substrate complex,

$$S + E \xrightleftharpoons[k_2]{k_1} (E\cdot S) \xrightarrow[k_3]{\quad} P + E$$

and $C$ is a product, $k_1$, $k_2$, and $k_3$ are appropriate rate constants. Let the total enzyme present, $E_0$, equal the free, $E$, plus the combined enzyme, $E\cdot S$ complex. These symbols will be used both for identification and for molar concentrations. Similarly, $S_0$, the total substrate
concentration is given by \( S_0 = S + E \cdot S \).

Then, in the steady state,

\[
\frac{d \; E \cdot S}{dt} = k_1 E \cdot S - k_2 E \cdot S - k_3 E \cdot S = 0
\]  \( (2) \)

and

\[
k_1 (E_0 - E \cdot S)(S_0 - E \cdot S) = k_2 E \cdot S + k_3 E \cdot S
\]  \( (3) \)

There are two extreme cases for which equation (3) can be simplified for solutions, namely one in which the substrate is in great excess, and the converse. The first case leads to the classical expression of Henri, as elaborated by Michaelis and Menten and the second becomes of importance if the substrate consists of colloidal particles in suspension.

**Case I. Soluble substrate in excess**

\[
k_1 (E_0 - E \cdot S) \frac{S_0}{S_0 + K_m} = k_2 E \cdot S + k_3 E \cdot S
\]  \( (3a) \)

and

\[
E \cdot S = \frac{E_0 S_0}{S_0 + K_m}
\]  \( (4) \)

**Case II. Soluble enzyme in excess**

\[
k_1 E_0 (S_0 - E \cdot S) = k_2 E \cdot S + k_3 E \cdot S
\]  \( (3b) \)

and

\[
E \cdot S = \frac{E_0 S_0}{E_0 + K_m}
\]  \( (5) \)
In both cases \( K_m = \frac{k_2 + k_3}{k_1} \), but the initial reaction velocities, \( v_1 \), are quite different. For case I we have

\[
v_i = k_3 \frac{E \cdot S}{K_m + S_0}
\]

and for case II

\[
v_i = k_3 \frac{E_0 S_0}{K_m + E_0}
\]

Equation (1) is an over-simplification and several important quantities, namely water, hydrogen ion concentration (or activity), ionic strength, cofactors including heavy metals, and redox potential, have been omitted. If these quantities are held constant they enter implicitly into the rate constants. If they vary or appear as reactants, explicit description is often possible. For example, in a study of the action of pepsin (E) on egg albumin (S) Bull and Currie write

\[
E + S \rightleftharpoons E \cdot S \\
E \cdot S + H^+ \rightleftharpoons E \cdot S \cdot H^+ \\
E \cdot S \cdot H^+ \rightarrow \text{peptides} + E
\]

Assuming, in the usual way, that the velocity of proteolysis in proportion to \( E \cdot S \cdot H^+ \), \( v = k_3 \cdot E \cdot S \cdot H^+ \), and being mindful of the conservation equation for total enzyme \( E_0 = E + E \cdot S + E \cdot S \cdot H^+ \), we have for the velocity of pepsin action
If the role of water, \( W \), is to be examined, one may use a set of formulas suggested by Haldane. 

\[
\begin{align*}
E + S & \longrightarrow E \cdot S \\
E \cdot S + W & \longrightarrow E \cdot S \cdot W \\
E \cdot S \cdot W & \longrightarrow E + \text{products}
\end{align*}
\]

Many enzyme reactions in nature take place on surfaces and in gels where both hydrogen ion concentration and water must be taken into account. Enzyme reactions on the surfaces of clays or in foods are examples. For such systems concentration with volume units are not applicable; the use of mole fractions allows comparisons to be made with corresponding enzyme-substrate reactions in solution.

For a generalized chemical reaction \( \Sigma y_s S \longrightarrow \Sigma y_p P \), equilibrium constants can be written as

\[
\frac{\pi(m_s)^{\gamma_s}}{\pi(m_p)^{\gamma_p}} = K
\]

\[
\frac{\pi(x_s)^{\gamma_s}}{\pi(x_p)^{\gamma_p}} = K_x = \frac{\pi \left( \frac{x_s}{55.5 + \sum m_i} \right)^{\gamma_s}}{\pi \left( \frac{x_p}{55.5 + \sum m_i} \right)^{\gamma_p}}
\]
X being mole fractions and m being molal concentrations. These constants are related by

\[ \left( \sum \frac{2}{3} \frac{E}{5} - \sum \frac{5}{7} S \right) \times \frac{K}{\gamma} = \frac{K}{\gamma} \]

and are obviously not generally proportional. X is a more desirable composition variable because it involves the mole number of every component in the system while m involves a given component and water only. Using mole fraction, \( v = k'X \frac{E}{S} \cdot H^+ \), and substituting the corresponding conservation equation, \( X = X_E + X_E \cdot S + X_E \cdot S \cdot H^+ \), we obtain

\[ v = \frac{k'X}{X + X + X +} \]

Multiplying through equation (8) and (9) by the volume, and substituting \( k'' = k' \left[ 55.5 + \sum m \right] \) we obtain the following pair of equations,

\[ v' = k'E_0 \left( \frac{K_1 K_2}{S \cdot H^+} + \frac{K_2}{H^+} + 1 \right) \quad (8a) \]

and

\[ v' = k'E_0 \left( \frac{K_1 K_2}{X \cdot X} + \frac{K_2}{X \cdot H^+} + 1 \right) \quad (9a) \]

where \( v' \) is the velocity in moles per second and \( E_0 \) is simply the total amount of enzyme. From (9a), as \( X \rightarrow 1 \), \( X_{H_2O} \) and \( X_{H^+} \) must decrease and \( v' \rightarrow 0 \), a result to be expected for gels and films of substrate which are undergoing dessication (McLaren).
B. Locus effects

In the above discussion the constants $k_1$, $k_2$, $k_3$, and $K_m$ are characteristic of the enzyme and substrate interaction, the strength of which may be strongly influenced by the local structure, be it substrate or carrier (Schurr and McLaren 1965). The variation of kinetic constants with different local environments will be referred to as the locus effect. The shift in the pH optima of some enzymes, which act in regions of electrostatic potential different from that of the bulk solution, is an example.

In 1937 Danielli wrote that "the bulk of the fluid contents of the cell are presumably of fairly uniform reaction, but the surfaces of granules, oil globules, mitochondria, and gel particles may well differ from the bulk reaction by up to 2 pH units, according to the constitution of the surfaces concerned. The cell may thus offer a much more diverse environment for enzyme reactions than has hitherto been supposed." For example, he showed that the pH at the interface of water and non-aqueous solutions of long-chain fatty acids is less than that of the bulk aqueous phase because of the concentration of negative charge at the interphase and a consequent concentration of $H^+$ ions at the interface to form an ionic double layer and he accounted quantitatively for this difference in pH by means of Donnan's theory ( ). Later Hartley and Roe ( ) derived an equation for the difference between the pH at a charged interface, $pH_s$, and that of an adjacent phase, $pH_b$, by an application of the theory of Debye and Hückel. They
pointed out that the electrokinetic potential (ξ) of the colloidal chemist can be identified with the potential ψ in the neighborhood of a simple ion at the distance of closest approach of another ion, as considered by Debye and Hückel. In this sense the ξ-potential determines the local concentration of ions near the surface of a particle, and the hydrogen ion concentration near the surface will be \( e^{-\xi/kT} \) times the \( H^+ \) concentration in bulk. The effective dissociation constant becomes

\[
K_s = K_{be} e^{-\xi/kT} = K_{be} F \xi / RT
\]

where \( K_s \) is the thermodynamic dissociation constant in bulk, \( e \) is the electronic charge, \( F \) is the faraday, \( T \) the absolute temperature, and \( k \) the Boltzmann constant.

At 25°C equation 10 may be rewritten as

\[
pK_s = pK_b - \xi/60
\]

or

\[
pH_s = pH_b + \xi/60
\]

where pH is in terms of \( H^+ \) concentration, not activities. Incidentally, no distinction between concentration and activity of \( H^+ \) is needed in bulk under physiological conditions, near neutrality. That an enzyme acting at a charged surface responds to hydrogen ion concentration rather than activity has been shown elsewhere ( ).
Equation 10b can be evaluated via electrophoretic measurements by substituting, for large particles, the Smoluchowski relationship

$$\mu = \frac{eD}{4\pi \eta}$$

We thereby arrive at the experimentally useful equation

$$\phi_H = \phi_{Hb} + 0.217 \mu$$

(12a)

For small particles, Hartley and Roe give at 25°C,

$$\phi_H = \phi_{Hb} + 0.325 \mu$$

(12b)

The mobility of a particle is reckoned negative for motion toward an anode, in microns/sec/volt/cm; D is the dielectric constant and η is the viscosity of the disperse medium at the same temperature.

In Table I are some values for $\Delta \phi = \phi_{Hb} - \phi_H$ evaluated by means of the equation of Hartley and Roe.

The attack of a protein substrate molecule by an enzyme most certainly involves an environment characterized more by $\phi_H$ than by $\phi_{Hb}$ (except if both proteins are at their isoelectric points, a most unlikely coincidence). The approach of Hartley and Roe to $\phi_H$ gives the surface pH at the plane of shear and this plane may be several Angstrom units away from the surface ionogenic groups (Davies, Haydon and Rideal), whereas proteolysis involves actual compound formation between enzyme and substrate and the functional group will be within the plane of shear of the two-protein molecule.

A comparison of the action of chymotrypsin on lysozyme (denatured) in solution and on the surface of kaolinite particles (about 1 μ in size)
Table I

Values of $\Delta pH = pH_b - pH_s$ for some biochemical and biological surfaces in aqueous systems at room temperature ($\chi$).

<table>
<thead>
<tr>
<th>Surface</th>
<th>Mobility* (microns/sec/V/cm)</th>
<th>$i^*$</th>
<th>$\xi$-potential, mV*</th>
<th>$pH_b$</th>
<th>$pH$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysozyme</td>
<td>4.0</td>
<td>0.05</td>
<td>52</td>
<td>8.05</td>
<td>-1.30</td>
</tr>
<tr>
<td>Kaolinite</td>
<td>-4.8</td>
<td>0.05</td>
<td>-62</td>
<td>8.05</td>
<td>1.04</td>
</tr>
<tr>
<td>Lysozyme on kaolinite</td>
<td>-2.5</td>
<td>0.05</td>
<td>-32</td>
<td>8.05</td>
<td>0.54</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>-2.0</td>
<td>0.02</td>
<td>--</td>
<td>6.9</td>
<td>0.44</td>
</tr>
<tr>
<td>Yeast</td>
<td>-1.2</td>
<td>0.01</td>
<td>--</td>
<td>4.1</td>
<td>0.30</td>
</tr>
<tr>
<td>Erythrocytes</td>
<td>-1.3</td>
<td>0.13</td>
<td>--</td>
<td>7.4</td>
<td>0.3</td>
</tr>
<tr>
<td>Mitochondria (lupine)</td>
<td>-0.77</td>
<td>0.12</td>
<td>--</td>
<td>6.9</td>
<td>0.2</td>
</tr>
</tbody>
</table>

* The ionic strength $i$ is of fundamental importance in governing, in part, these quantities. They are lower the higher the ionic strength.
showed that the pH optimum for the adsorbed enzyme is about 2 units higher than for the enzyme acting in solution. In other words, the enzyme behaves in accordance with a concentration of hydrogen ions at the surface of negatively charged kaolinite which is greater than in the surrounding buffer medium (McLaren and Estermann, 1957). Apparently the action of an enzyme at an interface can serve the role of a "molecular pH meter" in giving an idea of the acidity of an interface (McLaren and Babcock).

It must be remarked that the equations given above for reaction rates are applicable only to reactions which proceed so slowly that no molecular diffusion gradients, in the statistical sense discussed by Collins and Kimbol, appear ( ). That is, only reactions which are not diffusion controlled have been considered. Provided that enzyme reactions have equilibria lying far to the side of products \((E + P)\) and with \(k_2 \gg k_3\), it may be shown that the reaction velocity is not dependent on the viscosity of the medium (Schurr and McLaren, 1965). This assumption will have to be tested as more work is undertaken in structurally restricted systems. For example, an enzyme trapped within a substrate at low water content is subjected to an entirely different kind of environment than that in a dilute solution and the ease with which products can diffuse away from a complex will influence the overall kinetics, particularly if the reaction products are inhibitors (Dixon and Webb).
Suppose we have a substrate in the form of a coacervate droplet or gel particle in suspension which is so dilute in substrate that the rate of entry of an enzyme is scarcely impeded. Will the rate of hydrolysis contain constants $k_3$ and $K_m$ which are the same or similar to those for the same kind of substrate in solution? On the other hand, suppose the particles are not readily penetrable by enzymes and the surface area is so great that almost all the enzyme is adsorbed on the surface. Examples are fat droplets and starch granules in suspension as substrates. For such particles, from equation (7) one would not expect initial reaction rates to be directly proportional to enzyme concentration over much of a range. Furthermore, if such particles bear a net electric charge at the interface, the local, surface pH will not be the same as in bulk solution. It would be no surprise to find values for pH optima and $K_m$ rather far removed from values found for the same enzyme acting on chemically similar substrates in solution; Figure 2.

Many reactions at the cellular level, as in food preservation problems, and in cell particulates, will doubtless be found to be diffusion controlled. As an extreme case, consider the substrate concentration to be low and $k_2$ to be much less than $k_3$. Then equation (6) becomes simply

$$v = k_1 E_0 S_0$$

This situation can pertain in highly viscous media wherein the rate of collision of enzyme with substrate, limited by diffusion, becomes rate controlling (McLaren, 1963). The constant $k$ is equal to the encounter rate, namely $4 \pi N R_{12}^2 D_{12} 10^{-3}$ where $N$ is
Figure 2. The effect of pH on invertase activity of yeast cells and isolated enzyme (1, 2) and on chymotryptic activity in solution or adsorbed on kaolinite (3, 4).
Avogadro's number, $R_{12}$ is the sum of the radii of the reactants and $D_{12}$ is the sum of the diffusion coefficients. This encounter rate presupposes the model of Smoluckowski for the case of negligible electrostatic effects, such as in the case of catalase or invertase (Ackerman, Alberty and Hammer). For a small substrate molecule $k_1$ is approximately $k_1 \approx \frac{4}{3}\pi N R_1 D_2 10^{-3}$, that is, the size of the enzyme and the diffusion coefficient of the substrate are of paramount importance. If the enzyme is surrounded by other molecules in a membrane, however, a locus effect, either steric or electrostatic may be involved (see below, Romeo and Bernard). Experiments with cytochrome c and horse-radish peroxidase indicate that dipoles may be oriented to oppose the reaction (Ackerman, 1962), whereas the fumarase reaction is enhanced by electrostatic effects (Alberty and Hammer, 1958).

In the following discussion we will examine these questions and a number of others suggested by Figure 1 and for which data is available.

III. REACTIONS WITH COACERVATES, SWOLLEN GELS, OILS, ETC.

A. Action of soluble enzymes on insoluble substrates

1. Action of enzymes on proteins, starch, cellulose and chitin.

It is difficult to find a substrate which can be studied both in solution and in suspension. A rigorous comparison of enzyme action on both soluble and insoluble forms of the same substrate is complicated
by the fact that one cannot have two distinct equilibrium phases of a substrate under the same physical conditions, except at phase transitions where both phases exist. Otherwise one form of the substrate must be in a non-equilibrium state under the prevailing condition. A satisfactory situation is one in which both soluble and insoluble forms arise naturally as a consequence of hysteresis of the substrate with respect to variation of one or more thermodynamic variables. In this event substrate molecules of identical chemical composition and molecular weight comprise both forms.

Ordinary gelatin is suitable because, upon cooling sufficiently concentrated solutions, it readily forms gels which are essentially stable against dispersion into excess solvent, and which may be redissolved by mild heating. We can visualize two extreme cases. In one an enzyme can penetrate the gel in a time short compared to both (i) the duration of the kinetic experiment and (ii) the time required to digest the gel sufficiently to permit dispersion into the surrounding solvent. In the other the enzyme cannot penetrate the gel and can act only on the surface of the gel exposed to enzyme solution.

The action of trypsin on gel microspheres between 10 and 100 μ in diameter has been compared with its action on dilute gelatin sols which were obtained by melting out a corresponding suspension of gel spheres. The spheres were prepared by cooling a coacervate mixture of warm, dilute gelatin and ammonium sulfate, followed by removal of the salt; they were readily penetrated by trypsin (Schurr and McLaren).
The rates of digestion of gelatin microspheres and sols may both be described by equations of the form of equation (6) above. That is, the rate data in both instances may be fitted to

\[ v = \frac{k_B E S_0}{K_B + S_0} \]  

(13)

where \( k_B \) and \( K_B \) are empirically determined constants and \( v \) is in moles of peptide hydrolysed per minute. A relation between \( k_B \), \( K_B \), and fundamental physical constants of the system were established by analysis of a suitable model. Since there are a number of susceptible bonds per milligram of gelatin, falling into classes each characterized by a \( k_3 \) and a \( K_m \), \( k_B \) and \( K_B \) are appropriate averages (Schurr and McLaren), designated by \( \overline{k}_3 \) and \( \overline{K}_m \) respectively.

With the gel spheres it is necessary to allow also for the possibility of an equilibrium partition of the free enzyme between the bulk phase and the gel phase and appropriate average values for the gel (g) system are designated by \( k^g_B \) and \( K^g_B \). The Michaelis constant \( K^g_B \) is given by \( \overline{K}^g_m/K^g_E \) where \( K_E = E^g/E \) is the equilibrium constant for the partition of unbound enzyme between the outside solution (concentration \( E \)) and the interior of the gel (concentration \( E^g \)). Both \( k^g_B \) and \( K^g_B \) represent the same kinds of averages as \( k_B \) and \( K_B \), although the fundamental constants employed are those characterizing a gelatin molecule in the gel. It was found that the rate constants for dissociation of the enzyme-substrate complexes to hydrolysis products were essentially the same for both solution gelatin and spheres from
16° C to 24.5° C. This implies that the substrate structure in the gel has a negligible influence on the reaction rate once the enzyme forms a complex with substrate.

By contrast, the Michaelis constant for the gel system is greater than that for solutions below the swelling point of the gel, a difference that disappears as the melting point of the gel is approached, Table II. It was also concluded that $K_E$ is not appreciably greater than unity, so the difference is actually due to the fact that $K_{E}^{G}/K_{B}^{G}$ is greater than one below 27°. Evidently, the spheres possess a tertiary structure which must be disrupted to facilitate binding of the enzyme and this structure vanishes as the melting point (28° ± 1) of the gel is approached.

Gelatin spheres may also be chemically cross-linked by disulfide. These spheres closely resembled the plain spheres in size and shape and the visual (phase contrast optics) uniformity of digestion by trypsin is unchanged. Such spheres can stand a temperature of 100° for more than an hour without melting and the presence of covalent crosslinks reduces the susceptibility to digestion by trypsin and subtilisin.

By contrast, cross linking a concentrated gelatin gel results in a product ("Thiogel") impenetrable to trypsin. Thiogel can be digested from the outside, however, if blocks are exposed to dissolved trypsin (Tsuk and Oster). Under these conditions a cube of Thiogel with a surface area of 9.6 cm² was soaked in 200 ml of trypsin at a concentration of up to $3.2 \times 10^{-6}$ molar. This amount of trypsin, on close
Table II

Kinetic parameters for the hydrolysis of gelatin in sol and gel states by trypsin (Schurr and McLaren).

<table>
<thead>
<tr>
<th>Temperature</th>
<th>$k_B^g$</th>
<th>$k_B^g$</th>
</tr>
</thead>
<tbody>
<tr>
<td>spheres</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24.5</td>
<td>9.5</td>
<td>3.7</td>
</tr>
<tr>
<td>20.0</td>
<td>4.7</td>
<td>2.7</td>
</tr>
<tr>
<td>16.0</td>
<td>3.1</td>
<td>3.1</td>
</tr>
<tr>
<td>melted spheres</td>
<td></td>
<td></td>
</tr>
<tr>
<td>29.5</td>
<td>16</td>
<td>3.7</td>
</tr>
<tr>
<td>24.5</td>
<td>11</td>
<td>2.8</td>
</tr>
<tr>
<td>20.0</td>
<td>4.5</td>
<td>1.0</td>
</tr>
<tr>
<td>16.0</td>
<td>3.3</td>
<td>0.6</td>
</tr>
</tbody>
</table>
packing, could cover as much as 40,000 cm$^2$ of surface so $E_0 \gg S_0$ and equation 7 should apply (Figure 3).

For much the same reason this equation describes the action of elastase on elasten (Roberts and Samuel). Since one is dealing with digestion at the surface of a macroscopic substrate particle, or particles, we really need a modified form of equation 7 in terms of surface areas (McLaren, 1962).

Let the surface area of the particulate substrate, suspended in a solvent containing enzyme, be $A$ in cm$^2$/L and let $a$ be the surface of substrate occupied by enzyme. The rate of absorption is $k_a (A - a) E$ and the rate of desorption is $k_d a$. At equilibrium we have the Langmuir sorption isotherm

$$\frac{a}{A} = \frac{K_L E}{1 + K_L E}$$

(14)

where $K_L = k_a / k_d$. $K_L$ is analogous to $K_m^{-1}$. We have little information on the rate adsorption of enzymes on solids except that $k_a$ is probably faster in general than $k_3$ (McLaren and Peterson, 1965) and much faster than $k_d$ for charged adsorbents. The basis for this treatment is that "mass law" applies, i.e., the adsorbate does not interact with itself on the surface to form dimers, trimers, etc. at high concentrations so as to change the value of $k_d$ and the affinity of all surface sites for the solute is the same. At high values of $E$, $a \rightarrow A$ and the substrate may be said to be saturated. The Langmuir isotherm is a special case of the Frisch isotherm for a stiff macromolecule. Certainly
Figure 3-A. Variation of digestion rate, $v_i$, in mm/hr, of a cube of "Thiogel" as a function of enzyme concentration, $E_o$; cf equation 7 (Data of Tsuk and Oster). The line represents the empirical equation of Tsuk and Oster, namely $v = 0.74 E_o^{0.427}$. Figure 3-B. Reciprocal plot of data in Figure 3-A; cf equation (16c).
the adsorption of enzymes on clays does not follow equation (14) very well and Frick suggests that the data are more faithfully represented by equation (15)

\[ a/A = K_L E t(1 - a/A)^t \]  

(15)

where \( t \) is the number of segments or a macromolecule, all of which are deposited on the surface. Thus the initial slope of adsorption can be \( d(a/A)/dE > K_L \) as is observed experimentally (McLaren and Peterson). We will continue to use equation (14) as an approximation because the resulting rate equation (below) is cumbersome even in the simplest case.

Let us assume that the velocity of hydrolysis of the gel-solid phase is \( v = k' E_a \), where \( E_a \), the adsorbed enzyme, equals \( a/A_E \) and \( A_E \) is the area occupied per mole of adsorbed enzyme. \( E_a \) may be equal to or less than the amount of enzyme substrate complex (not all adsorbed enzyme may have all active sites in contact with susceptible bonds in the substrate surface). Then, by equation 14 and the conservation equation \( E = E + E_o \), we have (McLaren, 1963)

\[
v = k' E_o A \frac{k' K_L E}{A_E (1 + K_L E)} = \frac{k' A E_o}{A_E (E + \frac{1}{K_L}) + A} = \frac{k' E_o A}{A_E + A_E/K_L + A - a}
\]

(16)

Note that in experiments such as those of Tsuk and Oster, equation (16) reduces to

\[
v = k' E_o A/[A_E (E_o + K_L^{-1})] = k'' E_o A/[E_o + K_L^{-1}]
\]

(16a)
Equation (16a) can be compared with equation (7); for both situations enzyme is in excess of the available substrate and for particulate substrate we are interested in digestion at the surface. A useful form of equation 16a gives a reciprocal plot, viz.

\[
\frac{1}{v} = \frac{1}{k'} \left( \frac{A_E}{K_A} + 1 \right) \frac{1}{E_o} + \frac{A_E}{k'A} \cdot \frac{E}{E_o}
\]  

(16b)

If \( E_o \) is large (i.e., \( E \gg E_o \)) the intercept is \( A_E/k'A \), and the slope intercept quotient is \( (K_L^{-1} + A/A_E) \). Any departure from linearity of a \( 1/v \) vs. \( 1/E \) plot would indicate that the assumption \( E \approx E_o \) is not correct (Thomson, J.F., 1964). Of course, if we also know that \( A_E \gg A \), equation 16b may be reduced still further to

\[
\frac{1}{v} = \frac{1}{k'} \cdot \frac{A_E}{K_A} \cdot \frac{1}{E_o} + \frac{A_E}{k'A}
\]  

(16c)

with a slope intercept quotient of \( 1/K_L \). In Figure (3-B) we can see that only at the lowest enzyme concentrations do we have a departure from the trend predicted by the equation.

Actually Tsuk and Oster suggested an empirical equation for their experiments, figure 3-A, which is of the form \( v = KE^n \). This is a rough approximation for an equation of the form of equation 7 (McLaren and Peterson) although a geometrical interpretation has been given to the exponent \( n \) (McLaren, 1963), based on the Freundlich adsorption isotherm. For some examples the available adsorption data is better represented by this isotherm, namely \( a/A = KE^n \), than by that of Langmuir (Equation 14 above).
Walker and Hope measured the adsorption of salivary amylase on starch granules and found that the units enzyme adsorbed per gram of starch could be represented by \( 190 E^{0.6h} \). If we assume once again that \( v = k'E_a \), the rate equation becomes

\[
v = k'E_a^n
\]

The initial velocity is proportional to the total surface area of all suspended particles but to the enzyme concentration to a fractional power. Although this equation predicts the trend of the rate data of Walker and Hope for the digestion of starch, a closer fit is obtained by using \( n = 0.54 \) (McLaren, 1965). This departure from simple expectation can arise because of the probable surface mobility of adsorbed enzyme on the starch granules (McLaren and Estermann, 1956), and the adsorption of enzyme on starch is not rapid compared to the rate of hydrolysis so that equilibrium between dissolved and adsorbed enzyme is never quite reached. Also, the sites of adsorption of amylase on starch granules may include others besides those for which the union of enzyme and substrate leads to a hydrolyzable complex.

We can only anticipate that the rate of hydrolysis will be proportional to the surface area of the substrate and to the amount of enzyme adsorbed per unit of area. Further, the reactive sites may be partly at cracks or crevices on the granular rather than on smooth surfaces, which would lead to a value for \( n \) of less than 2/3*. Microscopic observations of

\* For surfaces, edges, and points, the values of \( n \) are 2/3, 1/3, and 0 for geometrical reasons. With \( n = 1 \) we have the solubility distribution isotherm for the distribution of a solute between two immiscible solvents (Gyani, 1945).
digesting starch in advanced stages indicates this (Balls and Schwimmer, 1944). It seems almost certain that in the experiments of Walker and Hope only a fraction of the total surface area of the starch was covered by enzyme; the total amount of enzyme was not enough for a monolayer even if all was adsorbed (McLaren, 1963).

Whitaker ( ) has studied the effect of enzyme concentration on the rate of hydrolysis of cellulose and swollen linters. His results are plotted in Figure 4: the data conforms to equation (17) with \( n = 0.66 \) for cellulose and \( n = 0.77 \) for swollen linters. According to theory, the latter value could mean that some penetration of linters by enzyme has taken place (McLaren, 1963).

A more complicated substrate is found in the form of bacterial cell wall; the hydrolysis of killed *Bacteria lactis aerogenes* cells by a proteolytic system from a soil *Actinomyces* also seems to be represented by equation 17 (Muggleton and Webb, 1952). Before lysis commences the enzyme is adsorbed onto the cells.

Chitin (poly-\( \beta \)-1→4-N-acetylglucosamine) is able to sorb lysozyme and chitinase selectively and can undergo catalytic changes in their presence. Lysozyme is also capable of depolymerizing cell-wall mucopolysaccharides of some microorganisms (Cherkasov, et al., 1966).

The course of action of lysozyme can be followed by decreases in viscosity or turbidity of substrate suspensions (Meyer and Hahnel, 1946) Smolelis and Hartsell, 1949). Reaction rates are highly sensitive to
Figure 4. Digestion of cellulose and swollen linters by cellulase (McLaren, Whitaker).
salt concentrations as is to be expected: ionic strength is not only important to the action of an enzyme in general, but in this case influences the binding to insoluble substrates (Smolelis and Hartsell, 1952).

There are, of course, many natural situations having features in common with the model systems discussed above. For example, an "escape hatch" from the cocoon of the moth is established by the hydrolytic action of an almost pure proteinase which the moth first secretes and then dissolves by means of a solvent which maintains the enzyme at optimum pH (Kafotos and Williams, 1964). Particulate food matter ingested by cells via phagocytosis, foods in the alimentary tract, and decomposition of plant litter and soil organic matter are other instances which might be analysed in this way. Of course the presence of organic solvents, emulsifying agents, exchangeable ions and the co-action of other enzymes can influence the rate of digestion of such substances as cell walls because such natural substrates consist of complicated structures containing more than one kind of polymeric material (Noller and Hartrell).

2. Action of enzymes on fats, polymeric esters and other nonporous substrates.

Another type of enzymatic process taking place in a heterogeneous medium is the action of pancreatic lipase on emulsified triglycerides. Schonheyder and Volquartz demonstrated that the activity of lipase on emulsified trihexanoyl glycerol depends upon the dimensions of the
liquid-liquid interface \( \Gamma \). The complete picture was somewhat complicated by the solubility of the substrate in water and therefore Benzonana and Desnuelle chose fats with longer acyl carbon chains having negligible solubility. Hydrolysis was a function of the number of enzyme molecules adsorbed at the interface. In a series of experiments containing particles of different size, it was shown that \( v \) depends on the area at the interface and not directly on the weight of the insoluble substrate. Lipase adsorption took place according to the Langmuir isotherm.

In the treatment of Sarda and Desnuelle \( (\) \) the data was summarized by the equation

\[
v = \frac{VA}{A + K_m}
\]

where \( V \) is the maximum rate and \( A \) is the molar surface concentration in moles per 1000 sq. cm. of surface. This is of the form of equation 6 with substrate in excess and of the form of equation (16) if \( A_{E \leq} \ll A \).

For similar experiments Benzonana and Desnuelle report that enzyme occupied but a few percent of the total surface.

The state of this enzyme at the surface is far from understood. As with the thiogel trypsin experiments, we can suggest that \( k' = f k_3 \)

where \( f \) is the fraction of adsorbed enzyme in a Michaelis-Menten complex with substrate. Lipase may be able to withstand surface denaturation - inactivation at oil-water interfaces. By contrast, chymotrypsin loses its activity at an octadecane-water interface.
(Ghosh and Bull). As the adsorption of chymotrypsin is increased, increasing the total enzyme in the suspension, some of the enzyme retains its activity. These phenomena may be related to the fact that chymotrypsin can readily be crystallized from aqueous solution, whereas fat splitting enzymes resist crystallization. The latter may have a greater proportion of non-polar amino acid residues on the exterior of molecules.

Desnuelle and coworkers have pointed out that such parameters as electric charges of emulsified particles may play a role during digestion and that bile salts seem to influence the extent of reversibility of lipase adsorption at the interface. Such salts stabilize lipase and cholesterol esterase from inactivation (Desnuelle, 1961); they are without influence on the action of chymotrypsin at physiological concentrations found in the intestine (Lippel and Olson).

For the digestion of tricaprin, Wallach ( ) has found a marked influence of detergent or pH optima and $K_m$ with rat adipose tissue lipase. With a cationic detergent, optimum pH $= 7.5$ and $K_m = 0.006$; with an anionic detergent pH $= 6.6$ and $K_m = 0.0045$; with a nonionic detergent pH $= 6.9$ and $K_m = 0.002$. Bangham has reported that the substrates of phospholipases can be converted to anionic or cationic forms by emulsification with appropriate surfactants. If the phospholipase is rendered oppositely charged by pH manipulation, hydrolysis rates of substrates can be increased. For an attack of
phospholipase \(E\) on lecithin, it is essential that the substrate particles carry a minimum net negative zeta potential (Bangham and Dawson). Evidently, before the enzyme can hydrolyse micelles (or high-pressure films) of phospholipids, the zeta potential at certain points on the interface must be such that it assists the enzyme to orientate at the surface so that its active center is in a favourable position for hydrolysis (Dawson and Bangham).

The influence of variations of surface tension on the stability and activity of enzymes (Shatoury) does not seem to have been studied extensively with emulsified substrates. A mobile liquid-liquid interface can assist in denaturation of enzymes, as we have noted. Conversely, a rigid solid-liquid interface does not seem to lead to enzyme molecule disorganization (unfolding) (Zittle, McLaren and Peterson) which explains in part the success of solid adsorbents in the purification of enzymes.

Poly-\(\beta\)-hydroxybutyrate is a very insoluble substrate for an extracellular enzyme from the soil organism \textit{Pseudomonas lemoignei}. The kinetics of hydrolysis of the polymer granules follows equation (16a) very well; the rate of hydrolysis is proportional to the surface area of the granules, to the amount of polymer initially present in the reaction mixture and to the amount of adsorbed depolymerase (McLaren and Delafield). A plot of \(1/v\) versus \(1/E_0\) is linear for three initial concentrations of polymer, Figure 5. Furthermore, replacing the area \(A\) by \([S/D]^{2/3}\), where \(D\) is density of the polymer, and integrating equation (16a) one finds that \(S^{1/3}\) is a linear function of digestion
Figure 5. Digestion of poly-β-hydroxybutyrate particles by the depolymerase of *P. lemoignei*.

A. Plot of equation (1ca)

B. Note that the reciprocal form of equation 6 does not apply to this insoluble substrate.
time with expected ratios of slopes $\Delta S^{1/3}/\Delta t$ for five different total enzyme concentrations examined.

Another pseudomonad enzyme, cell free, has been shown to etch single crystals of naphthalene in the presence of niacin adenine dinucleotide as cofactor. (Thomas et al.). The etched pits were flat-bottomed and hexagonal in shape and all of the same crystallographic outline. This was explained in terms of an anisotropy of reaction rates for the naphthalene lattice.

3. Action of enzymes in coacervates.

In addition to the action of enzymes on coacervates, a few papers have appeared on enzyme action within synthesized coacervates. It is believed that such studies may be useful models with which one may discover properties of enzymes the counterparts of which occur in living tissues (Oparin, et al., 1957).

One can, in principle, incorporate enzymes in coacervate droplets in order to mimic similar situations in protoplasm. Such droplets have been studied without an external membrane, although membranes can be added (Bungenberg de Jong, 1949). Coacervates formed from certain components can exist only within relatively narrow limits of pH and success can be assured only if this pH range includes an enzyme with a pH optimum near or in this region. Amylase action has been studied in a coacervate consisting of starch, protamine and gelatine; the products, amylopectins, erythrodextrins and eventually achrodextrines.
remained in the droplets. By comparison the activity of amylase was less in the coacervate than in solution. Presumably mobility of both enzyme and macro-molecular substrate are reduced by the macroscopic viscosity of the coacervate per se (McLaren, 1963). Further, the concentration of reducing sugar products was greater in than out of the droplets. This is a clear-cut example of the influence of structure on the nature of the process.

Similar experiments with bacterial catalase and hydrogen peroxide revealed that enzyme activity was confined to the droplets and that there was practically no enzyme in the equilibrium fluid.

4. **Action of enzymes on adsorbed substrates.**

In soil (Skujins, 1967) and for some industrial processes, including sewage disposal, high molecular weight substrates are adsorbed on sands and clays (Brock, 1966). Protein adsorbed on clays is readily digested by proteolytic enzymes (McLaren, 1954) and it was shown that this takes place by the formation of an enzyme-substrate complex in the adsorbed state (McLaren, 1955).

Rate measurements can be made provided the adsorbed protein is not readily detached from the clay and provided the hydrolysis products are readily liberated from the surface. The first may be achieved by adding a heat-denatured protein, with a high isoelectric point (such as lysozyme) to the negatively charged clay and the latter
by having dilute salt present as an eluting agent. Protein adsorption takes place partly by ion exchange and partly by non-ionic (hydrogen bonding?) bonding (McLaren, 1954). Proteolysis of the adsorbed complex by chymotrypsin occurs at about two-thirds of the rate obtained with an equivalent mixture of enzyme and substrate in solution in the absence of clay. Evidently adsorbed enzyme has a mobility about the surface of the adsorbant. The molecular weight of an enzyme is high and its diffusion rate is slow; consequently attachment of an enzyme to a surface should not drastically reduce a reaction rate if the substrate is soluble (Schurr, 1964). If the substrate is also adsorbed on a surface one or the other must have surface mobility and in the above described system it was shown that the enzyme has a slow but measurable mobility (McLaren and Esterman, 1956).

Protein substrates can also be absorbed between the sheets of expanding lattice type clay minerals, such as montmorillonite; this leads to an increase in d(001) crystallographic spacings of the clay. In fact, the increase can be used as a caliper for the size of protein molecules (McLaren and Peterson, 1961) and the subsequent decrease following proteolytic digestion can be used to follow enzyme action. For example a certain bentonite clay has a spacing of 11A. With absorbed protein the spacing is increased to 47A. Following digestion by enzymes of Pseudomonas sp. the spacing decreased to 17A showing that the bacterial enzymes can also penetrate the clay mineral
lattice. Incidentally, lignin and silicagel as well as clays reduce the velocity of digestion of protein (Estermann et al., 1959).

Since clays, being acidic colloidal particles, have surfaces 10 to 100 times more acid than a suspending medium at, say pH 7, i.e. \( \Delta pH = 1 \) to 2 (Harter and Ahlrichs, 1967), one would expect a shift in pH optimum for an enzyme acting at the surface. This has been found (McLaren and Estermann, 1957) and several other examples are now known. Consider, for example, the shift seen in Figure 2 for the action of chymotrypsin on adsorbed heat-denatured lysozyme; as already stated, it is about 2 units. The difference in pH between the surface of the substrate molecules in solution and that of the substrate or kaolinite may be calculated from the data in Table I. With \( pH_b = 8.05 \), \( pH_s \) for the surface of the substrate in solution is 9.4. For kaolinite covered with substrate, \( \Delta pH = 0.54 \) at an ionic strength of 0.05. Thus the effective pH at the surface of lysozyme molecules in solution is 9.4 whereas on kaolinite it is about 7.5. The zeta potential measurements serve to show that there should be a pH difference for optimum activity of chymotrypsin on adsorbed and unadsorbed lysozyme of about 1.9 units. Similar results have been found with this enzyme and substrate combination and bentonite (McLaren and Peterson, 1965).

The zeta potential values and \( \Delta pH \) for these systems should be reduced upon the addition of salt. Addition of salts causes elution of enzyme from the clay, however, thereby obviating the experiment. This approach to the problem has been successful under conditions in which the enzyme remains fixed to the carrier (Katchalski et al.), and will be discussed below.
Other examples of $\Delta p\phi H$ for enzymes are listed in Table III.

Trurnit carried out a thorough study of the action of chymotrypsin on three double layers of bovine serum albumin adsorbed on microscope slides pretreated with stearate. After adding enzyme to the liquid phase it was adsorbed onto the substrate, or removed substrate from the solid support, depending on buffer concentration and pH. The velocity of digestion was related to the enzyme concentration by an equation of the form of (17) with $n = 2/3$. He added, "this type of function should be expected if the enzyme concentration at the interface were a simple geometric function of the bulk concentration" (1953).

The apparent activation energy (Arrhenius coefficient) for the system was about half that known for similar reactions in homogeneous solution of substrate, but it could not be concluded whether this was due to the denatured state of the adsorbed substrate or to interfacial forces. This problem will come up again in experiments on enzyme-substrate-fibers where it will be shown that surface denatured and heat denatured forms of a protein substrate are not the same physically or chemically, and, therefore, such differences in kinetic quantities cannot be explicitly related to interfacial forces.

In the reactions described with clays above, it is known that the rate of adsorption of enzyme on to substrate coated clay particles is rapid compared to the rate of digestion in agitated suspensions. In fact, a complex of clay-substrate-enzyme reacts about as fast as does a complex of clay-substrate to which enzyme is added. Unlike the conditions used by Trurnit, surface areas of adsorbent and enzyme concentrations were relatively high. In these experiments, including
Table III

Shifts in pH optima ($\Delta$ pH) for enzyme action on carriers. An increase in pH optimum over that in solution is designated by +. $\Delta$ pH tends toward zero with increase in ionic strength.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Carrier</th>
<th>Substrate</th>
<th>$\Delta$ pH</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aminoacylase</td>
<td>DEAE-cellulose</td>
<td>acetylmethionine</td>
<td>-0.5 to -1.0</td>
<td>Tosa, 1967</td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>Kaolin</td>
<td>lysozyme</td>
<td>2</td>
<td>McLaren and Peterson</td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>bentonite</td>
<td>lysozyme</td>
<td>2</td>
<td>McLaren and Peterson</td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>carboxymethyl-cellulose</td>
<td>acetyltirosine</td>
<td>0</td>
<td>Mitz and Summakia, 1961</td>
</tr>
<tr>
<td>Ficin</td>
<td>carboxymethyl-cellulose</td>
<td>BAEE</td>
<td>0.3</td>
<td>Hornby et al, 1966</td>
</tr>
<tr>
<td>Invertase</td>
<td>DEAE-cellulose</td>
<td>sucrose</td>
<td>-2</td>
<td>Suzukiet al, 1966</td>
</tr>
<tr>
<td>Phosphatase</td>
<td>kaolin</td>
<td>naphthylphosphate</td>
<td>0.7</td>
<td>Ramirez and McLaren, 1966</td>
</tr>
<tr>
<td>Ribonuclease</td>
<td>cation exchange resin</td>
<td>ribonuclic acid</td>
<td>0</td>
<td>Burnett and Bull</td>
</tr>
<tr>
<td>Urease</td>
<td>bentonite</td>
<td>urea</td>
<td>0.6</td>
<td>Durand</td>
</tr>
</tbody>
</table>
those with preadsorption of enzyme, we would expect the rate to be
given by the equation \( \nu_1 = k'E_a \) and the amount of product by
\( P = k'E_a t \). This is observed (McLaren and Estermann, 1956) at values
of adsorbed enzyme low as compared with the amount of adsorbed
substrate. The model system of Trurnit has been analysed for
experiments with stirring (Trurnit 1954), based on the Nernst theory
of reaction kinetics in heterogeneous systems: if the reaction at the
interface is rapid as compared to the diffusion through the adherent
layer, interfacial reactions as a whole are governed by diffusion.
Under his conditions he showed that the amount of adsorbed enzyme
\( E_a = k_a E_t \), where \( k_a \) is now \( D/d \), the ratio of \( D \) the diffusion
coefficient and \( d \) the thickness of the undisturbed layer of solvent
next to the surface. The amount of reaction product is therefore
\[
P = \frac{k'_a}{2} E_0 t^2.
\]
Both rate measurements were established by experiment, Fig. (6).
Figure 6

Adsorption of chymotrypsin on stearate coated glass, upper curve, and digestion of bovine serum albumin multilayers by chymotrypsin, lower curve. Ordinate, thickness of layers, is a measure of the amount of protein at the solid-liquid interface. 

Upper curve equation:  \[ E_a = f(t) \]

Lower curve equation:  \[ S = f(t^2) \]
Adsorption of chymotrypsin on stearate coated glass, upper curve, and digestion of bovine serum albumin multilayers by chymotrypsin, lower curve. Ordinate, thickness of layers, is a measure of the amount of protein at the solid-liquid interface. Trurnit.

Upper curve equation: \( E_a = f(t) \)

Lower curve equation: \( S = f(t^2) \)
B. Action of insoluble enzymes on soluble and insoluble substrates.

Water insoluble derivatives of enzymes have been prepared by physical adsorption onto colloidal particles (Stone, 1955; McLaren, 1955; Mitz, 1956; Burnett and Bull, 1959; Nikolaev and Mardashev, 1961; Nikolaev, 1962), by entrapment of enzymes in insoluble matrices of cross linked polymer (Bernfield and Wan, 1963), by cross-linking of an enzyme by a bifunctional reagent (Quiocho and Richards, 1964), and by chemical attachment of enzymes to reactive polymer (Bar-Eli and Katchalski, 1960, 1961; Cebra et al. 1961; Mitz and Summaria, 1961; Manecke, 1962). They have been studied as models for some intracellular enzymes and for analytical procedures in chemical analysis (Hiks and Updike, Bauman et al.). This has become an active field (Katchalski, 1966), and some properties of these systems will be discussed in detail. A related topic, the activity of soluble enzymes in presence of soluble polyelectrolytes, has been reviewed (Mark and Morawetz, 1960).

It is significant that most adsorbed enzymes can be eluted in active form (Sober and Paterson, 1957; Schwimmer and Pardee, 1953; James and Augenstein, 1966) and adsorption by glass is much like that with clays. Work with clays is interesting because of the great versatility in surface area and properties available. Cation exchange resins have also been used; adsorbed ribonuclease on Dowex-50 shows no $\Delta pH$, however (Table III). The difference in pH between that of the surface of a negatively charged substrate molecule in solution (ribosenucleic acid) and that of the substrate on a negatively charged surface is expectedly small and so is $\Delta pH$. 
1. Properties of enzymes adsorbed on clays, glass and polymers.

A simple example of the action of an enzyme on a soluble substrate is that of urease, adsorbed on clays, on urea. This was investigated in a superficial way by Pinck and Allison (1961), who worked in a pH range where no adsorption of enzyme is really to be expected (McLaren, 1963). The problem was reinvestigated by Durand (1964) who found that bentonite reduces the reaction velocity and that a shift in pH optimum for urease occurs following adsorption (Table IV). Because adsorption alone should not greatly affect the reaction rate, since the rate of encounter of urea with enzyme is not greatly reduced (Schurr, 1964), we can rationalize the reduced rate by suggesting a covering of active sites on some enzyme molecules by the clay surface.

Similar results have been reported by Ramirez and McLaren for acid phosphatase, adsorbed on kaolin, that is a reduction in hydrolysis rate of 75 percent and a shift in pH optimum (Table III). Similar systems were studied earlier (Mortland and Gieseking, Kroll and Kramer), but comparisons in reaction rates did not take into account the ΔpH phenomenon (McLaren) as it had not been discovered.

Catalase is active on a cellulose anion exchange (Mitz, 1956); it is about 70 percent as active as soluble catalase. It can be eluted with other proteins and carbon dioxide can dissociate such insoluble protein-cellulose ion-exchange derivatives (Mitz and Yanari, 1956).

Aminoacylase has been studied in the presence of many adsorbents. Only partial adsorption was found with activated carbon and aluminum oxide and none with silica gel and anion-exchange resins. DEAE-sephadex
A-50 and DEAE-cellulose were strong adsorbents (Tosa et al., 1966a), and adsorbed enzyme was as high as 86 percent as active as soluble enzyme. Adsorbed enzyme could be eluted with 0.3 M sodium acetate (Tosa et al., 1966b).

DEAE-cellulose columns have also been prepared with adsorbed enzyme and suitable soluble substrates could be hydrolysed on passage through them (Tosa et al., 1966b). This technique has also been used with protease on clays (Skujins, et al., 1959). As might be expected, the pH optimum for the DEAE-cellulose-aminoacylase system was shifted toward the acid side of the optimum of soluble enzyme (Table III) (Tosa et al., 1967). The complex has a higher heat stability than the native enzyme and exhibits a lower Arrhenius activation energy (6,700 cal/mole) than found with free enzyme (11,100 cal/mole). Thus, complex formation amounts to the synthesis of a new enzyme, with altered kinetic constants, Table IV. Invertase has also been studied on DEAE-cellulose. It exhibits a ΔpH of 2 and a maximum activity of about half that of free enzyme. Heat stability is somewhat reduced (Suzuki, et al., 1966).

Earlier, it had been shown that catalase (Mitz, 1956) and asparaginase (Nikolaev and Mardashev, 1961; Nikolaev, 1962) can be adsorbed on ion-exchange cellulososes and that ribonuclease adsorbs to ion-exchange resins (Barnett and Bull, 1959; Mkrtumova, M.A. and Deborin, G.A., 1962). The soil contains a vast number of enzymes, some of which may be active in the adsorbed state and some in a covalently bound state with soil humus. In any case they are largely unextractable by any known means (Skujins, 1967).
A locus effect has long been known for the difference in reaction rates of invertase on sucrose in the presence of clays and soils (Joslyn, 1966). A difference in hydrogen ion concentration of soil particles and that of soil solution has been demonstrated by sucrose inversion.

2. Properties of covalently bound enzymes

a. Bound enzymes in suspension.

Levin et al. ( ) prepared insoluble trypsin by reaction with copolyethylene maleic anhydride. Reaction involved non-essential ε-amino groups and the resulting polymer-trypsin derivatives (P-T) were about half as active toward benzoylarginine ethylester (BAEE) as was free trypsin. On the other hand, the polymer chain network tended to exclude protein substrates such as casein and the reactions were lesser the greater the carrier-to-protein ratios (20:1 and 1:3). With heat denatured lysozyme as a substrate somewhat different peptide patterns are found with P-T as compared with free trypsin which suggests that a modified specificity has been superimposed on the bound trypsin molecules when acting on a high molecular-weight substrate. This observation may explain the results of Skujins and McLaren, who found such differences with subtilisin and Bacillus Subtilis adsorbed on kaolinite.

A low molecular weight (9,000) inhibitor can penetrate P-T complexes whereas a high molecular weight inhibitor (soybean trypsin inhibitor) cannot. Thus, STI inhibits the same P-T sites as are reached by the substrate casein but not all those reached by BAEE. Such P-T preparations are more stable to heat than free trypsin and retain some activity even in 8 M urea. Evidently the trypsin
molecules are permanently separated by the gel as autolysis is also blocked.

These preparations, particularly those of high carrier protein ratios showed a shift of pH optimum of as much as 3 units, at low ionic strength, Figure 7 (Goldstein et al.). This is as expected for trypsin embedded in a gel of negative charge (K at 4.35 and 7.5 for the free gel were noted), and the optimum returns to nearly that for soluble trypsin as the ionic strength is increased toward 2. That is, at high ionic strength the influence of a negatively charged ionic cage on the hydrogen ion concentration about a trypsin molecule is reduced. No displacement in the pH-activity curve of this type was found for derivatives in which the carrier is non-charged.

Goldstein et al. studied a P-T derivative consisting of 16.8 mg protein/100 mg dry weight in greater detail ( ). It was found, by comparison, that acylation alone (i.e., acetyltrypsin) produces a shift in pH optimum toward alkaline pH of over a unit, but this shift is not reduced by the addition of NaCl. By contrast, about 2/3 of the ΔpH of the P-T derivative can be deleted by adding salt, and at an ionic strength of 1 the pH activity curve is much like that of acetyl-trypsin (with or without salt).

With benzoylarginine amide (BAA) as substrate the apparent Michaelis constant for the P-T-BAA system was only \( 0.2 \times 10^{-3} \) (\( \mu = 0.04 \)), i.e., much less than \( K_m \) for the trypsin-BAA system, namely \( 6.9 \times 10^{-3} \) (\( \mu = 0.04 \)) Figure 8 (Goldstein). On adding salt \( K_m \) increased to
5.2 \times 10^{-3} \text{ for P-T, but no marked change in } K_m \text{ was noted for trypsin in solution. Qualitatively, "an enzyme bound to a charged polyelectrolyte acting on a substrate of opposite charge (BAA), will reach the limiting rate, } V_{\text{max}}, \text{ at lower bulk concentrations of substrate than those recorded for the corresponding unbound enzyme". The apparent Michaelis constant of the bound enzyme will therefore be lower than that of the free enzyme. From the expression}

\[ \Delta \phi = 0.43e\Psi/kT \]

and the data from the Figure 7 (Fig. 1 of Goldstein) for P-T we have the effect of ionic strength on the average electrostatic potential \( \Psi \) in the neighborhood of the enzyme molecules in the gel phase, Table V.

These values of \( \Psi \) are those to be expected for polyelectrolyte gels of the type ethylene-maleic acid and polymethacrylic acid, so here again we see that the enzyme can be used as a molecular electrode for characterizing the microenvironment. One would also expect to find that certain enzymes, the activity of which depends on the redox potential, could be used in a similar way to measure the oxidizing or reducing potential within a gel as well.

These displaced pH-activity curves of enzymes can be just as well explained by assuming a change in the ionization potential of the imidazole groups at the enzyme active site caused by the electrostatic field of the molecular environment surrounding the bound enzyme, incidentally. Either model of course leads to identical free energy terms (Goldstein et al., 1964; Schurr, 1964; Chattoraj and Bull, 1959).
Figure 7

pH-Activity curves for trypsin and polymer trypsin derivatives at different ionic strengths with BAEE as substrate (Goldstein et al., Figure 1, 1964).
Figure 8. Normalized Michaelis-Menten plots for trypsin and P-T acting on BAA (Goldstein et al., Figure 3, 1964).
Table IV

A comparison of some kinetic constants for soluble and insoluble enzymes.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Carrier</th>
<th>Substrate</th>
<th>$K_m$</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aminoacylase</td>
<td>DEAE-cellulose</td>
<td>Acetylimethionine</td>
<td>0.004</td>
<td>Tosa et al., 1967</td>
</tr>
<tr>
<td></td>
<td>none</td>
<td></td>
<td>0.006</td>
<td></td>
</tr>
<tr>
<td>Ficin</td>
<td>carboxymethyl-</td>
<td>BAEE</td>
<td>0.002</td>
<td>Hornby et al, 1966</td>
</tr>
<tr>
<td></td>
<td>cellulose</td>
<td></td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>Hexokinase</td>
<td>mitochondria</td>
<td>ATP</td>
<td>0.005</td>
<td>Li and Ch'ren, 1965</td>
</tr>
<tr>
<td></td>
<td>none</td>
<td></td>
<td>0.0013</td>
<td></td>
</tr>
<tr>
<td>Papain</td>
<td>co-polymer</td>
<td>BAEE</td>
<td>0.019 mole/L.</td>
<td>Silman et al., 1966</td>
</tr>
<tr>
<td></td>
<td>peptide</td>
<td></td>
<td>0.019 mole/L.</td>
<td></td>
</tr>
<tr>
<td>Trypsin</td>
<td>maleic acid-</td>
<td>BAA</td>
<td>0.002*</td>
<td>Goldstein et al., 1964</td>
</tr>
<tr>
<td></td>
<td>ethylene polymer</td>
<td></td>
<td>0.007</td>
<td></td>
</tr>
</tbody>
</table>

*At $\mu = 0.04$; on adding salt the value approaches that for trypsin.
Table V

Effect of Ionic Strength on the pH-Activity Profile of Bound Trypsin.
(Goldstein et al.)

<table>
<thead>
<tr>
<th>μ</th>
<th>Δ pH</th>
<th>ψ</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.006</td>
<td>2.4</td>
<td>0.15</td>
</tr>
<tr>
<td>0.01</td>
<td>2.0</td>
<td>0.12</td>
</tr>
<tr>
<td>0.2</td>
<td>1.3</td>
<td>0.08</td>
</tr>
<tr>
<td>1.0</td>
<td>0.4</td>
<td>0.03</td>
</tr>
</tbody>
</table>
Incidentally, whether or not $\Delta \mathrm{pH}$ is considered to be a measure of the difference of concentrations of hydrogen ion in and out of the gel phase or of the activity depends on the conventions used (McLaren and Babcock, 1959; Goldstein et al., 1964). Goldstein uses activity terms in discussing the hydrogen ion but switches to concentrations in discussing the distribution of substrate between the solution and gel phase. The Michaelis-Menten equation for solution

$$v = \frac{v_{\text{max}}(S)}{[K_m + (B)]}$$

is modified for enzyme action in the gel phase. It is written by Goldstein et al. as

$$v_g = \frac{v_{\text{max}}(S)}{[K_m^e - z\varepsilon\psi/kT + (S)]}$$

on the assumption that the true $K_m$ is the same in either phase and that the distribution of charged substrate between the gel phase and the external solution is given by $[S]_g = [S]e^{3\varepsilon\psi/kT}$. The experimental, apparent $K_{m,g} = K_m^e - z\varepsilon\psi/kT$. $z$ is the number (positive or negative) of charges $\varepsilon$ on the substrate and $k$ is the Boltzmann constant. A comparison of $K_m$ and $K_{m,g}$ is an alternate way of finding $\psi$.

$\psi$ may also be calculated from the theory of polyelectrolyte gels (Katchalsky et al., 1954); agreement with $\psi$ calculated [A. Katchalsky, N. Shavit, and H. Eisenberg (1954)] from the enzyme-kinetic data is very close and we may conclude that $k_m$ is about the same in or out of the gel. In addition we may infer that $k_3$ is also the same for both T and P-T systems.
Incidentally, the surface pH of micelles has also been studied with indicator dyes (Mukergee) and that of clays with adsorbed organic acids coupled with infrared spectroscopy (Harter).

Lowey et al. (1967) have used a P-T derivative to study the action of trypsin on myosin. With the water insoluble derivative the total number of peptide bonds available for enzymatic hydrolysis is markedly reduced. Soluble trypsin converts myosin into a large fragment (an ATPase) and a small one, the meromyosins. High concentrations of trypsin degrade the large fragment into small, globular subunits and a great deal of peptide material and non-protein nitrogen. The P-T derivative, by contrast, cleaves the large fragment under mild conditions with little loss of non-protein nitrogen, a feature which should be of value in the structural analysis of myosin to come.

By now a number of related systems have been prepared and for some of these kinetic constants have been obtained, Table VI.

Silman et al. report four water-insoluble papain derivatives prepared by three methods, namely, by covalent binding to a water-insoluble diazonium salt derived from a copolymer of p-aminophenylalanine and leucine, via binding to an insoluble preparation of collagen with bisdiazobenzidine-2, 2'-disulfonic acid, and by self crosslinking with bisdiazobenzidine. The copolymer derivative had a lower heat stability than that of native enzyme ( ).
Chymotrypsin and trypsin have been coupled with insoluble dextran (Sephadex) via a treatment with isothiocyanate and a reduction of activity was attributed to steric hindrance. This hindrance was most marked with amylase: evidently the substrate, starch, never makes contact with active regions of the enzyme (or else the active site is deactivated by the linking reaction) (Axen and Porath, 1966). Insoluble trypsin has been prepared by reaction with glutaraldehyde and by conjugation with aminoethylcellulose, also with the aid of glutaraldehyde (Habeeb, 1967). The cellulose derivative was 56 percent active as trypsin toward casein. A decrease in enzyme activity has also been observed with insolubilized urease (Riesel and Katchalski, 1964).

Cellulose reprecipitated from Schweizer's reagent is useful for the preparation of highly active, insoluble trypsin, chymotrypsin, and ribonuclease and cyanurcellulose is a useful intermediate. The altered properties of these insoluble enzymes are in the direction of reduced activity, reduced temperature stability and increased storability (Surinov and Manoilov, 1966). Chymotrypsin has also been insolubilized with carboxymethyl cellulose azide and the product has an increased heat stability. A derivative which had a tendency to swell in suspension was more active than a derivative that did not show this property. There appeared to be no shift in the pH optimum (Mitz and Summari, 1961).

Mitz and Summari were evidently the first to uncover a number of interesting properties of such systems. Insoluble trypsin and
chymotrypsin (carboxymethylcellulose) derivatives are more active on the smaller peptides as compared to protein substrates and are not inhibited by blood inhibitor, both of which suggest a locus effect. The trypsin derivative gives a controlled chymotrypsinogen to chymotrypsin conversion from which the product is uncontaminated by trypsin. Insoluble diazobenzylcellulose derivatives of ribonuclease and chymotrypsin were not very active.

Hornby et al. (1966) have used the procedure of Mitz and Summari to chemically attach ficin to carboxymethyl cellulose. The derivative shows a ΔpH of ca 0.3, with BAE E (and none with casein as substrate), a value which decreases with ionic strength. Such a small value and tendency have been overlooked in the work of Mitz and Summari. The derivative had only about 10 percent of the free esterase activity of ficin, but the product is stable for months, and has a greater heat stability than native enzyme. The derivative revealed only about 5% of free ficin activity as a protease. This was shown not to be due to loss of a vital SH group during coupling. The insoluble enzyme was less dependent on cysteine for activation than the free enzyme. As may be seen in Table IV, the apparent Kₐ value for the ficin derivative is less than with free ficin. Benzoylarginine ethyl ester is positively charged in the range of enzyme activity, and should be attracted to the carrier exchange sites electrostatically with a resultant increase in k₁ and a decrease in k₂. Once the enzyme substrate complex is formed it can react to produce a zwitter ion and an alcohol, neither of which is attracted by the derivative, so
should not be different in these homogeneous and heterogeneous systems.

Insoluble trypsin and ribonuclease have also been prepared by coupling with carboxymethylcellulose hydrazide by Epstein and Anfinsen (1962). Complete reduction of the trypsin derivative with urea and mercaptoethanol followed by reoxidation resulted in recovery of \( \frac{1}{4} \) percent of the original activity. The corresponding figure for ribonuclease activity was 40 percent.

Diazotized polyaminopolystyrene has been used to insolubilize pepsin, diastase, carboxypeptidase and ribonuclease (Brandenberger 1956). Grubhofer, N. and Schleith, L., 1953, 1954).

b. **Bound enzymes in columns.**

Once it had been found that enzymes are quite active on adsorbents and other polymeric carriers, it was obvious that systems had become available for obtaining reactions in columns and from which reaction products could be readily separated without contamination by enzyme. Clay-bacterial enzyme columns have been studied as an analogue of soil (Skujins et al.) and the enzyme activity of lichen cells entrapped in acrylamide gels has been recorded (Mosbach and Mosbach, 1966). Reaction kinetics in columns have been reviewed (Malek and Fencil).

Solutions can be continuously passed through columns of adsorbed enzyme, and the choice of carrier is great (positive, negative zwitterionic and neutral adsorbents and chemically binding gels and
action solids). The simultaneous role of enzyme-carrier and chromatographic separation in such columns allow one to separate reactants and products, and in principle, to give very high yields, even greater than those obtainable in equilibrium homogeneous systems. Hornby et al. ( ) reports yields of 19-99 percent for the hydrolysis of BAEE, depending on flow rates.

On passing through a trypsin column, polylysine was converted to lysine and di-, tri-, and tetrlysine was converted to lysine and di-, tri, and tetrlysine. Protamine and oxidized insulin give normal tryptic digests (Bar-Eli and Katchalski, 1960).

Small amounts of enzyme can be used over and over without much loss in activity which is delightful if the enzyme is precious (Mosbach and Mosbach). A flavoprotein D-oxynitrilase combines with a cellulose-based ion exchanger to form an active, stable catalyst. Columns of the catalyst have been used for the continuous synthesis of D-α-hydroxynitrites from aldehydes and hydrocyanic acid. The products can be prepared in kilogram quantities with milligram quantities of enzyme (Becker and Pfeil).

Bernfield and Wan have immobilized chymotrypsin and trypsin in acrylamide gels. Of course research and analyses with columns can be mechanized and automated, and gels of enzyme can be handled in the form of particles, blocks, strings, rods, coatings and tubes, all containing the same amount of enzyme per unit volume of gel. Glucose oxidase and lactic dehydrogenase activity in polyacrylamide
gel have been controlled in this matter (Hicks and Updike, 1966). The apparent $K_m$ for the dehydrogenase system was higher in the gel than in free solution, which may reflect a reduction in $k_1$ owing to a reduced (diffusion limited) rate of combination of enzyme and substrate in the gel.

Chlorine esterase has been immobilized with a starch matrix on a urethan foam pad (Bauman et al., 1965; Guibault and Kramer, 1965). The activity can be monitored electrochemically or fluorimetrically, depending on the substrate.

3. **Bound enzymes in films and membranes.**

We have thus far encountered a number of examples of extra-cellular digestion. Solids ingested by phagocytosis and perhaps transient subcellular particles such as fat globules and starch granules are subject to intra-cellular digestion, doubtless by nearly identical mechanisms. In some cases enzymes are probably stabilized by micelles (Ugolev, 1955). A third consists of membrane-contact digestion (Ugolev, 1965).

As soon as we begin to encounter enzyme action in films, mitochondria, cell surfaces and the like we encounter in a dramatic way the question of the meaning of concentration in volume elements so small as to raise doubt about the adequacy of statistical averages of numbers of molecules and ions (McLaren and Babcock). This in turn raises questions about the validity of mass action, equilibrium, steady
state assumptions, etc., when ions, particularly hydrogen, are
being formed or consumed, or local charges are altered in position
and numbers by virtue of reorganization of subcellular structures.
Mitchell (1967) has reviewed the kinetics of translocation of
biological membranes. One model that has been studied is of great
heuristic value. It is the action of an enzyme attached to a
semipermeable membrane. Goldman et al. prepared a papain-collodion
membrane matrix by adsorption of papain, followed by cross-linking
the papain with bisdiazobenzidine 3, 3'-disulfonic acid. Microscopic
examination of the film showed that the two outer layers contained
all the protein to a depth of 70 μ. With proper treatment the enzyme
could be attached to only one side of the film; with such a film,
activated with cysteine, diffusion-reaction studies may be performed.
For example, when permeability studies were carried out with BAEE on
the enzyme-free side, BAEE diffused through the film and only the
product BA emerged on the papain side. This is an example of vector
chemistry (Mitchell). Curiously, at pH 6 the papain membrane had
only about 5 percent of the activity of an equivalent amount of free
enzyme on BAEE but 40 percent of the activity on benzoylarginine
amide (BAA). The expected ΔpH was observed with these systems with
BAA (Fig. 9) as substrate; the net charge of both products, summed,
does not differ from BAA in the microenvironment and the influence of
membrane charge is manifest (ΔpH = 2). The pH optimum for soluble
papain is at about 6 with both substrates, but in the film the
optimum appears to be above 9.6 with BAEE and is never reached. This will-o'-the-wisp behavior can be accounted for qualitatively by noting that the carboxyl-group proton cannot be transferred to ethanol in the way it can transfer to ammonia released from BAA and the increase in hydrogen ion concentrations in the membrane during hydrolysis tends to depress the activity of the papain. This depression can only be counteracted by adding base. If the membrane is dried and powdered to destroy some of its structure, a pH optimum is observed, at pH 8 with BAEE. Further, the behaviour with BAEE approaches that with BAA if high concentrations of buffer are employed, and calculations of the hydrogen ion concentration in the membrane based on assumptions of diffusion coefficients and Fick's law, at low buffer capacity, indicated that the internal value of hydrogen ion concentration could be several orders of magnitude greater than in the external solution. It should be pointed out that "not infrequently the addition of either suspended solids or polymeric material to a homogeneous liquid increases the diffusive transport rates of some third species in this medium" (Metzner, 1965) and even the simple assumptions are subject to doubt as to quantitative validity in structured media.

Membrane digestion occurs in yeasts, microorganisms and plant roots (Brown, 1954), as already mentioned. It may be difficult experimentally to localize the site of splitting of food-stuffs and to be sure that a liberated, soluble enzyme is not involved as well as a localized membrane-containing enzyme (Ugolev, 1965). An existence of a Δ pit locus effect is certainly indicative if the enzyme can be
Figure 9. Activities of crystalline papain and of a collodion matrix papain membrane on the low-molecular weight synthetic substrates BAEE and BAA (Goldman et al. 1965).
studied in both the surface and solubilized state. Other techniques have been used including a) specific enzyme inhibitors that do not penetrate cells (Rothstein and Meier, 1948; Ugolev), b) failure of digestion to vary with variation of external pH, suggesting that the process is intracellular, and c) histochemical localizations of enzyme activity (e.g., Jensen). In the intestine many macromolecules seem to be digested by membrane contact in addition to the occurrence of such process in the bulk content of the gut. The problem of self protection of living cells from such digestive processes is still mysterious (Montagu, 1966); the subject of mammalian and plant tissue cell surfaces has been reviewed (Weiss, 1962)(Miller, 1962).

A self-digesting membrane system, studied by Mazia and Hayashi, is that of "fibers" of pepsin and albumin. These are actually folded, compressed mixed monolayers of pepsin and albumin that have been spread on a Langmuir trough (Mazia)(Mazia and Hayashi). The films under autodigestion when placed in buffer of low pH and the kinetics has been partially analysed (Schurr and McLaren, 1966; McLaren and Babcock, 1961). Mazia and Hayashi compared the rates of action of pepsin on ovalbumin in fibers with the rate on free, heat denatured ovalbumin but it is probable that the substrate molecules do not have the same chemical composition and molecular weight in both soluble and insoluble forms, as judged from both thermodynamic reasoning and chemical tests (Schurr and McLaren, 1966). Since in the fibers the usual concentration terms, such as moles per liter, are meaningless, the mole fraction equations were used and the results were compared with reaction in homogeneous solution. The assumptions were steady
Figure 10  Plot of equation 8a and 9a for the action of pepsin on ovalbumin in solution and as fibers (McLaren, 1962).
state kinetics in both systems and that Bull and Currie's equilibrium
constants apply to both native and denatured ovalbumin. In Figure 10
are plotted the data in terms of the equations (8a) and (9a). By
equation (8a) the maximum velocity is $v' = 0.11 E_0$ for pepsin in
solution, and by equation (9a) $v'/E_0 = 0.09$ in the fiber, which is
only a little less. However, the ratio of found rates in fiber and
in solution with Mazia's compositions is near 50 whereas the calculated
ratio is only 3.2. Either the assumed constants are not applicable to
surface denatured ovalbumin, or the composition of heat denatured
ovalbumin (used by Mazia for comparison) and surface denatured
albumin are too different for comparison in an enzyme-substrate
reaction, or else the influence of "fiber" structure per se is profound.
All these reasons may be important and they serve to show that any
attempt to study enzyme reactions of a digestive nature in the living
cell at the present time, where the structure plays a part, will have
to solve such problems. Here locus effects extend beyond ambiguities
of concentration and diffusion to those of configuration; form becomes
equally as important as function (McLaren, 1960).
B. STEADY STATE STUDIES OF NITRIFICATION IN SOIL:
THEORETICAL CONSIDERATIONS

Erh, Elrick, Thomas and Corke (3) and Macura and Func (8) have outlined the methods for study of nitrification in soil and have pointed out that a continuous flow method in an open system is more closely related to field conditions than are batch or reperfusion procedures. Other ways for study include the chemostat in which the flow rate of a nutrient is determined by growth rates of organisms (internal control) or by the low concentration of a growth factor (external control) (11).

In columns of soil, nitrifying organisms can grow, i.e., increase in number, after substrate is furnished to the column until a steady state, maximum population is reached (12). The soil can be incubated with substrate until this population is reached, followed by analysis of effluent from freshly perfusing substrate solution, or else the effluent can be analysed from the very beginning. In the latter case organisms will grow and the makeup of the effluent solution will continually change. In the former, the relative amounts of products may remain constant, i.e., the makeup of the effluent can become time invariant and a steady state pertains. In both cases the evidence suggests that the maximum population continues to carry out nitrification with very little multiplication, except to replace those organisms that die, but it is not known how the rate with a constant population compares with those of a growing population on a per cell basis. It is recognized that mutation can also take place during continuous flow (11).

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1 Submitted for publication.
For the simple system depicted in Fig. 1, I have indicated a series of consecutive reactions for nitrification in a column of soil, including a transport of fluid into the column at a rate of \( k_0 \text{ cm/day} \). The entering fluid contains ammonium at an initial concentration \( (\text{NH}_3^+) \). Within the soil ammonium is transformed to nitrite by \textit{Nitrosomonas} species at a specific rate, \( k_1 \), and nitrite is oxidized to nitrate with a rate constant, \( k_2 \), by \textit{Nitrobacter} species. Finally, products and any unreacted ammonium exit. This is an idealized situation; a host of factors which could modify the specific rates have been set aside. Some of these factors are known and will be discussed later.

The specific rate constants, \( k_1 \) and \( k_2 \), include the concentrations of enzymes responsible for the respective reactions, and these concentrations may be taken as proportional to the numbers of respective organisms present. Consecutive reactions of time \( (\tau) \); however, we will want to express concentrations as functions of distance in a soil column, that is to say we have a vectorial chemical reaction sequence.

\section*{MODELS}

We can begin with the simple case in which the soil column is treated with a solution of ammonium and allowed to stand without flow until a maximum population of nitrifying organisms has been reached. [To avoid details of ionic elution, simultaneous reactions


by other denizens in the column, etc., one could use glass beads as the carrier (7) to simplify the model. By choice of ion exchange beads, ion exchange could be reintroduced to the model (9). Fresh substrate solution is then leached through the column. Although this solution enters the soil at a rate \( k_0 \), the rate is faster in the soil column since the water content is only of the order of one-fourth gram per cm\(^3\). Let the flow rate in the soil be \( X \) cm/day. The flow rate, \( f \), in the soil is then \( E k_0 \), where \( E \) is an expansion factor. Then \( X = ft = E k_0 t \).

The rate of disappearance of the ammonia, for a constant amount of catalyst, is given by

\[
\frac{d(NH_4^+)}{dt} = -k_1(NH_4^+) \tag{1}
\]

and the variation in concentration with distance is

\[
\frac{d(NH_4^+)}{dx} = -k_1(NH_4^+) \tag{2}
\]

where \( k_1 \) is \( k_1/Ek_0 \). Similarly, the rate of change of nitrite concentration is

\[
\frac{d(NO_2^-)}{dt} = k_1(NH_4^+) - k_2(NO_2^-) \tag{3}
\]

and

\[
\frac{d(NO_2^-)}{dx} = k_1(NH_4^+) - k_2(NO_2^-) \tag{4}
\]

where \( k_2 = k_2/Ek_0 \).

From these equations, the concentration of \( NH_4^+ \) at any distance in the soil column is given by

\[
(NH_4^+) = (NH_4^+)_0 e^{-k_1x} \tag{5}
\]
The concentration of nitrite is given by

\[
(NO_2^-) = \frac{K_1(NH_4^+)_o}{K_2 - K_1} \left[ e^{-K_1X} - e^{-K_2X} \right]
\]  \hspace{1cm} (6)

and

\[
(NO_3^-) = (NH_4^+)_o - [(NH_4^+) + (NO_2^-)]
\]  \hspace{1cm} (7)

Incidentally equation (5) can be applied to the reperfusion situation by setting \( X = L \), the total length of the soil column. If the solution is recycled once the concentration becomes

\[
(NH_4^+) = (NH_4^+)_0 e^{-\frac{K_1}{L}} = (NH_4^+)_0 e^{-2K_1L}
\]

and for \( n \) cycles

\[
(NH_4^+) = (NH_4^+)_0 e^{-nK_1L} = (NH_4^+)_0 e^{-\alpha t}
\]

Here \( \alpha = rK_1L \), and \( r \) is the rate of cycling, \( n \) per day.

In order to plot equations 5-7, some numbers have been arbitrarily chosen from the literature for illustration. From Knowles et al., the growth rate constant for Nitrosomonas can be interpolated to be 1.43/day at \( 25^\circ \) and that of Nitrobacter as 1.65/day, and from experiments of Erh et al., reasonable values are \( k_o = 3.85 \text{ cm/day} \) and \( E = 3.9 \) for a column 10 cm in diameter. \( (NH_4^+)_o \) is chosen as 100 ppm nitrogen. Further, we arbitrarily take \( k_1 \) and \( k_2 \) as numerically equal to the growth rate constants simply for purposes of illustration of nitrification curves.

The relationships among these constants will be discussed below.
Values of nitrate, nitrite and ammonia as functions of distance of travel in this hypothetical column of soil are plotted in Fig. 1.

In the general case, additional features must be taken into account. In a resting soil there will be an initial numerical population of nitrifiers, $n_1$ and $n_2$, which begin to multiply following entry of ammonium. The constants $k_1$ and $k_2$ are actually $k'_1(C_1)$ and $k'_2(C_2)$ respectively, where $C_1$ and $C_2$ are enzyme systems involved in the oxidations. These concentrations, $(C_1)$ and $(C_2)$, may be taken as proportional to the biomasses, $m_1$ and $m_2$, of *Nitrosomonas* sp. and *Nitrobacter* sp. respectively. Further, these biomasses are related to substrates by the Monod equations (7), namely

$$- \frac{d(NH_4^+)}{dt} = Ad(m_1)/dt$$

and

$$- \frac{d(NO_2^-)}{dt} = Bd(m_2)/dt$$

where $A$ and $B$ are proportionality constants. It is assumed, of course, that $n_1$ is directly proportional to $m_1$ and $n_2$ to $m_2$. For the growth of *Nitrosomonas* sp. we write, in the usual way (9)

$$m_1 = m_{o1} e^{\gamma_1 t}$$

and for *Nitrobacter* sp.

$$m_2 = m_{o2} e^{\gamma_2 t}$$

as first approximations for the initial increases in biomasses. $\gamma = 0.693/\tau$, where $\tau$ is the mean generation time and is
FIGURE 1

EFFLUENT at L = 40 cm
COMPOSITION of

X = LENGTH of COLUMN, cm

(NH4+) NO2 - NO3
(NH4)0 k0 k1 k2 k3

NH4+ NO3 - NO2

NITROGEN (ppm)
FIGURE 1. Continuous flow of nitrogen in a soil column.

\( k_0 \) is the rate of inflow of ammonium, and \( k_1 \) and \( k_2 \) are the specific reaction rates for the chemical oxidations shown at the top of figure. For a steady state of flow \( k_0 \) is also the exit flow rate. \( \Sigma N_1 \) is the sum of all forms of nitrogen in the effluent and equals \( (NH_4^+)_o \) in the steady state, whereas the population is at a maximum, and nitrogen is no longer being retained by the biomass. The horizontal dashed lines give the compositions indicated at soil column lengths of \( L = 20 \text{ cm} \) and \( L = 40 \text{ cm} \). The nitrite concentration is computed with equation (6).

Note that the near coincidence of concentrations of \( NO_2^- \), \( NO_3^- \) and \( NH_4^+ \) at \( X = 11.6 \text{ cm} \) is a coincidence for the values of \( \gamma_1 \) and \( \gamma_2 \) chosen and is not general. Also, the reaction is assumed to be at about neutrality, whereas \( NH_4^+ \gg NH_3 \).
characteristic of the species ($\gamma$). An important feature of $\gamma$, however, is that it depends upon the concentration according to a second Monod equation, namely

$$\gamma_1 = \frac{\gamma_{\infty} (NH_4^+)}{K_m + (NH_4^+)}$$

for Nitrosomonas

and

$$\gamma_2 = \frac{\gamma_{\infty} (NO_2^-)}{K_m + (NO_2^-)}$$

for Nitrobacter.

$\gamma_{\infty}$ is the maximum growth rate constant at "infinite" (high) substrate concentration, provided the nitrogen source is the limiting nutrient ($\gamma$), and $K_m$ is the corresponding Michaelis-Menten concentration (the concentration at which the ratio $\gamma/\gamma_{\infty} = 0.5$), characteristic of the organism. The constants $K_m$ are generally very low compared to $(NH_4^+)_o$ (1) and therefore $\gamma \approx \gamma_{\infty}$.

These relationships give, for the rate of disappearance of ammonia, from equation (1)

$$- \frac{d(NH_4^+)}{dt} = k'_1 \beta_1 m_0 \gamma_1^t (NH_4^+) = K'_1 e^t (NH_4^+)$$

(12)

where

$$K'_1 = k'_1 \beta_1 m_0 \quad \text{and} \quad \beta_1 = c_1/m_1.$$
The situation is further complicated by the fact that the population does not increase indefinitely, but rises to a maximum value; at least the nitrification data from perfusion experiments seems to indicate this ( ). Consequently, we need growth equations which will encompass this feature in place of equations (10) and (11).

Let \( \frac{dn}{dt} = \gamma n(1 - n/n_{\text{max}}) \)  \hspace{1cm} (13)

where \( n_{\text{max}} \) is proportional to the fraction of surface covered with nitrifier at time \( t \) and \( n_{\text{max}} \) is the total number when the soil "surface" is saturated with nitrifying organism. Integration of equation (13) for each species leads to

\[
n_1 = \frac{n_{o_1} n_{\text{max}_1}}{n_{o_1} + n_{\text{max}_1} e^{-\gamma_1 t}} \hspace{1cm} (14)
\]

and

\[
n_2 = \frac{n_{o_2} n_{\text{max}_2}}{n_{o_2} + n_{\text{max}_2} e^{-\gamma_2 t}} \hspace{1cm} (15)
\]

if \( n_o \ll n_{\text{max}} \). Note that for long times \( n = n_{\text{max}} \) as needed.

For the non-steady state model the rate of disappearance of ammonium now becomes

\[- \frac{d(NH_4^+)}{dt} = k_1 \beta_1 m_1 (NH_4^+) \hspace{1cm} (16)\]

provided that we ignore the small amount of nitrogen fixed as microbial mass [a correct assumption ( )]. At some fixed time we may write

\[
\frac{d(NH_4^+)}{(NH_4^+)} = - \frac{K_1 dX}{[1 + (\frac{m_{\text{max}_1}}{m_1}) e^{-\gamma_1 t}]} \hspace{1cm} (17)
\]
Note that when \( m_{\text{max}} \) is reached, equation (17) becomes equation (2).

Upon partial integration, for any given time, we obtain ammonium concentrations as a function of distance \( X \) in the column, i.e.,

\[
(NH_4^+)_T = (NH_4^+)_o e \left( - \exp \frac{K_1 X}{1 + \left( m_{\text{max}} / m_o \right) e^{-\gamma_1 t}} \right)
\]

This can only be a rough approximation unless the flow rate is fast compared to the growth rate. Otherwise the amount of catalyst will vary as the ammonium passes any plane at \( X \). A plot of equation (18) at any time, and for relatively rapid flow, will simply be a curve similar to the ammonium curve of Fig. 1, but with a more shallow slope.

In general, as the ammonium front moves forward, for a total travel time \( T = \frac{X_x}{f} \), organisms behind the front will be stimulated to grow, and at any distance \( X_t \), the time since the solution reached that distance is \( T - t = \frac{(X_T - X_t)}{f} \).

Substitution of \( X_T = L \) for the length of the column and \( (L - X)/f \) for the time that *Nitrosomonas* has had to grow from the commencement of flow of ammonia into the column into equation (17) and integrating, we obtain as a general solution of the following equation,

\[
\frac{-d(NH_4^+)}{dX} = \frac{k_1 \beta_1 (NH_4^+)^{m_{\text{max}}}}{E_k \left( 1 + M e^{-7_1 (X_T - X)/f} \right)}
\]
namely

\[
(NH_4^+) = (NH_4^+_o) \left[ \frac{x \gamma_1}{e^x} \frac{(1 + M')}{(1 + M'_e)^2} \right] \gamma_1
\]

where \( M' = \frac{M - x}{e^x} \) and \( M = \frac{m_{max}}{m_{o1}} \).

Again, simply for purpose of illustration, we let \( \gamma_1 = k_1 \), so that \( k_1 e / \gamma_1 \) is unity. (This will generally not be the case, but the important result of equation (19) is not altered by this simplification.) Equation (19) thereby reduces to

\[
(NH_4^+) = (NH_4^+_o) \left[ \frac{-\gamma_1(L - x)}{e^x + M} \right] \gamma_1(L - x)
\]

for the variation of ammonium concentration within the column, and for the concentration at the exit where \( X \) is \( L \). The equation is plotted in Fig. 2 with \( M = 10 \) and \( L = 20 \) and 40 cm. It is important to note that as \( L \) becomes large in comparison with \( X \), for example \( L = 100 \) cm, equation (19a) becomes almost identical with equation (5), both of which are plotted as the lower curve in Fig. 2. (Here \( \gamma_1 = k_1 \ ).

Note also that for \( X = 0 \), \( (NH_4^+) = (NH_4^+_o) \), for \( X = L \),

\[
(NH_4^+) = (NH_4^+_o)(1 + M)/(e^x + M)
\]

and for \( X = L = infinity \),

\( (NH_4^+) \) is zero. Further, in general from equation (13)
\[ M = \frac{m_{\text{max}} - 1}{m_c} \] and it follows that for \( M = 1 \), \( K_1 = k'_1 \beta (1 + 1) m_0 \).

In Fig. 2, the upper curve is a plot of \( (\text{NH}_4^+) = (\text{NH}_4^+)_o e^{-\frac{K_1 X}{10}} \), showing the course of oxidation of ammonium if the population of Nitrosomonas is not allowed to multiply during perfusion, for example by means of radiation or by the presence of a growth inhibitor deliberately added to the ammonium solution. Here \( M = 0 \) and \( K_1 = k'_1 \beta_1 m_0 / f = k'_1 \beta_1 m_{\text{max}} / 10 f \).

The equation describing the path of the fluid flow front may be had by setting \( L = X \) in equation (19) or (19a). For the special case of \( k_1 = \gamma_1 \), equation (19a) becomes

\[
(NH_4^+) = \frac{(NH_4^+)_o}{\left[ e^{(7/2) L} + M \right]} \quad \text{(19b)}
\]

It is drawn as the broken line in Fig. 2 for \( M = 10 \) as before.

Equation (19b) is further instructive: if \( f \) is very large compared with \( L \), there is no reduction of \( (\text{NH}_4^+) \) with distance and if \( f \) is very small, at any value of \( L \) the concentration of \( (\text{NH}_4^+) \) falls toward zero.

Equations for the concentrations of \( \text{NO}_2^- \) and \( \text{NO}_3^- \) as functions of \( X \) can in principle be found by the procedure used for the first model. That is to say, for the rate of change of nitrite concentration we write

\[
\frac{d(\text{NO}_2^-)}{dt} = k'_1 \beta_1 m (\text{NH}_4^+) - k'_2 \beta_2 m_0 (\text{NO}_2^-) .
\]
where \( m_2 \) is the biomass of Nitrobacter species, and with the aid of equation (19a)

\[
d(NO_2^-) \Delta X + \frac{K_2(NO_2^-)}{1 + M_2' e^{\gamma_2/f \cdot X}} = \left[ \frac{K_1(NH_4^+)}{1 + M_1'} \right] e^{-\gamma_1/f \cdot X}
\]

(20)

Here \( M_2' = M_2 e^{-(\gamma_2/f)L} \) and \( K_2 = K_2' \beta_{2,\text{max}} / E'k_0 \).

Even by letting \( K_2'f/\gamma_2 \approx 1 \) by analogy with the transformation of equations (19) to (19a), equation (20a) requires a solution for which there is no standard indefinite integral, namely

\[
(NO_2^-) = \left[ \frac{e^{(\gamma_2/f)X}}{1 + M_2' e^{(\gamma_2/f)L}} \right] ^{-K_2'f/\gamma_2} \left[ \frac{K_1(NH_4^+)}{1 + M_1'} \right] \int \frac{dX}{M_2' e^{(b-a)X} + e^{-ax}}
\]

(20a)

Here \( b = \gamma_2/f \) and \( a = (\gamma_2 - \gamma_1)/f \).

Fortunately, for purposes of illustration, and without loss of the picture qualitatively, under certain growth conditions \( \gamma_2 \neq \gamma_1 \) (6).

Our integrated equation is then simply

\[
(NO_2^-) = \left[ \frac{K_1(NH_4^+)}{1 + M_1'} \right] \left[ e^{-(\gamma_2/f)X / M_2'} - M_2' \right] X + f/\gamma \log \left( \frac{\gamma_2/f}{1 + M_2' e^{(\gamma_2/f)L}} \right)
\]

(20b)
For convenience in plotting we make the not unreasonable assumption
that \( M_1 = M_2 \) and, with the values of \( \gamma \) and \( M \) already chosen the
concentration of nitrite leaving a column of length \( L = 20 \text{ cm} \) as
a function of time (or of multiples of column length \( L \)) are plotted
in Fig. 3 with the aid of equation (20b).

For the limiting case when the column exhibits maximum growth
equation (20b) becomes (20c) by setting \( L = \infty \),

\[
(\text{NO}_2^-) = K(\text{NH}_4^+) o x e^{-\left(\frac{L}{L}\right)X}
\]

and this equation is plotted as the lower curve in Fig. 2. Again
the concentration of \((\text{NO}_3^-) = (\text{NH}_4^+) o - [(\text{NH}_4^+) + \text{NO}_2^-] \), Fig. 3.

DISCUSSION

The usefulness of these models will depend on how well they
predict the kinds of data reported in the literature. For the
models sufficient oxygen and auxiliary nutrients (\( \text{CO}_2 \), cations and
anions) are assumed to be present so as not to be rate limiting.
In the literature concentrations are measured in the effluent
whereas Figures 1 and 2 give the distribution of metabolites within
a column as well. Sometimes data has been reported as concentrations
as functions of total time of flow through a column (e.g., Macura
et al.) and sometimes as multiples of pore volumes (Erh, et al.).
FIGURE 2. Variation of ammonium concentration in a column as a function of column length and total time of flow (expressed as multiples of length of column \( L = 20 \text{ cm} = f \cdot t \)). Upper and lower (5L) curves are plots of equation (17) with \( K_f/10 \) (no growth of organisms) and \( \gamma_1 \), respectively. Curves marked L, 2L, and 5L are plots of equation (17a) and the dashed curve gives the composition of the solution front by equation (17b), which falls below the uppermost curve owing to microbial growth. Here \( M = 10 \) and \( \gamma/f = 0.005 \). The curve labeled \( \text{NO}_2^- (L = \infty) \) gives the variation of nitrite concentration with distance of the long flow times with \( \gamma_1 = \gamma_2 \) (equation 20c).
FIGURE 3. Composition of effluent at \( L = 20 \text{ cm} \) with the position of the front at times \( T = L/f \) in multiples of \( L \). The curve for \( \text{NO}_2^- \) is a plot of equation (20b) and the curve for ammonia is for values at \( L = 20 \), Fig. 2. Solid lines are for perfusion through unconditioned columns. Horizontal dotted line gives concentration of ammonium effluent from a preconditioned column (from Fig. 1).

Note that with \( K_2 \gg K_1 \), there is almost no free nitrite in the effluent.
Preconditioned Soil Column

Influence of the constants. By equation (6) if $K_2$ is much greater than $K_1$, $(K_2 - K_1)$, the concentration of nitrite is never very large compared to $(NH_4^+)$ and quickly vanishes with distance, $X$. Further, $(NO_2^-)$ is generally much less than $(NO_3^-)$ except at the beginning of the vector reaction. On the other hand, with $K_2 < K_1$ the concentration of $(NO_2^-)$ can be much larger than $(NO_3^-)$ at small values of $X$ and greater than $(NH_4^+)$ at larger values of $X$. The maximum value of $(NO_2^-)$ is given by

$$\frac{K_2}{K_1 - K_2}$$

and the position of this maximum in the column can be found with the aid of equations (6) and (20). By inspection of Fig. 1 it can be seen that the relative concentrations of the three forms of nitrogen, $NH_4^+$, $NO_2^-$ and $NO_3^-$ depend on the length of the column for a steady rate of flow. Since the population of nitrifiers is fixed and maximal, the concentrations in the solution leaving the column will appear to be constant at some value $L$, i.e., the column may be said to be in a steady state. These remarks might pertain to a column with the following properties.

a. The column is inert, say of quartz beads or resin beads. There is no ion exchange with the metabolites. A column of soil is more complicated; it shows an elution of ions with time as can be expected as $NH_4^+$ replaces other cations and nitrous and nitric...
ions replace soil anions. Strictly speaking such replacements will prevent a true steady state from ever developing in so far as metabolism depends on microenvironmental conditions of surface pH, soil structure, and redox potential, all of which will evolve as the system tends toward a steady state.

b. Heterotrophic organisms are absent. Heterotrophs can grow on nitrogen waste products and other catabolic products produced by the nitrifiers, and these will contribute to the factors just mentioned.

Distributions of nitrifiers in the column will overlap depending on the relative rate constants \( K_1 \) and \( K_2 \). If \( K_2 \gg K_1 \) most Nitrosomonas species will be near the front of the column, whereas if \( K_2 \approx K_1 \) populations of both nitrifiers will nearly coincide.

c. A source of insoluble carbonate is present to buffer the system. Otherwise a reduction in pH along the column carrier can prevent the organisms from metabolizing.

Inspection of equation (5), for example, [or equation (7) which mirrors equation (5) at large values of \( X \) (compare Fig. 1)]

\[
\left( \frac{\text{NH}_4^+}{\text{NH}_4^+} \right) / \left( \frac{\text{NH}_4^+}{\text{NH}_4^+} \right)_0 = e^{-(k_1 X/l)}
\]

shows that if the flow rate is increased the distance at which the same amount of oxidation is observed will also increase. Provided \( L \) is great enough, however, the oxidation will be almost complete regardless of the initial concentration of ammonia or of the flow
rate. In other words, the input concentration $(NH^+)_o$ and the output concentration $(NO_3^-)_L$ will be equal and independent of $f$ if the column is long enough, as found by Erh et al. for a twofold change in flow rate, i.e., by equations (5) and (7)

$$(NO_3^-)_X = (NH^+)_o(1 - e^{-KL}).$$

**Influence of increasing initial ammonium concentration.** For a given value of flow rate and length of column, an increase in $(NH^+)_o$ will lead to a proportional increase in $(NH^+)_o$ at L. Under the conditions described for this situation by Ehr et al., no nitrite was observed in the effluent and consequently, by equation (7) and (5) an increase in $(NH^+)_o$ will increase $(NO_3^-)_L$ proportionally by

$$(NO_3^-)_L = (NH^+)_o(1 - e^{-KL}).$$

Of course, as one increases the value $(NH^+)_o$ one pour volume of effluent would need to pass the column before the increased concentration of $(NO_3^-)_L$ would appear constant, in order to allow for the time required for the front of increased total nitrogen content to reach L. [This neglects any diffusional broadening of concentrations at the front (3).] Any increase in this time would suggest that the maximum possible population of nitrifiers had not been present prior to the increase in $(NH^+)_o$. Prior to the increase the population may have been more or less steady, in keeping with the nutrient level available ([l]). Any increase in populations would increase $K_1$ and $K_2$. 
**Virgin Soil Columns**

The composition of the solution leaving the column depends again on flow rates and rate constants. The concentration of ammonia leaving a column of length L, based on curves in Fig. 2 is plotted in Fig. 3 as a function of time and of multiples of L, which amounts to the same thing for either parameters. \((L = ft)\) is a measurement of the time elapsing since perfusion began. The lower dotted line in Fig. 3 represents the concentration of ammonium leaving a preconditioned column. It may be nearly zero if the time or length \(L\) is great enough.

In order to calculate the concentration of nitrate (or of nitrite) leaving the column we have proceeded to find the concentration of nitrite as a function of \(X\), with aid of the calculus along the lines by which equations (6) and (7) were obtained. We have limited the present discussion to the instance that \(\gamma_2 = \gamma_1\), \(M_2 = M_1\) and \(K_2 = K_1 = \gamma/\ell\). This limited picture is nevertheless not without value as the general features of nitrification are encompassed by it. A comparison of Figures 1 and 2 shows that the concentration of nitrite within a column is only slightly higher at any distance, if \(\gamma_2 = \gamma_1\), instead of differing by \(\gamma_1 = 1.43/\text{day}\) and \(\gamma_2 = 1.65/\text{day}\) as chosen for the preconditioned soil. Comparison of Figures 2 and 3 reveals that the preconditioned "soil" and the "soil" perfused for a time equivalent to several multiples of \(L\) are essentially the same provided the "soil" behaves somewhat like glass beads.
On the other hand the two soils will differ drastically if the virgin soil is perfused for a time during which ions can be exchanged and eluted from the column. In the preincubated soil the population can multiply and nitrogen can be metabolized without loss of such ions and we have no way of knowing a priori how much the two soils would actually differ; the models are severely limited from the point of view of handling all the chemical variables in a real soil. In spite of this short coming, the first model seems to be comprehensive enough to match the general trend of results reported in the literature for recyclical perfusion (/2). The more recent results of Macura and Kunc are encompassed by the second model as may be seen in Fig. 4 wherein some of the data of Fig. 3 is plotted as cumulative yields of nitrogen with the aid of equations

\[ \text{NH}_4^+ = \int (\text{NH}_4^+) dL = \int (\text{NH}_4^+) \frac{A}{n} dT \]

and

\[ \text{NO}_2^- = \int (\text{NO}_2^-) dL = \int (\text{NO}_2^-) \frac{A}{n} dT \]

where A is the cross sectional area required to convert n multiples of L into volumes of effluent. From equation (20c) we note that at large values of \( X \), i.e., more soil in the column in terms of Macura's results, the concentration of nitrite falls toward zero and the cumulative curves for nitrite would level off with time of flow. Ehr et al. describe a number of features of nitrification which point up the insufficiencies likely to be encountered in any
FIGURE 4. Cumulative yields of nitrogen from a column of \( L = 20 \) cm. Data of Fig. 3.
model as an analog of a real soil, for a true steady state system can never be achieved. Even though a maximum population of nitrifiers may be achieved for a short time, elution of soil ions by the products of the nitrification reaction will result in changes in microenvironments that inevitably will tend to new quasi-steady states of indefinite duration.
LITERATURE CITED


C. ESTIMATION OF SURFACE pH

Harter and Ahlrichs (2) have published a new method for estimating pH at the surface of clays and found that the surface of bentonite seems to be about 100 times more acid than ambient solution at a pH of about 7.0. Their rational has been mildly criticized by Mortland (5) and rebutted by the authors (1). Although I have nothing to add to Mortland's specifics, I wish to call attention to two other procedures in addition to those mentioned by Harter and Ahlrichs.

1. Surface pH and electrophoretic mobility, μ.

According to the Hartley-Roe equation, surface pH, pH_s, is related to bulk pH for a suspension of particles by

\[ \text{pH}_s - \text{pH}_b = 0.217 \mu = \Delta \text{pH} \]

for large particles and \( \Delta \text{pH} = 0.325 \mu \) for small particles. Strictly speaking this pertains to the plane of shear. For kaolinite \( \Delta \text{pH} \) is about 1 in sodium phosphate-borate buffer at an ionic strength of 0.05 and \( \text{pH}_b = 8.05 \), as measured by G.V.F. Seaman [in (3)].

2. An enzyme as a molecular pH meter (3).

The action of chymotrypsin on denatured lysozyme as a substrate, in solution and adsorbed on bentonite and kaolinite reveals differences in pH optimum for activity of ca. 2 pH unites (3). For free lysozyme \( \Delta \text{pH} = -1.3 \) and for lysozyme on kaolinite \( \Delta \text{pH} = 0.54 \) for the complex, both being calculated from electrophoretic measurements. Thus the effective pH at the surface of lysozyme molecules in solution is 9.4 and that on kaolinite in suspension is somewhere between 7.05 and 7.51 in the same

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1 Submitted for publication.
solvent, pH\textsubscript{b} = 8.05. Alternately, subtracting 1.3 units from 2 units for the shift in enzyme optima leaves a Δ pH of about 0.7 for both bentonite and kaolinite (4).

Although these acidity differences between surface and solution are smaller than those reported by Harter and Ahlrichs, Mortland is of course correct in stating that a whole range of acidities are possible with clays, depending on water content, ionic strength, and the particular electrolytes present. This could account for the smaller values for Δ pH found by us.

References

II. EXPERIMENTAL

A. PERSISTENCE OF ENZYMATIC ACTIVITIES IN STORED AND GEOLOGICALLY PRESERVED SOILS

The considerable volume of literature about enzymatic activities in soils has recently been reviewed (1,2). Answers to basic questions concerning the origin, localization and persistence of soil enzymes are still wanting. Several difficulties have been recognized, including inherent differences in natural soil samples, changes in activities during handling, and need for better methods (1,3). Almost all prior studies on soil enzymes have been with fresh or freshly air-dried soils. This report, on the other hand, describes some results obtained with about 60 years old, stored arid area soils, and with geologically preserved, 3,715 to 32,000 years old, permafrost peat and soil samples wherein the numbers of live organisms have diminished considerably.

Materials and Methods

Soils. The characteristics of soils are shown in Table I. Aiken, Columbia, Dublin, Yolo, Fresno alkali, and the saline Ravenswood Point, Jarvis Landing and Dumbarton Bridge soil samples have been stored air-dry at room temperature for the periods indicated. The Staten, Oxford Tract, and Strawberry Creek soil samples were examined fresh. Soils in the Hilgard Collection (located in Hilgard Hall, University of California, Berkeley) were collected in California during the first

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<table>
<thead>
<tr>
<th>Soil</th>
<th>Description</th>
<th>pH</th>
<th>Organic C</th>
<th>Microorganisms millions/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aiken</td>
<td>California, clay, B horizon, stored 6 yrs.</td>
<td>5.8</td>
<td>0.18</td>
<td>0.20</td>
</tr>
<tr>
<td>Columbia</td>
<td>California, fine sandy loam, stored 6 yrs.</td>
<td>6.8</td>
<td>1.03</td>
<td>0.48</td>
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<td>Dublin</td>
<td>Contra Costa County, California, adobe clay loam, A horizon, stored 12 yrs.</td>
<td>5.7</td>
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<td>2.2</td>
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<td>Yolo</td>
<td>California, silt loam, stored 6 yrs.</td>
<td>7.3</td>
<td>0.67</td>
<td>1.4</td>
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<tr>
<td>Staten</td>
<td>Lodi, California, peaty muck, fresh.</td>
<td>7.4</td>
<td>2.36</td>
<td>18</td>
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<tr>
<td>Strawberry Creek</td>
<td>Berkeley, California, sandy loam, fresh</td>
<td>6.7</td>
<td>1.39</td>
<td>30</td>
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<tr>
<td>Oxford Tract</td>
<td>Berkeley, California, loam, fresh</td>
<td>5.8</td>
<td>1.80</td>
<td>40</td>
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<td>Fresno, alkali</td>
<td>Eastern Fresno County, California, fine sandy loam, top 15 cm, salinity 2.6%, mostly carbonates and bicarbonates, stored 8 yrs.</td>
<td>9.4</td>
<td>0.38</td>
<td>1.3</td>
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<tr>
<td>Ravenswood Point, saline</td>
<td>San Mateo County, California, salt evaporator levee surface soil, salinity 3%, mostly Na⁺, Cl⁻, SO₄²⁻, stored 1 yr.</td>
<td>6.6</td>
<td>1.2</td>
<td>0.95</td>
</tr>
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<td>Jarvis Landing, saline</td>
<td>Alameda County, California, salt evaporator road levee surface soil, salinity 10%, mostly Na⁺, Cl⁻, SO₄²⁻, stored 1 yr.</td>
<td>7.8</td>
<td>0.3</td>
<td>0.30</td>
</tr>
<tr>
<td>Soil</td>
<td>Description</td>
<td>pH</td>
<td>Organic C</td>
<td>Microorganisms millions/g</td>
</tr>
<tr>
<td>----------------------------------</td>
<td>-----------------------------------------------------</td>
<td>-----</td>
<td>-----------</td>
<td>----------------------------</td>
</tr>
<tr>
<td>Dumbarton Bridge, saline</td>
<td>Alameda County, California, salt evaporator levee surface soil, salinity &gt; 10%, mostly Na+, Cl-, SO₄²⁻, stored 1 yr.</td>
<td>6.7</td>
<td>0.8</td>
<td>0.19</td>
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<tr>
<td>Hilgard Collection No. 1</td>
<td>Coachella Valley, loam, top 30 cm.</td>
<td>7.0</td>
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<td>0.0</td>
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<tr>
<td>Hilgard Collection No. 2</td>
<td>Same as No. 1, 60-90 cm deep</td>
<td>8.0</td>
<td>traces</td>
<td>0.039</td>
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<td>8.1</td>
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<td>0.038</td>
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<td>Hilgard Collection No. 4</td>
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<td>8.3</td>
<td>0.19</td>
<td>0.011</td>
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<td>Hilgard Collection No. 5</td>
<td>Bishop-Owens River Valley, sandy, top 30 cm.</td>
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<td>Hilgard Collection No. 6</td>
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<td>8.3</td>
<td>0.11</td>
<td>0.030</td>
</tr>
<tr>
<td>Hilgard Collection No. 7</td>
<td>Bakersfield-Kern River Delta, loam, top 30 cm.</td>
<td>6.8</td>
<td>1.49</td>
<td>5.0</td>
</tr>
<tr>
<td>Hilgard Collection No. 8</td>
<td>Same as No. 7, 60-90 cm deep</td>
<td>7.2</td>
<td>0.27</td>
<td>1.8</td>
</tr>
<tr>
<td>Hilgard Collection No. 9</td>
<td>Tulare Experiment Station, sandy, alkaline, top 30 cm.</td>
<td>9.9</td>
<td>0.50</td>
<td>0.39</td>
</tr>
<tr>
<td>Hilgard Collection No. 10</td>
<td>Same as No. 9, 60-90 cm deep</td>
<td>8.6</td>
<td>0.50</td>
<td>0.16</td>
</tr>
<tr>
<td>Hilgard Collection No. 11</td>
<td>San Bernardino-Victoria Tract, sandy loam, top 30 cm.</td>
<td>7.0</td>
<td>0.48</td>
<td>0.36</td>
</tr>
<tr>
<td>Hilgard Collection No. 12</td>
<td>Same as No. 11, 60-90 cm deep</td>
<td>7.8</td>
<td>0.23</td>
<td>0.16</td>
</tr>
<tr>
<td>Soil</td>
<td>Description</td>
<td>pH</td>
<td>Organic C %</td>
<td>Microorganisms millions/g</td>
</tr>
<tr>
<td>-----------------------</td>
<td>-----------------------------------------------------------------------------</td>
<td>-----</td>
<td>-------------</td>
<td>----------------------------</td>
</tr>
<tr>
<td>Pt. Barrow, No. I-1182</td>
<td>Alaska, peat, 8715 ± 250 yrs. old, 45 cm deep in permafrost</td>
<td>6.2</td>
<td>19.06</td>
<td>0.0022</td>
</tr>
<tr>
<td>Pt. Barrow, No. 4</td>
<td>Alaska, loam, overlaying No. I-1182, 5-30 cm deep, subject to freeze-thaw cycle.</td>
<td>4.6</td>
<td>1.27</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Pt. Barrow, No. 714</td>
<td>Alaska, humic sandy silt, 5.50 m deep in permafrost, approx. 32,000 yrs. old.</td>
<td>7.2</td>
<td>3.33</td>
<td>0.0014</td>
</tr>
<tr>
<td>Pt. Barrow, No. I-700</td>
<td>Alaska, peat, 1.40 m deep in permafrost, 9550 ± 240 yrs. old</td>
<td>6.3</td>
<td>24.0</td>
<td>34</td>
</tr>
<tr>
<td>Annavah</td>
<td>Iraq, desert, silt loam, stored 1 yr.</td>
<td>7.4</td>
<td>1.13</td>
<td>7.6</td>
</tr>
<tr>
<td>Twairaj</td>
<td>Iraq, desert, silt loam, stored 1 yr.</td>
<td>7.8</td>
<td>0.96</td>
<td>7.8</td>
</tr>
<tr>
<td>Lahaina</td>
<td>Island of Oahu, latosol clay, top 25 cm.</td>
<td>5.7</td>
<td>2.5</td>
<td>0.92</td>
</tr>
<tr>
<td>Mahukona</td>
<td>Island of Hawaii, latosol silty clay loam, top 15 cm.</td>
<td>5.4</td>
<td>2.7</td>
<td>0.34</td>
</tr>
<tr>
<td>Molokai</td>
<td>Island of Oahu, latosol clay, top 25 cm.</td>
<td>6.6</td>
<td>1.6</td>
<td>1.0</td>
</tr>
<tr>
<td>Wahisawa</td>
<td>Island of Oahu, latosol clay, top 25 cm.</td>
<td>4.7</td>
<td>2.1</td>
<td>0.081</td>
</tr>
<tr>
<td>Kawaihae</td>
<td>Island of Hawaii, Red Desert latosol loam, top 5 cm.</td>
<td>5.9</td>
<td>4.0</td>
<td>4.7</td>
</tr>
<tr>
<td>Nipe I</td>
<td>Puerto Rico, latosol clay, top 2.5 cm.</td>
<td>5.1</td>
<td>5.41</td>
<td>0.06</td>
</tr>
<tr>
<td>Nipe II</td>
<td>Same as Nipe I but 15-25 cm deep</td>
<td>5.2</td>
<td>5.35</td>
<td>0.27</td>
</tr>
</tbody>
</table>
decade of this century under the direction of Professors E.W. Hilgard and R.H. Loughridge and have been stored undisturbed since their collection. The selected soils No. 1 to No. 12 are non-cultivated desert and arid area samples.

The Point Barrow, Alaska samples were collected in 1964, air-dried and examined a year later; their radiocarbon age has been established by the U.S. Army Cold Regions Research and Engineering Laboratory, Hanover, New Hampshire.

The Hawaiian latosol samples were obtained through the courtesy of Dr. O.R. Younge from the Department of Agronomy and Soil Science, University of Hawaii, in 1964. The Puerto Rico latosol Nipe clay was obtained through the courtesy of Dr. R.A. Luse, Agricultural and Biosciences Division, Puerto Rico Nuclear Center.

The organic carbon content is the difference between the total CO₂ obtained by an improved dry combustion method (6) and the amount of carbonate-CO₂ as determined by Williams' method (7).

The pH values of the soil samples were measured in a 1:1 water suspension.

The determination of phosphatase activity followed a published procedure (8). To a one-gram sample of soil was added 2 ml of 0.005 M (i.e., 10 μm) Na-β-naphthyl phosphate and 3 ml of water. The mixture was incubated without shaking for 6 hours at 25°C. Following incubation the solution was filtered; 1 ml of the filtrate was added to 2 ml of 0.2 N NaOH in a 100 ml volumetric flask and diluted to the mark. The concentration of β-naphtol was determined by fluorescence in a Brice-Phoenix photometer. The excitation wavelength was 360-370 μm and the emission wavelength was measured at the 425 μm peak.
Dehydrogenase activity was determined following the method of Kozlov (9); Thunberg tubes for anaerobic incubation were utilized. To a 1 g sample of soil was added 5 ml of a 0.3% water solution of 2,3,5-triphenyltetrazolium chloride and glucose. The reaction mixture was placed in Thunberg tubes, filled with nitrogen gas, and incubated for the desired length of time at 25°C without shaking. After incubation the resulting triphenyl formazan was dissolved in 10 ml methanol, filtered, and determined photometrically. Estimates were corrected for controls; the procedure included washing soils with methanol and a measure of the contribution of any naturally occurring colored compounds.

Catalase activity was determined following the method of Johnson and Temple (10). To a 1 g sample of soil was added 5 ml of 0.3% H₂O₂ and 5 ml of H₂O. After incubation at room temperature for the desired length of time, 5 ml of 3 N H₂SO₄ was added to stop further enzyme activity. To determine the amount of the residual H₂O₂, the slurry was filtered and a 5 ml aliquot was titrated with 0.1 N KMnO₄ to the equivalence point.

Esterase activity was determined according to the method of Haig (11) with phenyl acetate as the substrate. The substrate solution was prepared by adding 1 gram of phenyl acetate to 10 ml of 95% ethyl alcohol. This was mixed with 250 ml of 0.2 M phosphate buffer, pH 7.2, and the volume was made up to one liter with water. To a one-gram sample of soil was added 3 ml of the 7.35 x 10⁻³ M phenyl acetate solution and the mixture was incubated at room temperature for periods up to one hour. After incubation, 1 ml of the slurry was added to a 50 ml
volumetric flask. Five ml of the Folin-Ciocalteu reagent and 15 ml of 20% NaCO₃ were added and the solution was diluted to volume with water, 30-35°. After titration the concentration of free phenol was determined photometrically.

Urease activity \( ^{14} \text{O}_2 \) analysis. To a 1 g soil sample in a planchet was added 10 mg \( ^{14} \text{urea} \) (Calbiochem, Los Angeles) containing 10 μc \( ^{14} \text{C} \), and 0.5 ml 5% acetate, pH 5.5, 0.05 M. The planchet was placed in a radioactive gas counting chamber and the increase of \( ^{14} \text{O}_2 \) in the chamber was monitored with a Geiger-Mueller gas-flow tube connected to a decade scaler and to a count ratemeter. Integrated amounts of \( ^{14} \text{O}_2 \) in the chamber were strip-chart recorded. Results were expressed as the rate of increase of counts per minute (Δ cpm/m) during the first 100 minutes; 1 μc of \( ^{14} \text{O}_2 \) in the chamber gave a count of 12,250 cpm.

The enzyme activity determinations described above are improved procedures of methods described previously in our project reports.

For the determination of urease activity in soils at various atmospheric humidities, the soil samples (1 g) were equilibrated at the desired humidity in a closed container. Upon equilibration 10 mg (10 μc) of dry \( ^{14} \text{urea} \) was added to the soil, mixed and placed in the radioactive gas detection chamber. The atmosphere in the chamber was equilibrated at the desired humidity before introducing the soil-urea mixture, and maintained at the desired level by placing in the chamber a container with appropriately diluted sulfuric acid according to the International Critical Tables.
Urease activity in a synthetic soil. A crystalline urease preparation UR (Worthington Biochemical Co.), suspended in water, was adsorbed on a synthetic soil (50% kaolin, NF, acid washed + 50% celite, analytical grade), dried in vacuum at room temperature and treated with C\textsuperscript{14}-urea similarly to natural soil and examined as described above.

C\textsuperscript{14}-labelled urea was obtained from Calbiochem, Los Angeles and Volk Radiochemical Company, Burbank, California. The C\textsuperscript{14}-urea was mixed with purified urea to obtain a stock containing 1 mc/g.

Urease. Precrystalline urease (Code: URC), Worthington Biochemical Corporation, Freehold, New Jersey, was used.

The NF grade urease was obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio. It was determined that the preparation had 3 Worthington units per mg.

Enumeration of microorganisms. The numbers of microorganisms (bacteria and streptomycetes) in all but the Hilgard Collection soils were determined by dilution plating on yeast extract - soil extract agar. The medium consisted of: glucose, 1.0 g; K\textsubscript{2}HPO\textsubscript{4}, 0.5 g; KNO\textsubscript{3}, 0.1 g; soil extract, 100 ml; yeast extract, 1.0 g; Bacto-agar, 15 g. The medium was made to one liter with tap water and autoclaved. Soil extract solution was made from 1 kg of fresh garden soil suspended in 1 liter of water, autoclaved for 30 minutes at 15 psi and after addition of CaCO\textsubscript{3} filtered with bacteriological filters. Plate counts of all colonies were made after 4-day incubation at room temperature.

Hilgard collection samples were plated on trypticase soy agar (Baltimore Biological Laboratory, Inc.) (cf. 3rd Progr. Rep., January 14, 1966, NASA-CR-70058).

The sensitivities of enzyme assays, showing the lowest amounts of products detectable per gram of soil by the methods described are given in Table II.
TABLE II

Sensitivity of Assays

<table>
<thead>
<tr>
<th>Activity</th>
<th>Product</th>
<th>Minimum amount μm per g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catalase</td>
<td>H₂O₂</td>
<td>0.1</td>
</tr>
<tr>
<td>Dehydrogenase</td>
<td>triphenyl formazan</td>
<td>0.001</td>
</tr>
<tr>
<td>Esterase</td>
<td>phenol</td>
<td>0.005</td>
</tr>
<tr>
<td>Phosphatase</td>
<td>β-naphthol</td>
<td>0.02</td>
</tr>
<tr>
<td>Urease</td>
<td>CO₂</td>
<td>0.008</td>
</tr>
</tbody>
</table>

Results

Results of the determination of phosphatase, urease, dehydrogenase, catalase, and esterase activities in soils are shown in Table III and Figures 1, 2, and 3.

Phosphatase (hydrolysis of Na-β-napthyl phosphate) was detected in measurable amounts in all soils; only trace amounts appeared in Point Barrow soil No. 714 and in several soils from the Hilgard Collection.

The problems involving detection of phosphatase in soils have been critically evaluated (3). As with other enzymes in soil, pretreatment of soil (including moisture regime), incubation temperature, stirring of the samples during incubation, and other manipulative variations influence the values of activities detected. Our samples were not stirred on a rotating wheel during the incubation and, consequently, gave lower values for these soils (Table IV).
<table>
<thead>
<tr>
<th>Soil</th>
<th>Phosphatase β-naphthol</th>
<th>Urease CO₂</th>
<th>Denhydrogenase H⁺</th>
<th>Catalase H₂O₂ decomposed</th>
<th>Esterase phenol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µm/g/hr.</td>
<td>µm/g/hr.</td>
<td>µm/kg/hr.</td>
<td>µm/g/min.</td>
<td>µm/g/hr.</td>
</tr>
<tr>
<td>Aiken</td>
<td>0.23</td>
<td>0.013</td>
<td>0.0014</td>
<td>1.5</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Columbia</td>
<td>0.23</td>
<td>0.027</td>
<td>0.0016</td>
<td>8.5</td>
<td>0.15</td>
</tr>
<tr>
<td>Dublin</td>
<td>1.35</td>
<td>1.156</td>
<td>0.0027</td>
<td>15.0</td>
<td>2.25</td>
</tr>
<tr>
<td>Yolo</td>
<td>0.20</td>
<td>0.234</td>
<td>0.0031</td>
<td>14.0</td>
<td>0.45</td>
</tr>
<tr>
<td>Staten</td>
<td>0.26</td>
<td>0.495</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxford Tract</td>
<td>0.67</td>
<td>0.553</td>
<td>0.0040</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Strawberry Creek</td>
<td>0.21</td>
<td>0.293</td>
<td>0.0055</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Annanah</td>
<td>0.14</td>
<td>0.082</td>
<td>0.0021</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Twairaj</td>
<td>0.01</td>
<td>0.082</td>
<td>0.0028</td>
<td></td>
<td></td>
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<tr>
<td>Pt. Barrow I-1182</td>
<td>0.84</td>
<td>0.084</td>
<td>0.0013</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pt. Barrow 4</td>
<td>0.27</td>
<td>0.023</td>
<td>none</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pt. Barrow 714</td>
<td>traces</td>
<td>none</td>
<td>0.0008</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pt. Barrow I-700</td>
<td>1.20</td>
<td>0.793</td>
<td>traces</td>
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<td></td>
</tr>
<tr>
<td>Hilgard 1</td>
<td>0.11</td>
<td>0.005</td>
<td>none</td>
<td>traces</td>
<td>traces</td>
</tr>
<tr>
<td>Hilgard 2</td>
<td>0.02</td>
<td>none</td>
<td>none</td>
<td>traces</td>
<td>traces</td>
</tr>
<tr>
<td>Hilgard 3</td>
<td>0.02</td>
<td>0.025</td>
<td>none</td>
<td>traces</td>
<td>traces</td>
</tr>
<tr>
<td>Hilgard 4</td>
<td>traces</td>
<td>none</td>
<td>none</td>
<td>traces</td>
<td>traces</td>
</tr>
<tr>
<td>Hilgard 5</td>
<td>0.12</td>
<td>0.063</td>
<td>traces</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hilgard 6</td>
<td>0.07</td>
<td>0.017</td>
<td>traces</td>
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<td></td>
</tr>
<tr>
<td>Hilgard 7</td>
<td>0.22</td>
<td>0.174</td>
<td>0.0010</td>
<td>traces</td>
<td></td>
</tr>
<tr>
<td>Hilgard 8</td>
<td>0.13</td>
<td>0.038</td>
<td>traces</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hilgard 9</td>
<td>0.13</td>
<td>traces</td>
<td>0.0012</td>
<td>traces</td>
<td></td>
</tr>
<tr>
<td>Hilgard 10</td>
<td>0.04</td>
<td>traces</td>
<td>traces</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hilgard 11</td>
<td>0.20</td>
<td>0.049</td>
<td>traces</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hilgard 12</td>
<td>0.09</td>
<td>traces</td>
<td>traces</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fresno alkali</td>
<td></td>
<td>0.065</td>
<td>none</td>
<td>traces</td>
<td></td>
</tr>
<tr>
<td>Ravenswood Pt.</td>
<td></td>
<td>0.025</td>
<td>none</td>
<td>traces</td>
<td></td>
</tr>
<tr>
<td>Jarvis Landing</td>
<td></td>
<td>none</td>
<td>none</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dumbarton Bridge</td>
<td></td>
<td>none</td>
<td>none</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lahaina</td>
<td></td>
<td>0.308</td>
<td>none</td>
<td>traces</td>
<td>traces</td>
</tr>
<tr>
<td>Mahukona</td>
<td></td>
<td>0.083</td>
<td>traces</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Molokai</td>
<td></td>
<td>0.040</td>
<td>traces</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wahiawa</td>
<td></td>
<td>0.154</td>
<td>traces</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kawaihae</td>
<td></td>
<td>0.905</td>
<td>traces</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nipe I</td>
<td></td>
<td>0.144</td>
<td>traces</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nipe II</td>
<td></td>
<td>0.113</td>
<td>traces</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 1.

Enzymatic and Biological Activity in Air-dried Agricultural Soils in Comparison with the Organic Carbon Content.

- Relative Enzymatic Activity, µm Product/gm/hr.
- Organic Carbon Content, %
- Number of Microorganisms

MICROORGANISMS

ORGANIC CARBON

UREASE

PHOSPHATASE

AIR-DRIED SOILS

DUBLIN
COLUMBIA
YOLO
AIKEN
Enzymatic and Biological Activity in Geologically Preserved Permafrost Soils (I-700: 9550 ± 240 years old; I-1182: 8715 ± 250 years old; 4: loam, overlaying I-1182; 714 - approx. 32,000 years old) in Comparison with the Organic Carbon Content.
<table>
<thead>
<tr>
<th>Soil Location</th>
<th>No.</th>
<th>Depth, cm</th>
<th>Number of Microorganisms, Log</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bakersfield-Kern River Delta</td>
<td>7</td>
<td>0-30</td>
<td></td>
</tr>
<tr>
<td>San Bernardino</td>
<td>11</td>
<td>0-30</td>
<td></td>
</tr>
<tr>
<td>Victoria Tract</td>
<td>12</td>
<td>60-90</td>
<td></td>
</tr>
<tr>
<td>Bishop</td>
<td>5</td>
<td>0-30</td>
<td></td>
</tr>
<tr>
<td>Owens River Valley</td>
<td>6</td>
<td>60-90</td>
<td></td>
</tr>
<tr>
<td>Coachella Valley</td>
<td>1</td>
<td>0-30</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>60-90</td>
<td></td>
</tr>
<tr>
<td>Victorville</td>
<td>3</td>
<td>0-30</td>
<td></td>
</tr>
<tr>
<td>Mohave River Mesa</td>
<td>4</td>
<td>60-90</td>
<td></td>
</tr>
<tr>
<td>Tulare Experiment, Alkali</td>
<td>9</td>
<td>0-30</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>60-90</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 3.**

Relative Enzymatic Activity \( \mu \text{m product/gm/hr.} \)

Organic Carbon Content, %

Enzymatic and Biological Activity in Approx. 60 Years Old Stored Arid Area Soils in Comparison with the Organic Carbon Content.
Dehydrogenase. A measurable dehydrogenase activity was found in reasonably fresh soils and soils with high microbial counts, e.g., Pt. Barrow 1-700. In the other geologically preserved and in stored Hilgard Collection samples the dehydrogenase activity was small and often negligible. Although a nonbiological reduction of triphenyl tetrazolium chloride occurs in samples at temperatures higher than 65°C, the formazan released at the temperatures used in assays, 30 to 37°C, is due to biological activity only (12).

Catalase and esterase activities, determined in 4 six-years old stored soils were found to be considerable (Table III).

Urease activity was present in most of the soils. It was not detected in some stored desert soils from the Hilgard Collection and in the 32,000 years old Pt. Barrow soil No. 714.

It seems possible that urease in such stored and air-dried soils exists in an oxidized and, therefore, inactive state. Extensive pretreatment of several old soils with H₂S or cysteine, however, did not increase the urease activity.
At atmospheric humidities between 100% and 80% the reaction rate retained its zero order character for more than 8 hours (Figure 4).

The reaction rate in Dublin soil at 100% atmospheric humidity was 68% of that at the water holding capacity.

The enzymatic behaviour of added crystalline urease on a synthetic model soil is shown in Figure 5. With increasing amounts of urease added the apparent activity rate more than doubled: 0.1 µg of added urease released 0.075 µm CO\textsubscript{2} per gram of synthetic soil per hour from 10 mg C\textsubscript{14}-urea present, at the water holding capacity of the soil, whereas 1 µg urease released 1.61 µm CO\textsubscript{2} at the same conditions.

During a period of 20 months four of the Hawaiian soils lost urease activity between 3% and 28% and the activity in Kawaihae soil increased by 8.5%; the Puerto Rico Nipe clay samples lost their urease activity by more than 50% (Table V). All soils were stored air-dry at room temperature during the 26 months between the tests.

**TABLE V**

<table>
<thead>
<tr>
<th>Soil</th>
<th>Urease Activity, CO\textsubscript{2} µm/g/hr.</th>
<th>Percent change in 26 months</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Examined July, 1965</td>
<td>Examined Sept., 1967</td>
</tr>
<tr>
<td>Lahaina</td>
<td>0.325</td>
<td>0.308</td>
</tr>
<tr>
<td>Mahukona</td>
<td>0.116</td>
<td>0.083</td>
</tr>
<tr>
<td>Molokai</td>
<td>0.051</td>
<td>0.040</td>
</tr>
<tr>
<td>Wahiawa</td>
<td>0.159</td>
<td>0.154</td>
</tr>
<tr>
<td>Kawaihae</td>
<td>0.834</td>
<td>0.905</td>
</tr>
<tr>
<td>Nipe I</td>
<td>0.292</td>
<td>0.144</td>
</tr>
<tr>
<td>Nipe II</td>
<td>0.312</td>
<td>0.113</td>
</tr>
</tbody>
</table>
FIGURE 4.
UREASE ACTIVITY IN SOIL AT LOW ATMOSPHERIC HUMIDITIES
The activities of native enzymes in soil and of purified enzymes in solution in vitro are by no means comparable with respect to the amounts of enzymes present because of the differences in physical and chemical environment. Nevertheless, some semiquantitative values regarding the enzymatic content in soils may be estimated. For example, the enzymatic activity values for one gram of Columbia soil (Table III) are equivalent to the activity of 140 μg potato acid phosphatase (Pentex, Inc.), 0.033 μg urease (Worthington, UR), 0.0009 μg dehydrogenase (Worthington, ADHS, estimated), and 0.34 μg catalase (Worthington, CTR, estimated). The amount of protein based on the bacterial biomass in a fertile soil is about 100 μg per gram.

Discussion

Phosphatase activity in soils has been studied by many investigators (1); the published reports are abundant in contradictory observations and interpretations. Most of the observations show that the maximal activity occurs near a neutral pH value and not necessarily at the natural pH of the soils. In some soils the activity may increase with increasing pH. It has been noted that phosphatase activity is greater in soils containing larger amounts of organic matter. This trend is also evident in some soil samples examined here (Figures 1 and 2). It appears that phosphatase activities in the stored Hilgard soils reflect the number of microorganisms, rather than the amount of organic matter present (Figure 3). Of course, generalization is difficult as the organic matter rich soils as a rule also have significantly higher numbers of microorganisms.
Perhaps direct correlation cannot be expected. It has been shown that phosphatase activity in soil is inversely proportional to the biologically available phosphate: adding inorganic phosphate usually diminishes it and even in soils rich in organic matter it is associated with phosphate availability (13).

Our data (Figures 1 and 2) suggest that phosphatase activity may not be directly correlated with microbial numbers in some soils. The data are consistent with previously reported findings (3) indicating that most of the soil phosphatase is extracellularly bound to the soil organic matter. It is of interest to note, for example, that there was no phosphatase (and no urease) activity in the Pt. Barrow soil No. 714, although a large number of microorganisms was recovered. It is likely that the soil was contaminated while in transit from the source to the laboratory, as, unfortunately, there were no specific aseptic procedures attempted during the handling of soil samples by the collecting agency. The results indicate that the large number of microorganisms present in this soil did not produce measurable phosphatase (or urease) activity. The activities were lost during the 32,000 year long burial time in permafrost.

Most investigators have shown (1) that phosphatase accumulates as a result of microbial activities in soil, but that the enzymatic activity does not represent solely the immediate microbial activity.

Dehydrogenases. The measurement of dehydrogenase activity in soil has been used to obtain correlative information on the biological activities of microbial populations in soil. Measurable activity may be obtained without any additions of metabolites, and the results in
such cases reflect endogeneous respiration (12). Experimental results have shown repeatedly that the dehydrogenase activity in soil reflects the metabolic rate rather than the microbial numbers (12)(14). Usually the activity does not correlate with plate counts in nonamended soils, but upon the addition of nutrients it increases with increasing microbial numbers. Generally, cultivated soils exhibit high dehydrogenase activity, whereas in saline and high pH soils it is considerably lower (15).

A measurable dehydrogenase activity was shown to exist in the reasonably fresh soils examined (Table III). There was very little or no dehydrogenase activity in the stored Hilgard Collection soils and in the old Pt. Barrow soils with the exception of No. I-700; the latter reflects the high microbial activity in this sample. No other correlation between dehydrogenase and other factors (organic carbon content, pH, other enzymatic activities) was evident.

Although Stevenson (14) has reported high rates of dehydrogenase activity in soils, apparently his data include effects of microbial proliferation resulting from prolonged incubation periods. Our results show that incubation of a soil sample with water overnight increases the microbial count by a factor of three and incubation with triphenyl tetrazolium chloride by a factor of eight. This increase is accomplished without addition of any organic matter and under limited oxygen availability.

Urease. Notwithstanding the presently available methods and apparatus used in the soil respiration studies, there exists a need for a precise and sensitive method for measuring small amounts of CO$_2$ released from soil during short experimental periods. Often $^{14}$C-labeled compounds (instead of "cold" organics) may be added to soil and the release of carbon dioxide may be determined by monitoring the appearance of $^{14}$CO$_2$. 
For this purpose a self-contained radioactive gas detection chamber has been developed in this laboratory (cf., previous reports). Specifically, the chamber was designed for the studies of urease activity in soils: $^{14}$C-urea $\rightarrow$ $^{14}$CO$_2$ + ammonia, but it may be adaptable for other types of investigations where $^{14}$C-compounds may be used, e.g.:

- $^{14}$C-glucose $\rightarrow$ $^{14}$CO$_2$ + products
- $^{14}$C-humus $\rightarrow$ $^{14}$CO$_2$ + products
- $^{14}$C-plant residues $\xrightarrow{\text{waterlogged}}$ $^{14}$CH$_4$ + products

By using the above described apparatus for the examination of urea hydrolysis in soils we have obtained results which show that there exists a linear $^{14}$CO$_2$ development rate in all soils for at least 3 hours after which time a sigmoidal increase of rate indicates microbial proliferation. The urease activity varies from non-detectable levels in saline soils (Jarvis Landing, Dumbarton Bridge) and some stored deeper horizon desert soils (Hilgard 2, and Hilgard 4) to low levels in organic matter poor agricultural soils (Aiken, Columbia) and some lateritic soils (Molokai) to very high levels in organic matter rich lateritic type (Kawaihae), or adobe clay (Dublin).

The maximal activity of urease in most soils is found at pH 6.5 to 7.0. It has been reported that in alkaline soils the activity decreases considerably and it is especially low in carbonate-rich soils, apparently because of the detrimental effect of Ca$^{++}$ on the urease-producing organisms (16). We found no activity in the alkali Hilgard Collection samples No. 9 and 10, but the alkali (pH 9.4) carbonate-rich Fresno soil showed a reasonable level of activity. On the other hand there was no
activity in highly saline (sodium sulfate and chloride, 10% or more) but otherwise neutral (pH 6.7, Dumbarton Bridge) or only slightly alkaline (pH 7.8, Jarvis Landing) soils. There was a measurable activity in the pH 6.6, 3% saline Ravenswood Point Soil.

Published reports (1) show that urease activity in soils usually correlates with the number of microbes in soil, but its increase with increasing organic-matter content also has been noted; normally soils with higher organic matter content also have higher numbers of microorganisms. Much of urease activity is present in the rhizosphere and considerable seasonal variations have been observed (1). In soil fractionation studies the highest urease activity remained associated with the clay (below 2 μ diameter) fraction (17). It is evident that urease exists in a free state in the soil and it is the only enzyme to date which has been extracted from soil and characterized (18).

A correlation of organic-matter content and microbial numbers in the diverse soils examined here is not evident (Table I) and our data indicate that the urease activity may be better correlated with the organic carbon content than with the number of organisms (Figures 1, 2, and 3). There was no urease activity in the Pt. Barrow soil No. 714, although a large number of microorganisms was recovered. It is likely that the organisms proliferated in this soil after its collection and the exposure to the ambient atmosphere. The results indicate that the large number of microorganisms present in this soil did not produce measurable urease activity which was lost during the 32,000 year long burial time in permafrost. On the other hand, reinvasion of an autoclaved and urea-amended Dublin soil by microorganisms was clearly
reflected in the increase of urease activity in this soil (Figure 6). It is evident that the addition of urea to the soil favored the growth of ureolytic organisms.

A measurable urease activity was evident in an organic-matter poor, 8,715 years old, buried permafrost silt, and a considerable activity was found also in a 9,550 years old buried permafrost peat.

In the 60 years old Hilgard Soil Collection samples urease activity was higher in the surface (0-30 cm) than in the deeper (60-90 cm) layer (Figure 3). These data probably reflect the ratio of the respective activities at the time of collection. It is evident that even in a very little differentiated soil, as in recently collected Nipe I and Nipe II, the urease activity is lower in a deeper layer as compared to the surface. The characteristics of phosphatase activity in the Alaska and Hilgard Collection soils was rather similar to the urease activity, whereas the dehydrogenase activity reflected the metabolic activities of the microorganisms present.

Incidentally, the surviving organisms recovered in these soils belong mostly to the genus *Bacillus*; besides the sporeformers, a *Pseudomonas* sp. and several "soil diphtheroids" have been recovered (19).

An attempt was made to estimate the amount of urease in the tested soils. For this purpose very small amounts (0.1 to 1 µg per gram of adsorbent) of crystalline urease were adsorbed on a "synthetic soil", e.g., kaolinite-celite mixture. The relative urease activity, however, did not follow linearly to the amount adsorbed on the kaolinite-celite mixture. The apparent urease activity increased with increasing amounts of urease adsorbed (Figure 5). The mechanism for such behaviour is not clear. It might be possible, for example, that the strongly adsorbing
sites are covered first and that such an action has certain denaturing effects, or that an inhibitor present in the system might be selectively adsorbed by the enzyme.

Urease activity in soils below 100% relative humidity reflected the same characteristics as pure urease in the same conditions (20), i.e., the activity response followed the water vapor adsorption isotherm by urease, except that the rates had a lower magnitude (Figure 7). These results indicate that considerable amounts of urea may be hydrolized in "air dry" field soils.
FIGURE 7.

RELATIVE UREASE ACTIVITY IN SOIL AND IN PURE STATE

COMPARED WITH WATER VAPOR ADSORPTION ISOTHERMS OF UREA AND UREASE
References


(5) J. Brown, Arctic, 18, 36 (1965).


B. ADSORPTION AND REACTIONS OF CHITINASE AND LYSOZYME ON CHITIN.

The currently reported phase of the investigation of chitinase and lysozyme activity in adsorbed state on chitin is a continuation of the previously described project under the same title (19,25,26,27) on enzyme kinetics in structurally restricted systems.

It is known that the addition of Ca\(^{++}\) during the process of purification shows preserving and stabilizing effect on some enzyme activities. For example, use of Ca\(^{++}\) in elastase extraction (30) and the markedly increased pH and thermal stabilities of proteolytic enzymes in presence of Ca\(^{++}\). Ca\(^{++}\) ions have a role in determining the electro-kinetic potential of the colloidal substrate particles (2) and thus increasing the enzymatic reaction rate (16). We have examined the effect of Ca\(^{++}\) on the chitin-chitinase system.

Adsorption of proteins on Ca-phosphate is responding to slight changes in pH and to buffer salt concentrations, and the adsorbed protein may be released easily and gradually by changing the conditions in the column (26).

Ca-phosphate gel and hydroxylapatite has been used in many separations of enzymes, including the separation of *Aspergillus niger* chitinases (20). We have explored the use of Ca-phosphate gel for further purification of streptomycete 2B chitinase. Similarly, celite, polyacrylamide gels, CM-cellulose and hydroxylapatite gel were further evaluated for column chromatographic purification. Also, the conditions for sorption of chitinase, lysozyme and ribonuclease on chitin and the effect of Mg\(^{++}\), Co\(^{++}\) and Zn\(^{++}\) ions on chitinase activity were investigated further.
Materials and Methods

Most of the materials and methods have been described in the previous reports (19,25,26,27).

Dispersed chitin. Prepared as described (19).

Chitinase (19) was obtained from the streptomycete strain 2B. The DEAE-cellulose purified preparations were fractionated through Sephadex G-50 (coarse) column and the aliquots containing the first eluted peak of chitinase activity were collected and used for adsorption and activity experiments.


Ribonuclease - Cryst., salt free, Lot R 541 and cryst. from EtOH, Lot R 568-9 from Worthington Biochem. Corp., Freehold, N.J.

Absorptivity (extinction coefficient). Following values were used:

- Chitinase \( A_{280}^{mg/ml} = 1.10 \) (19)
- Lysozyme \( A_{280}^{mg/ml} = 2.64 \) (9,18)
- Ribonuclease \( A_{280}^{mg/ml} = 0.734 \) (23)

Chitinase and lysozyme activities were based on the amount of the released N-acetylglucosamine, as determined by the DMAB method (19,24).

Proteinase activity was determined by using a modification (12,31) of Kunitz's method (15).

Reagents:

1. 0.6% casein solution, pH 7.2: isoelectric casein (Difco Laboratories) was dissolved in slightly alkaline water. KOH was added to pH 6.4 and the solution was adjusted to pH 7.2 with 1.0 M \( \text{H}_3\text{PO}_4 \) and filtered.
2. TCA solution (12): 0.11 M CCl₃COOH, 0.22 M CH₃COONa, 0.33 M CH₃COOH.

Procedure:
Mix 5.0 ml of casein solution with 1.0 ml of sample. Upon incubation for 10 min. on Ferris wheel at 30°, 5.0 ml of TCA solution was added and similarly incubated for 30 min. The resulting precipitate was removed by filtration and the absorbptivity of the supernatant was measured at 275 ml.

Microcel, a synthetic Ca silicate (Johns Manville Co.).
Particle size < 0.1 μ, surface area 175-200 m²/g.

Celite - diatomaceous silica product (Johns Manville Co.).

DEAE cellulose, CM cellulose, Polyacrylamide gels (Bio Gel P-150 and Bio Gel P-300), Hydroxylapatite gel (Bio Gel HT) were obtained from Bio-Rad Laboratories, Richmond, California. According to the manufacturer, Bio Gel HT was prepared by the method of Keilin and Hartree (Proc. Roy. Soc. London, Ser. B, 124, 397 [1938]).

Electrophoresis apparatus - Research Specialities Co., model no. 1400.
Total distance along paper strip between the levels of buffer was 48 cm.
For each run two 5 x 17 cm oxoid cellulose acetate strips were used with two 11 cm wide Whatman No. 1 filter paper wicks. Distance between wicks: 15.0 cm. Voltages applied: 400 to 600 v.

Barbital (veronal) buffer was prepared according to Cramer and Tiselius (6): 1.84 g diethylbarbituric acid and 10.30 g sodium barbital were dissolved in water to make 1 liter. The ionic strength of this buffer was 0.05. The pH of the buffer was adjusted to pH 8.6 with NaOH.

Tris-EDTA-borate buffer (1): 60.5 g Tris, 5.0 g EDTA, and 1.6 g borate acid were dissolved in 1 liter water. The pH of the buffer was 8.9 and conductivity 3.0 mmhos.
Sodium phosphate buffer, 0.033 M, was used in electrophoresis at various pH values.

Ponceau S stain, 0.2% in 3% aqueous TCA, used with 200 μg or more protein applied to the electrophoresis strip.

Chitinase - purified streptomycete 2B chitinase, lyophilized and dissolved in distilled water was used in all electrophoresis experiments.

A sample of purified streptomycete 2B chitinase was extensively dialysed in cold against distilled water and divided in two parts. To one part diluted CaCl$_2$ solution was added, calculated to add 0.0204 mg CaCl$_2$ per 1.0 mg of enzyme protein. Both chitinase solutions were lyophilized and kept at 2°C.

The following procedure was used for elution of proteins from electrophoresis strips. The sample was applied across the strip leaving about 4 mm clear margins at edges. After the run a 8-10 mm wide marginal strip was cut off and stained. The protein bonds on the remaining strip were cut out according to the spots on the marginal strip and immersed in 3.0 ml, 0.05 M, Na acetate buffer, pH 7.0, in screw cap tubes and placed in a refrigerator overnight.

Electrophoresis. 20 to 200 μg of protein in 0.02 ml distilled water were applied on each strip. The average duration of each run was 1 hour 40 minutes.

Cooled buffer was used and a tray with crushed ice was placed inside the apparatus beneath the strip-supporting board and the apparatus was placed in a refrigerator at 2°C.
Results and Discussion

Effect of Mg++, Zn++ and Co++ ions on chitinase activity (Table VI).

Various concentrations of Mg, Zn and Co-chloride salts were added to reaction mixtures. Depending on the amount added they changed the pH and interfered with DMAB color reaction. As a check parallel series were run without the enzyme but with a known amount of N-acetylglucosamine added and the pH was checked and adjusted to 9.2 after adding borate buffer. All results were corrected for corresponding blanks. From the three cations tested, Co++ was the most inhibitory while the presence of Co++ and Zn++ in concentrations below 0.001 M had an activating effect. In concentrations up to 0.2 M the sequence, with respect to inhibition, was as follows: Mg++ > Co++ > Zn++.

Under the same conditions but without chitinase present Mg++ was not adsorbed in detectable amounts on chitin in concentrations from 0.0001 M to 1.0 M.

The adsorption of lysozyme, chitinase and ribonuclease on chitin.

Cherkasov et al. (7) reported that practically no lysozyme was adsorbed on chitin in distilled water, and likewise it was not adsorbed in 0.1 and 1.0 M urea solutions and in 0.1 and 0.5 M ethanol solutions.

Using distilled-water-dialyzed lysozyme and chitinase solutions, and similarly dialysed chitin suspension, the adsorption characteristics of both enzymes on chitin were compared and gave the following results. Adsorption time for chitinase was 1 min. at 0°, but for lysozyme 30 min. at 25°. Due to the high activity chitinase was not tested at 25°. The pH of the reaction mixtures was 7.0 in all cases. Instead of a buffer,
TABLE VI

Effect of $Mg^{++}$, $Zn^{++}$ and $Co^{++}$
on chitinase activity (added as chlorides)

<table>
<thead>
<tr>
<th>Molarity of the salt in 0.03 M Na-acetate buffer pH 5.5</th>
<th>Relative activity, in %, with added ions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$Mg^{++}$</td>
</tr>
<tr>
<td>none</td>
<td>100</td>
</tr>
<tr>
<td>0.0001</td>
<td>100</td>
</tr>
<tr>
<td>0.0002</td>
<td>*</td>
</tr>
<tr>
<td>0.0005</td>
<td>*</td>
</tr>
<tr>
<td>0.001</td>
<td>99</td>
</tr>
<tr>
<td>0.002</td>
<td>*</td>
</tr>
<tr>
<td>0.005</td>
<td>*</td>
</tr>
<tr>
<td>0.01</td>
<td>83</td>
</tr>
<tr>
<td>0.02</td>
<td>*</td>
</tr>
<tr>
<td>0.05</td>
<td>34</td>
</tr>
<tr>
<td>0.1</td>
<td>29</td>
</tr>
<tr>
<td>0.2</td>
<td>27</td>
</tr>
<tr>
<td>0.5</td>
<td>*</td>
</tr>
</tbody>
</table>

* - not determined.
solutions of NaCl in glass distilled water were used. In agreement with previously obtained results (25) the amount of chitinase adsorbed was the same in distilled water as in 0.00001 M to 0.2 M NaCl solutions, and, as expected, lysozyme in distilled water was not adsorbed. With 0.10 mg/ml lysozyme and 1.0 mg/ml chitin present an increasing quantity of lysozyme was adsorbed in solutions containing 0.0001 to 0.001 M NaCl. In 0.01 M NaCl solution the same amount was adsorbed as in 0.1 M and 0.2 M NaCl solutions (or the difference was within limits of a possible error, ± 5 μg/ml.

Previous work indicated (25) that the desorption of lysozyme from chitin showed a "hysteresis" pattern. Experiments with chitinase showed that the desorption of chitinase from chitin followed a similar "hysteresis" pattern (Figure 8).

McLaren (18) used ethylamine HCl to desorb proteins from clays. Lysozyme could be eluted from kaolinite without loss of activity. Our results showed that ethylamine desorbs lysozyme from chitin at pH 10. After washing the same chitin adsorbed new lysozyme in the same weight ratio.

All attempts to adsorb ribonuclease on chitin failed. Experiments were carried out at 25°. From 0.16 to 0.44 mg/ml of ribonuclease was incubated for 15-20 minutes with 0.25 to 1.0 mg/ml chitin. The tests were performed in 0.03 M Na-phosphate buffer and in universal buffer at pH range from 2 to 9. In another series 0.006 M solutions of Na-phosphate, Na-acetate and NaCl, and universal buffer at pH from 7 to 9 were used. There was no adsorption of ribonuclease on chitin in either case.

The sorptive characteristics of chitinase, lysozyme are summarized in Table VII.
FIGURE 8

DESORPTION of CHITINASE from CHITIN

EFFECT of ENZYME CONCENTRATION in MEDIUM

TEMPERATURE : 0°

P H PHOSPHATE–ACETATE BUFFER 0.03 M, pH 5.5

CHITIN : 10 mg per ml.
<table>
<thead>
<tr>
<th></th>
<th>Effect of pH</th>
<th>Effect of ion concentration</th>
<th>Saturation</th>
<th>Spontaneous desorption</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chitinase</td>
<td>No clear adsorption maximum; most adsorbed</td>
<td>No effect. The same amount adsorbs in dist. water and a wide range of ion concentrations.</td>
<td>Not reached</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>at pH 5 to 8.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lysozyme</td>
<td>Adsorption increases from pH 3 to 9:</td>
<td>No adsorption in dist. water. 0.0001 to 0.001 M NaCl - increasing 0.01 to 0.2 M NaCl - same amount adsorbed.</td>
<td>Reached</td>
<td>Present</td>
</tr>
<tr>
<td></td>
<td>maximum at pH 9.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ribonuclease</td>
<td>No Adsorption</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The adsorption of proteins on chitin has been studied by Hackman (11) who investigated effects of pH, ion concentration, and temperature on adsorption of water-soluble insect cuticular protein to chitin. Results indicated that adsorption was dependent on pH, the highest amount was adsorbed at the isoelectric point of the protein. Increase in ion concentration decreased adsorption, but changes in temperature had little effect. Tyrosine-rich protein fractions were preferentially adsorbed. Adsorption was partly irreversible and an increase to pH 9 was necessary before all the adsorbed protein was removed. It was concluded that there was only a weak bonding between chitin and water-soluble insect cuticular protein. In a further work Hackman (10) pointed out that tyrosine, as well as free α-amino groups appeared important in the binding of N-acetyl-D-glucosamine by insect cuticular proteins.

Adsorption of chitinase on celite and micro-cel.

Various clays, metallic oxides, silicates and other substances were tested by Berger (3) and Berger and Reynolds (5) in attempts to adsorb selectively either chitinase or chitobiase. Between pH 5 and pH 7 part of the protein was adsorbed on "magnesol" (a preparation of Mg oxide - Westvaco Corp.). The activity was partly eluted at pH 12 to 14, but the ratio of the activities remained the same. The enzyme system was irreversibly adsorbed to "florex", a purified clay, and reversibly adsorbed in Zn hydroxide gels.

Celite, a diatomaceous silica product is used as a filter-aid in the preparation of ammonium sulfate precipitated chitinase. Experiments at 25° and with an incubation time of 30 min. showed that chitinase was adsorbed on celite and that adsorption was strongly pH dependent.
adsorption was about 1/3 of maximum at pH 7 and the highest at pH 4. Reaction mixture contained 0.005 M Tris and 0.001 M Ca-acetate made to the desired pH by adding 0.01 M acetic acid (Figure 9). Chitinase could be desorbed from celite by pH or concentration gradient. However, celite columns did not separate streptomycete 2B chitinase in distinct peaks or in usable fractions of proteinase and chitinase activities. As eluants 1) 0.005 M Tris and 0.001 M Ca-acetate buffer, pH 7, 2) 0.005 M Na-acetate, 0.001 M Ca-acetate, 0.001 M Ca-acetate, pH 5 to 7, and 3) 0.005 M Tris, 0.001 M Ca-acetate, pH 8 to 9 were used.

Results of 2 experiments are shown in figures 10 and 11.

Micro-cel, an artificial Ca-silicate, is another filter aid. Chitinase adsorbs on it in large amounts and it was not possible to desorb it without a nearly complete loss of activity. Fractionated on a micro-cel column, chitinase showed some peaks of proteins and peaks of chitinase and proteinase activities, but a workable separation was not achieved. Proteinase activity was more stable than chitinase activity. Results are presented in figures 12 and 13.

Fractionation on CM-cellulose and hydroxylapatite columns.

Tsuru et al. (31) used CM-cellulose column and 0.001 M Tris-maleate, 0.002 Ca-acetate buffer at pH 6.4 in purification of B. subtilis protease.

It was hoped that proteinase activity from streptomycete chitinase may be adsorbed and retained in CM-cellulose column. However, the chitinase and proteinase activities of the streptomycete 2B chitinase preparation were not separated; 0.01 M Na-acetate, 0.001 M Ca-acetate, pH 6.4, was used as an eluant.
**FIGURE 9**

**ADSORPTION of STREPTOMYCETE CHITINASE on CELITE**

**DIATOMACELLS SILICA PRODUCT**

- **CELITE**: 10 mg./ml.
- **NO acetate, 0.005 M**
- **Ca acetate, 0.001 M**
- **pH 7.0**
- **TEMPERATURE**: 25°C
- **INCUBATION TIME**: 30 min.

---

**EFFECT of pH on ADSORPTION of STREPTOMYCETE CHITINASE on CELITE**

- **CELITE**: 4.0 mg./ml.
- **TRIS, 0.005 M** + **0.001 M AcOH**
- **pH**
- **TEMPERATURE**: 25°C
- **INCUBATION TIME**: 30 min.
- **CHITINASE**: 88 µg./ml.
FIGURE 10

FRACTIONATION of STREPTOMYCETE CHITINASE on CELITE®
COLUMN USING pH GRADIENT

CELITE COLUMN: 12 x 50 mm
FLOW RATE: 1.0 mL/min.
ELUANT: 0.005 M Na acetate
  0.001 M Ca acetate
  0.005 M TRIS
  0.001 M Ca acetate
  pH 5.7
  pH 8.9
  pH 9

LOAD: 3.0 mL, 1 mg, PURIFIED
CHITINASE (on DEAE CELLULOSE
at pH 8.9 and on SEPHADEX
at pH 7.0)

- A280 RELATIVE AMOUNT of PROTEIN
- A585 RELATIVE CHITINASE ACTIVITY
- A275 RELATIVE PROTEINASE ACTIVITY
- pH of ELUTE

A BUFFER of pH 4.0 was used to
prepare the COLUMN, and to
pH 4.0 ADJUSTED CHITINASE
was ADSORBED on it. ELUTION
FOLLOWED with pH 5.0 BUFFER
**Figure 11**

Fractionation of Streptomyces Chitinase on Celite Column at pH 7.0

Celite column: 12 x 50 mm.
Flow rate: 1.0 ml per min.
Eluent: 0.005 M Tris
0.001 M Ca acetate pH 7.0
Load: 3.0 ml, ~ 9.45 mg.
Purified on DEAE cellulose at pH 3.9 and on Sephadex G-50 fine column at pH 7.0 - Streptomyces Chitinase

- $A_{280}$ Relative amount of protein
- $A_{585}$ Relative chitinase activity
- $A_{275}$ Relative proteinase activity

![Graph showing the fractionation results](attachment:graph.png)
FIGURE 12

FRACTIONATION of STREPTOMYCES CHITINASE
on "MICRO-CEL" COLUMN at pH 8.9

ARTIFICIAL SILICATE COLUMN: 12 X 63 mm.
FLOW RATE: 1.0 ml/min.
ELUANT: 0.005 M TRIS
0.001 M CaCl₂
pH 8.9
+ 0.0 to 1.0 M NaCl
LOAD: 2.0 ml, 2.3 mg purified chitinase
(on DEAE CElLULOSE COLUMN at
pH 8.9 and on SEPHADEX G-50
fine COLUMN at pH 7.0)

A585
A275
A280

RELATIVE AMOUNT OF
PROTEIN

RELATIVE CHITINASE
ACTIVITY

RELATIVE PROTEINASE
ACTIVITY

MILLILITERS

0.2 0.4 0.6 1.0 M in NaCl

pH of BUFFER at START: 8.9
FIGURE 13

FRACTIONATION OF STREPTOMYCETE CHITINASE ON MICRO-CEL COLUMN OF pH 7.0

ARTIFICIAL Ca SILICATE COLUMN: 12 x 52 mm
FLOW RATE: 1.0 ml./min.
ELUANT: 0.005 M TRIS
       0.001 M Ca acetate) pH 7.0
LOAD: 2.0 ml., 8.3 mg, purified chitinase
(on DEAE cellulose at pH 8.9 and on
SEPHADEX G 50 at pH 7.0)

- - - - - A280 (RELATIVE AMOUNT OF PROTEIN)
- - - - - A585 (RELATIVE CHITINASE ACTIVITY)
- - - - - A275 (RELATIVE PROTEINASE ACTIVITY)
Hydroxylapatite has been widely used in enzyme work. Sörbo (20) investigated the adsorption of proteins on Ca-phosphate. In general, maximum adsorption was observed at the isoelectric point and at a comparatively low ionic strength. When ionic strength was increased by addition of phosphate adsorption was diminished and a shift of the point of maximum adsorption to the acid side of isoelectric point occurred. On addition of NaCl the adsorption decreased.

Oyakura (20) separated chitobiase and chitinase activities in Aspergillus niger chitinase by using hydroxylapatite gel.

A separation of purified streptomycete 2B chitinase on hydroxylapatite column was successful: two activity peaks emerged. The first peak had about 2 times higher chitinase activity and about 5 times higher proteinase activity as compared with the second peak. Results are shown in Figure 14. The nature of both fractions are being investigated.

Electrophoretic properties of streptomycete 2B chitinase.

Streptomycetes 2B chitinase was separated on cellulose acetate strips in three separate bands, which are moving toward the cathode at pH 8.6 in veronal buffer (6) or at pH 6.9 in Tris-EDTA-boric acid buffer (1). Chitinase activity was extracted from the fastest moving band, from sample with Ca^{++} added. No activity was extracted from the parallel run strip, where the chitinase preparation did not contain calcium chloride.

In a Tris-EDTA-boric acid buffer, pH 5.5 (adjusted with HCl) the chitinase moved toward anode but did not separate. There was no movement in a 0.033 M Na phosphate buffer at pH 6.7-7.0 but a 3- to 4-fold widening of the spot was observed.
FIGURE 11
FRAGMENTATION OF STREPTOMYCESTE CHITINASE ON HYDROXYLAPATITE COLUMN AT pH 10 USING CONCENTRATION GRADIENT

G.L. GEL HT COLUMN: 12 x 150 mm.
ELUANT: NO PHOSPHATE BUFFER, 0.001 M TO 0 M
PH: 5.8 TO 7.2
FLOW RATE: 10 ml/min.
LOAD: 5.0 ml, ~ 9.4 mg, PURIFIED STREPTOMYCESTE CHITINASE (LUMIÈRE CULTURE CONCENTRATE AT pH 8.9 AND ON SEPHADEX G-20 FINE COLUMN AT pH 7.0)

- - - - - A280 RELATIVE AMOUNT OF PROTEIN
- - - A585 RELATIVE CHITINASE ACTIVITY
- - - - - A275 RELATIVE PROTEASE ACTIVITY

A280

0.001 0.005 0.05 0.1 0.2 M NO PHOSPHATE BUFFER
At more acid pH values of the Na-phosphate buffer the mobility of chitinase protein was reversed: it moved toward cathode again, with a considerable widening of spot but without any clear separation.

**Purification of chitinases.**

Chitinases are widely distributed in nature: bacteria, plants and animals, including mammals, are known to produce chitinases. Depending on the source the extracted chitinase actually is a chitinolytic enzyme system containing in most cases chitobiase, more than one chitinase and related enzymes. The homogeneity and purity of the obtained enzyme depend not only on the source of enzyme but also on every detail in purification procedures. Chitinase may also have an activity of another enzyme, for example, chitinase activity of lysozyme (4).

Takeda et al. reported (29) that two enzyme fractions obtained from *Helix pomatia* digestive juice were free of proteinase activity and did not show any contamination on electrophoresis. First fraction (M.W. 21,000) had lysozyme and chitinase activities. Second fraction (M.W. 24,000) had lysozyme activity with little or no chitinase activity.

Chitinase preparations from cockroaches, bean seeds, and the puff balls were acting on cell walls of *Micrococcus lysodeicticus*, behaving similarly to lysozymes (22).

It seems that *Streptomyces antibioticus* chitinase is the only one that has been highly purified in sufficiently large quantities to obtain detailed data on its physical and chemical properties (13). It has been shown to be homogeneous in the ultracentrifuge and in solubility tests in ethanol and ammonium sulphate, and the preparation was devoid of any trace of other enzymes (chitobiase, amylase, cellulase, proteolytic
enzymes). It separated, however, in three distinct fractions migrating to the cathode at pH 8.2 in a veronal-HCl buffer. Other data given for *St. antibioticus* chitinase: a single absorption band at 280 μm; 

\[ E_{280}^\text{mg/ml} = 1.24; \text{ Sedimentation constant: } 3.42 \text{ Svedberg units; M.W. } = 30,000, \]

Michaelis constant: 0.010 - 0.011 (g/100 ml).

*Streptomyces 2B* chitinase was always obtained from the same strain of microorganisms and by the same method of preparation. Therefore it is assumed that all lots of chitinase used in various experiments did have the same properties and characteristics. Our preparation of the *streptomyces 2B* chitinase is a heterogeneous mixture of at least three proteins as shown by electrophoresis; in this respect it behaves like *St. antibioticus* chitinase. Also the lack of a sharp pH adsorption peak on chitin may be caused by the same reason. Our *streptomyces 2B* chitinase possesses proteinase activity, which could presumably be separated by improved methods.

Most of the properties and characteristics of chitinases have been investigated by working with crude and with partially purified enzymes, and only in few cases highly purified chitinases have been used (13,20,24).

Ammonium sulphate precipitation of the crude enzyme should be done in cold. The sedimented protein should be dissolved in a cool buffer immediately as any exposure to air (oxygen) is harmful (8). Ammonium sulphate from chitinase solutions can be removed by dialysis in cold:

to the chitinase solution, pH 7, some drops of 0.01 M CaCl₂ or Ca-acetate are added and then dialyzed against 0.005 M Tris or Na-acetate and 0.001 M Ca acetate solution adjusted to pH 7.0. It is important to have Ca⁺⁺
in the chitinase solution as well as in the buffer solution outside the dialyzing tube to avoid loss of activity.

Saturation of streptomyces culture filtrate to 0.7 with ammonium sulfate at pH 5.0 did not precipitate chitobiase and precipitated only about 25% of chitinase. The same saturation at pH 7.0 yielded both chitinases in full quantity (5). To obtain pure Streptomyces antibiotic chitinase Jeuniaux (15) used an extensive ammonium sulphate precipitation cycle following adsorption and desorption from chitin. The final precipitate was obtained between 0.3 and 0.5 ammonium sulphate saturation at 0° and pH 5.2.

Skujins et al. (24) obtained a highly active enzyme, chitinase, from streptomyces culture filtrates at pH 5.5 and 0.9 saturation.

Lloyd et al. (17) saturated pervaporated culture filtrates to 0.7 at pH 7.0. Powning and Irzykiewicz (21) added a cold saturated ammonium sulphate solution, pH 4.5 to a raw chitinase extract of plant origin; 0.5 final saturation.

Otakara (20) investigated the effect of ammonium sulphate saturation with respect to amount of chitobiase and chitinase precipitated in Aspergillus niger culture filtrates (Table VIII).

TABLE VIII

<table>
<thead>
<tr>
<th>Ammonium sulfate saturation</th>
<th>Percent of total activity precipitated chitinase</th>
<th>chitobiase</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 - 0.3</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>0 - 0.4</td>
<td>53</td>
<td>20</td>
</tr>
<tr>
<td>0 - 0.5</td>
<td>86</td>
<td>60</td>
</tr>
<tr>
<td>0 - 0.6</td>
<td>90</td>
<td>95</td>
</tr>
<tr>
<td>0 - 0.7</td>
<td>92</td>
<td>96</td>
</tr>
<tr>
<td>0 - 0.8</td>
<td>92</td>
<td>97</td>
</tr>
</tbody>
</table>
At 0.7 saturation 8% of the chitinase activity was precipitated which was in agreement with the results of Berger and Reynolds (5).

The extended saturation range for precipitation as well as differences in saturation and pH for chitobiase and chitinase precipitation shows the heterogeneous nature of chitinases in raw material.

More chitinase activity was precipitated from streptomyces 2B culture filtrates at 0.7 saturation and pH 7.0 than at 0.85 saturation and pH 5.5. After purification on DEAE-cellulose and sephadex, there was no difference in activity between chitinases from both precipitates, expressed for a unit weight of protein.

Ethanol precipitation, as a simple and reliable one, was preferred by Lloyd et al. (17). Cold (-15°) ethanol was slowly added to chilled pervaporated streptomyces culture filtrate, adjusted to pH 3.5 to give a final concentration of 70% ethanol. The precipitate was immediately collected by centrifugation and redissolved in a minimum volume of 0.005 M phosphate buffer, pH 6.8, and dialyzed against a large volume of cold buffer.

The method worked well with streptomyces 2B culture filtrates. The activity of chitinase was not affected when all the work was done in the cold room with properly cooled (-18°) ethanol.

Ca-acetate precipitation. Addition of Ca-acetate as a powder or as a cold concentration solution to cooled culture filtrate (pH 7.65), to make 0.02 M - 0.03 M saturation caused a slight drop of pH and an appearance of a fluffy and threadlike precipitate. The dialyzed precipitate showed a very high proteinase activity and a presence of chitinase. However, the remaining Ca-acetate in culture filtrates
interfered with a subsequent ammonium sulphate precipitation and the possibilities of Ca-acetate precipitation to separate proteinase and chitinase activities was not investigated further.

Column chromatography and separation methods based on molecular sieving may be used as the next step in purification of chitinases.

Amberlite CG-50 ion exchange resin has been used as one of the final steps in obtaining 2 enzyme fractions from Helix pomatia digestive juice (29). Although free of proteinase activity, the first fraction showed lysozyme and chitinase activities and the second fraction - lysozyme activity with little or no chitinase activity. Both fractions showed only little contamination on electrophoresis and their molecular weights were 21,000 and 24,000 respectively.

DEAE-cellulose column and the use of 0.01 M Na-phosphate buffer at pH 8.4, separated the streptomycete chitinase of many proteins and pigmented substances (24). Dialed 50% ammonium sulphate fraction of been chitinase applied to a DEAE-cellulose column in 0.005 M citrate buffer at pH 6.0 was separated in two distinct fractions (21): zero to 1 M NaCl gradient yielded an acetylglucosaminidase fraction. Chitinase is only weakly adsorbed on DEAE-cellulose. Therefore the purification is based mostly on the retention of the other proteins in column. The procedure requires use of low ionic strength buffers and dilute chitinase solutions. Even small volumes of raw chitinase solutions (adjusted to pH 8.4 with 1 M H₃PO₄) show a pH drop to 8.2 after passing through the column, and some proteins and colored compounds are not retained in the column any longer. It was found that purification of streptomycete 2B chitinase on DEAE-cellulose at pH 8.9 was more effective than at pH 8.4;
Chitinase activity did not suffer and the colored compounds remained in the column. Chitinase, purified at pH 8.9 on DEAE-cellulose did not show the second activity peak when fractionated further on Sephadex G-50 (coarse) indicating that more homogeneous preparation may be obtained at pH 8.9 than at pH 6.4 although some chitinase may be retained in this column. Only dialyzed chitinase solutions are suited for use on DEAE-cellulose as the presence of ammonium sulfate rapidly exhausts the ion exchange capacity of column.

Polyacrylamide gel proved to be quite useful in the purification of chitinase: about 2/3 of proteinase activity with 1/2 of the protein can be separated from chitinase on Bio-gel P-150, with a loss of only 1/3 of chitinase activity (Figure 15). As an eluant 0.005 M Tris and 0.001 M Ca-acetate, pH 7.0 were used. Separation of proteinase and chitinase activities on Bio-gel P-300 was less effective. Bio-gel P-30 has been used as first step in separation of lytic enzymes in digestive juice of Helix pomatia (29).

Hydroxylapatite gel was used successfully by Otakara (20) in separating chitobiase and chitinase activities in Aspergillus niger chitinase; 0.0001 M to 0.2 M phosphate buffer, pH 6.8, was applied. Streptomyces 2B chitinase - after purification on DEAE-cellulose and on Sephadex - was separated in two activity peaks on the hydroxylapatite gel using 0.001 M to 0.2 M Na-phosphate buffer, pH 6.8 - 7.2. The first peak shows about two times higher chitinase activity and about 5 times higher proteinase activity than the second peak.

Electrophoresis is a very useful technique to investigate the homogeneity of chitinase.
FIGURE 15
FRACTIONATION OF STREPTOMYCES 2B CHITINASE ON POLYACRYLAMIDE GEL

BIO-GEL P-150, 50 - 150 mm
COLUMN: 1.5 x 5 cm.
FLOW RATE: 10 ml/hr.
ELUANT: 0.005 M TRIS
0.001 M DTT ACETATE, pH 7.0
LOAD: 10.0 ml 4% PURIFIED CHITINASE - PASSED THROUGH CHROMATOGRAphIC CELLULOSE at
pH 8.2 AND RESIN SORBENT SEPHADEX G-25, pH 2.0

A585
A275
A280

1.2
.6
.9
.4
.6
.2
.3

10 20 30 40 50
MILLILITERS
Berger and Reynolds (5) used zone electrophoresis on starch beds at pH 6.3 and separated *Streptomyces griseus* chitinase in 2 chitinases and a chitobiase.

Jeuniaux (13) could separate *Streptomyces antibioticus* chitinase in three distinct chitinolytic fractions, which migrated to the anode in veronal, pH 8.2, buffer.

*Streptomyces* 2B chitinase also separated in three fractions moving toward the cathode. Cellulose acetate strips and Tris (1) and veronal (6) buffers at pH 6.6 and pH 8.9 were used.

**Adsorption of chitinase on chitin** for purification purpose was considered by Berger (3). The idea was dismissed because of difficulties in desorbing chitinase from the chitin.

Jeuniaux (13) successfully adsorbed *Streptomyces antibioticus* chitinase from its culture filtrates on colloidal chitin at pH 5.2 and 0°C. Chitin, with the chitinase adsorbed, was sedimented and washed with cold 0.02 M citric acid Na₂HPO₄ buffer, pH 5.2. The sedimented chitin-chitinase material was hydrolized at 36°C and chitinase was precipitated with ammonium sulfate.

Lloyd et al. (17) used a modification of the procedure described by Jeuniaux and obtained chitinase with a very high specific activity.

**Adsorption on bauxite** (crude aluminum oxide) was first used for the separation of chitinase and emulsion of *Helix pomatia* digestive juice by Zechmeister, Toth and Balint (32,33). Bauxite selectively adsorbed chitobiase from chitinase solution, but desorption was not possible (5).
Summary

The stabilizing effect of Ca\(^{++}\) ion on chitinase activity was found to be of practical value in purification procedures. The influence on activity of Mg\(^{++}\), Zn\(^{+}\), and Co\(^{++}\) ions also was examined.

Attempts to adsorb ribonuclease on chitin gave negative results. It was shown that the desorption of chitinase from chitin followed a similar 'hysteresis' pattern as for lysozyme.

Electrophoretic properties of streptomycete 2B chitinase were examined and compared with those of other streptomycete strains (reported in literature).

The streptomycete 2B chitinase preparations showed considerable proteinase activity. Celite and artificial Ca-silicate filter-aids, CM-cellulose and hydroxylapatite gel columns were examined for the use in further purification of chitinase. Hydroxylapatite gel column separated streptomyces 2B chitinase in 2 fractions, but both of them showed chitinase and proteinase activities. With respect to the unit weight of protein, the ratio of chitinase activity to proteinase activity was 4.5:2 and 2.1:0.4, respectively.
References


(27) Skujins, J.J. and A.D. McLaren. Sixth Semiannual Progress Report, Space Sciences Laboratory, University of California, Berkeley, July 14, 1967; NASA-CR-


(30) Thuillier, Y. C.A. \textbf{67}, 102772-d (1967); Brit. patent 1,076,776.


C. STUDY OF ORGANIC-MATTER-EXHAUSTED SOILS

Jenkinson (1) followed the mineralization of organic carbon in soils after repeated treatments consisting of a partial sterilization by heating or by chloroform vapor followed by inoculation with soil organisms. With successive treatments the amount of organic carbon mineralized gradually decreased but even after five treatments less than five per cent of the total organic carbon had been mineralized, indicating that a large portion of the organic carbon consisted of material that was not degradable by microbial action nor was rendered susceptible to microbial degradation by the treatments used.

We have initiated a study to determine whether this resistant organic matter contained any enzymatic activity; that is, whether enzymes from microorganisms or plant residues in the soil can enter this resistant fraction and remain insusceptible to the denaturing effects of microbial activity, of natural phenomena such as drying, heating, etc., or of sterilizing agents such as chloroform.

It is important to differentiate between enzymatic activity residing in this resistant fraction and the activity arising from the microorganisms in the soil or from material susceptible to microbial degradation. Therefore, the microbial content of the soil and the treatments themselves had to be studied in detail to determine their effect on the enzymatic activity of the soil. The hydrolysis of urea to ammonia and carbon dioxide by ureases was chosen as the enzymatic activity to be followed.
Materials and Methods

Soils: The soils were obtained from Dr. D.S. Jenkinson, Rothamsted Experiment Station, England, and they were the ones used in his experiments (1); they had the code letter "c". We used a sample of untreated "c" soil and a sample of "c" soil which had undergone five treatments of chloroform sterilization, inoculation, remoistening and incubation, the fifth treatment followed by a final inoculation, incubation for ten days and drying. These two soil samples were designated "untreated" and "treated" respectively.

Soil dilution: About 120 mg of soil is weighed into a sterile metal-capped test tube, 5.0 ml sterile water is added, and the tube let stand undisturbed for 15 minutes to wet the soil. The contents are then mixed thoroughly. The tube is allowed to stand about 15 sec. to let the heavier particles settle and the supernatant suspension is used to make dilutions or is pasteurized. All dilutions are made in sterile water using sterile pipets.

Pasteurization: The supernatant suspension of the soil is carefully placed in the bottom of a sterile metal-capped test tube to avoid wetting the sides. The tubes are heated in a boiling water bath for five minutes and then cooled immediately in ice water. The tube is shaken vigorously before making dilutions.

Plating procedure: A medium of 0.01% Difco Bacto-peptone, 0.01% Difco yeast extract, 0.05% KH₂PO₄, and 0.02% MgSO₄·7H₂O in tap water is adjusted to pH 7.0 with NaOH. Difco agar is added to 1.5%. The medium is sterilized 15 minutes at 120°C and then cooled to 45°C. 1.0 ml of inoculum is added to a petri dish, then about 20 ml of the nutrient agar is added. The dish is rocked to mix the inoculum with the agar. After the agar has set the plates are incubated at room temperature for ten days.
Results and Discussion

The initial tests on the soil "c" show the following results:

<table>
<thead>
<tr>
<th></th>
<th>untreated</th>
<th>treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>organisms/g</td>
<td>$2.5 \times 10^6$</td>
<td>$2.5 \times 10^7$</td>
</tr>
<tr>
<td>spores/g</td>
<td>$1.5 \times 10^4$</td>
<td>$4.0 \times 10^6$</td>
</tr>
<tr>
<td>urease activity, Δcpm/m</td>
<td>4.0</td>
<td>0.7</td>
</tr>
</tbody>
</table>

The untreated soil contained one spore per 167 organisms and the treated soil contained one spore per 6.25 organisms; that is, the ratio of spores to total organisms in the treated soil was about 27 times that of the untreated soil. The reason for this is obscure. The exact treatments which these soil samples have undergone is unknown. Perhaps the chloroform treatments provide a selective advantage for the growth of spore-forming microorganisms. If this is true and the spores are chloroform-resistant as well as heat-resistant, they may accumulate in the soil with successive treatments.

The treated soil had a ten-fold higher microbial count than the untreated soil but had only 17.5% of the urease activity. The experimental data indicate that the treatments have eliminated a large portion of the urease activity but they do not indicate how much activity was lost (since the treated soil was reinoculated before the activity was measured) nor do they indicate the location of the urease activity in the soil samples. Compared to the untreated soil, little activity is associated with the microorganisms in the treated soil, but since the types of organisms in the soil samples are different, one cannot conclude that activity associated with microorganisms in the untreated soil accounts for a negligible amount of the total activity of that soil.

Further studies on these soils are in progress.