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INTERACTIONS AMONG MICROBIAL CELLS, ENZYMES, SUBSTRATES AND CLAY MINERALS

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

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* * * * *

The Ohio State University
1971

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PUBLICATIONS


FIELD OF STUDY

Microbial Physiology
TABLE OF CONTENTS

ACKNOWLEDGMENTS ........................................... ii
VITA. ...................................................... iii
TABLE OF CONTENTS .......................................... v
LIST OF FIGURES ........................................... ix
LIST OF TABLES ............................................. xii
INTRODUCTION ................................................. 1
LITERATURE REVIEW ........................................... 5
MATERIALS AND METHODS ..................................... 19

Organisms .................................................. 19
Media ......................................................... 19
Substrates Tested ........................................... 23
Mineral Particulates ........................................ 23
Isolation of Organisms ....................................... 24
Preparation of Standard Inocula ................................ 25
Tests of Substrate Utilization ................................ 26
Spectrophotometry ........................................... 26
Estimation of Protein ........................................ 27
Assay of Enzyme Activity ................................... 27
Density Gradient Centrifugation ................................ 28
Estimation of Kaolinite-Cell Aggregation
by Determination of Sedimentation Velocity ................. 30
Statistical Analyses ......................................... 30

RESULTS ..................................................... 32

Preliminary Studies of Streptomyces Fradiae
and Micromonospora Chalcea ................................ 32
Substrate Utilization by Streptomyces
Fradiae and Micromonospora Chalcea ......................... 33
Adherence of Kaolinite to Mycelia of
S. Fradiae and M. Chalcea ................................ 35
Adsorption of Protein in General and
Protease in Particular to Kaolinite
and Bentonite .............................................. 37
Degradation of Dye-Conjugated Collagen Substrates by Cell-Free Enzyme Preparations ................................................................. 48

Isolation of Streptomyces and Micromonospora Strains from Lake Erie ................................................................. 51

Studies of Organisms Isolated from Lake Erie .......... 53

Screening of Isolated Actinomycetes for Culture Characteristics and Substrate Utilization ................................. 53

Further Studies of Substrate Utilization by Streptomyces sp. S-l ........... 56

Collagen .................................................. 56

Chitin .................................................. 59

Test for growth promoting factor associated with chitin .... 64

Cellulose .................................................. 66

Comparisons of the Appearance of S-l Mycelia Cultured in Various Media ........................................................................... 74

Adherence of Mineral Particulates to Cells .............................. 77

Photography ..................................................................... 77

Effect of pH at Constant Ionic Strength .................................. 77

Effect of Time at Which Mineral was Introduced to Culture .......... 85

Comparison of Sedimentation Velocities of Cells, Particulates and Mixtures of the Two ............................................. 89

Effect of Cell-to-Particulate Ratio at Various pH's, at Constant Ionic Strength of Buffer ............................................. 89

Effect of Cell Age at 5 pH's, at Constant Kaolinite and Cell Concentrations ............................................................... 89

Effect of Buffer Concentration, at 5pH's, at a Constant Cell-to-Kaolinite Ratio ............................................................... 97

Density Gradient Centrifugation ............................................ 98

Adherence of S-l Mycelium to Substrates ................................. 109

Collagen .................................................. 109

Chitin .................................................. 109

Cellulose .................................................. 109

Adherence of Active Enzyme to Mineral Particulates ............. 116

Mineral Unwashed After Exposure to Enzyme ....................... 116

Mineral Washed After Exposure to Enzyme Solution .................. 117

Effect on pH on Enzyme Activity ........................................ 125
<table>
<thead>
<tr>
<th>Topic</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration of Enzyme from Dilute Solution</td>
<td>131</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>135</td>
</tr>
<tr>
<td>SUMMARY</td>
<td>145</td>
</tr>
<tr>
<td>BIBLIOGRAPHY</td>
<td>148</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>157</td>
</tr>
<tr>
<td>Table</td>
<td>Description</td>
</tr>
<tr>
<td>-------</td>
<td>-------------</td>
</tr>
<tr>
<td>1</td>
<td>Substrate utilization by <em>Streptomyces fradiae</em> and <em>Micromonospora chalcea</em></td>
</tr>
<tr>
<td>2</td>
<td>Distribution of <em>Streptomyces fradiae</em> and <em>Micromonospora chalcea</em>, blood fibrin and kaolinite in discontinuous density gradients</td>
</tr>
<tr>
<td>3</td>
<td>Enzyme activity, estimated by rate and extent of chromophore release from Azocoll, (1) in culture medium filtrate (CMF₁) from <em>Micromonospora chalcea</em> cultured 3 days in chemically defined medium; (2) on particulates added to CMF₁, removed by centrifugation, and resuspended in Tris buffer; and (3) in supernatant fluid (CMF₂) after addition and removal of particulates</td>
</tr>
<tr>
<td>4</td>
<td>Isolation of actinomycetes from surface or bottom water and mud from the Western Basin of Lake Erie</td>
</tr>
<tr>
<td>5</td>
<td>Substrate utilization by actinomycetes isolated from Lake Erie</td>
</tr>
<tr>
<td>6</td>
<td>Significance of ionic strength, pH, and their interaction in the sedimentation of S-l cells and kaolinite. Two-way analysis of variance</td>
</tr>
<tr>
<td>7</td>
<td>Estimation of protein (Lowry method) in cell-free filtrate of <em>Streptomyces</em> sp. S-l culture medium (CMF); in supernatant media (KS₁, IS₁, and BS₁) after addition and removal of the mineral particulates kaolinite (K), illite (I) and bentonite (B); and in supernatant media (KS₂, IS₂ and BS₂) after addition and removal of a second sample of the same mineral particulate</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Estimation of protein: (a) in dilutions of cell-free culture medium and (b)</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>remaining in culture medium after absorption with kaolinite and bentonite</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Adsorption of protein by kaolinite</td>
<td>40</td>
</tr>
<tr>
<td>3</td>
<td>Adsorption of protein by bentonite</td>
<td>41</td>
</tr>
<tr>
<td>4</td>
<td>Azocoll chromophore released by proteolytic activity of <em>Micromonospora chalcea</em></td>
<td>43</td>
</tr>
<tr>
<td>5</td>
<td>Azocoll chromophore released by proteolytic activity of medium in which <em>M. chalcea</em> had been cultured and homogenized</td>
<td>45</td>
</tr>
<tr>
<td>6</td>
<td>Adsorption of enzyme by kaolinite</td>
<td>46</td>
</tr>
<tr>
<td>7</td>
<td>Azocoll chromophore released by proteolytic enzyme adsorbed to kaolinite</td>
<td>47</td>
</tr>
<tr>
<td>8</td>
<td>Comparison of growth rates of 3 Actinomyces, isolated from Lake Erie</td>
<td>55</td>
</tr>
<tr>
<td>9</td>
<td>Comparison of <em>Streptomycyces</em> sp. S-1 cultured in (a) chemically defined medium containing glucose, asparagine and CH₃COONH₄; and (b) minimal salts medium containing 0.01M CaCl₂ and native collagen</td>
<td>57</td>
</tr>
<tr>
<td>10</td>
<td>Macroscopic appearance of <em>Streptomycyces</em> sp. S-1 cultured in minimal salts medium (MSM); MSM + glucose + NH₄SO₄; MSM + chitin, and MSM + chitin + kaolinite</td>
<td>60</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>-----------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>11</td>
<td>Microscopic appearance of <em>Streptomyces</em> sp. S-l cultured in minimal salts</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>medium (MSM); MSM + glucose + NH$_4$SO$_4$; MSM + chitin and MSM + chitin</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+ kaolinite</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>Hydrolysis of cellulose by <em>Streptomyces</em> sp. S-l</td>
<td>68</td>
</tr>
<tr>
<td>13</td>
<td>Dissolution of cellulose by <em>Streptomyces</em> sp. S-l</td>
<td>70</td>
</tr>
<tr>
<td>14</td>
<td>Growth of <em>Streptomyces</em> sp. S-l in cellulose agar after complete dissolution of the cellulose</td>
<td>72</td>
</tr>
<tr>
<td>15</td>
<td>Microscopic appearance of <em>Streptomyces</em> sp. S-l cultured in media containing chitin or native collagen</td>
<td>75</td>
</tr>
<tr>
<td>16</td>
<td>Adherence of kaolinite to <em>Streptomyces</em> sp. S-l at various pH's</td>
<td>79</td>
</tr>
<tr>
<td>17</td>
<td>Adherence of illite to <em>Streptomyces</em> sp. S-l at various pH's</td>
<td>81</td>
</tr>
<tr>
<td>18</td>
<td>Adherence of bentonite to <em>Streptomyces</em> sp. S-l at various pH's</td>
<td>83</td>
</tr>
<tr>
<td>19</td>
<td>Effect of the time at which illite was introduced to cultures of <em>Streptomyces</em> sp. S-l</td>
<td>87</td>
</tr>
<tr>
<td>20</td>
<td>Sedimentation velocities of homogenized suspensions of <em>Streptomyces</em> sp. S-l at various pH's</td>
<td>90</td>
</tr>
<tr>
<td>21</td>
<td>Sedimentation velocities of homogenized suspensions of <em>Streptomyces</em> sp. S-l and kaolinite (0.2 mg/ml) at various pH's</td>
<td>91</td>
</tr>
<tr>
<td>22</td>
<td>Sedimentation velocities of homogenized suspensions of <em>Streptomyces</em> sp. S-l and kaolinite (0.4 mg/ml) at various pH's</td>
<td>92</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>Sedimentation velocities of homogenized suspensions of Streptomyces sp. S-l and kaolinite (0.6 mg/ml) at various pH's.</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>Sedimentation velocities of homogenized suspensions of Streptomyces sp. S-l and kaolinite (0.8 mg/ml) at various pH's.</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>Sedimentation of suspensions of 4 and 5 day cultures of Streptomyces sp. S-l added to kaolinite at various pH's.</td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>Sedimentation of suspensions of Streptomyces sp. S-l added to kaolinite in 0.01 and 0.02M Tris buffer at various pH's.</td>
<td></td>
</tr>
<tr>
<td>27</td>
<td>Formation of cell-mineral aggregates of Streptomyces sp. S-l demonstrated by density gradient centrifugation on a linear water:Renografin gradient.</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>Adherence of kaolinite to Streptomyces sp. S-l demonstrated by density gradient centrifugation.</td>
<td></td>
</tr>
<tr>
<td>29</td>
<td>Adherence of kaolinite to Hide Powder Azure demonstrated by density gradient centrifugation.</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>Adherence of Streptomyces sp. S-l to a particulate substrate: collagen.</td>
<td></td>
</tr>
<tr>
<td>31</td>
<td>Adherence of Streptomyces sp. S-l to a particulate substrate: chitin.</td>
<td></td>
</tr>
<tr>
<td>32</td>
<td>Adherence of Streptomyces sp. S-l to a particulate substrate: cellulose.</td>
<td></td>
</tr>
<tr>
<td>33</td>
<td>Dissolution of Hide Powder Azure (HPA) by Streptomyces sp. S-l enzyme absorbed to kaolinite.</td>
<td></td>
</tr>
<tr>
<td>34</td>
<td>Adsorption of active enzyme to kaolinite, illite and bentonite.</td>
<td></td>
</tr>
<tr>
<td>Figure</td>
<td>Page</td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>------</td>
<td></td>
</tr>
<tr>
<td>35</td>
<td>133</td>
<td></td>
</tr>
</tbody>
</table>

Concentration of protease from Streptomyces sp. S-1 culture medium filtrate by kaolinite
INTRODUCTION

In aquatic environments, large quantities of organic materials, some very complex, are recycled by the enzymatic activities of micro-organisms. Microbial extracellular enzymes responsible for decomposing organic substances have been extensively investigated under laboratory conditions, but one natural factor rarely considered in enzymological studies is the ubiquity of mineral particulates in aquatic ecosystems. That clay minerals do influence microbial metabolism and enzyme activity in soils has been well documented, but little is known about the overall effect that such particulates have on the decomposition of refractory organic compounds in lakes.

Collagen, chitin and cellulose are synthesized abundantly by aquatic organisms. For producers, the durability of these compounds is advantageous; for those organisms, man included, which would recycle the components of such compounds, durability presents an obstacle. The endurance of an organic compound is a function of the abundance of hydrolyzing agents and
the accessibility of the compound to those agents. Man considers collagen, chitin and cellulose refractory only because relatively few organisms produce the requisite hydrolytic enzymes necessary to decompose them.

As a group, the actinomycetes are considered able to degrade these complex organic materials. No data are available as to the absolute importance of actinomycetes in lakes, but their high numbers relative to the total bacterial count, the broad range of their enzymatic competence, the rapidity with which some strains convert complex organic materials into cell mass under laboratory conditions, and the absence of large deposits of arthropod shells, animal hides, dead leaves and similar detritus in lake bottom sediments, suggest that actinomycetes may contribute significantly to degrading these materials. If actinomycetes are active in recycling refractory compounds in lakes, they may be valuable for recycling the same materials as they become the waste products of man's industry.

In composting, man has long exploited the cellulolytic capability of soil actinomycetes to convert unwanted plant products into usable soil amendment. He may well be able to harness the proteolytic and chitino-
lytic facility of aquatic actinomycetes to convert hide, hair, feathers, fish scales and arthropod shells into usable carbohydrates and amino acids.

Since mineral particulates significantly influence microbial metabolism and enzyme performance, I undertook a study of some aspects of this influence with a view to its pertinence to problems of waste disposal and lake eutrophication. I have demonstrated, usually qualitatively, occasionally quantitatively, five interactions which affect the enzyme activity of microorganisms of the family *Streptomycetaceae* and com-mittantly affect the degradation of the organic materials which they utilize. These five interactions are:

1. Adherence of mineral particulates to cells.

2. Adherence of microbial cells to three particulate substrates: collagen, chitin and cellulose.

3. Adherence of kaolinite to collagen.

5. The activity of adsorbed enzyme when the adsorbent mineral adheres to a particulate substrate, collagen.
LITERATURE REVIEW

Decades ago microbiologists were aware that addition of particulates to liquid cultures of bacteria could stimulate growth (Heukelekian and Heller, 1940). Conn and Conn (1940) suggested that increased surface for growth provided by mineral colloids, or their adsorption of inhibitory waste products, rather than just a contribution of ions to the medium, might explain the increase in microbial growth. Bigger and Nelson (1941) inferred that talc, serving as a surface for adsorption of CO₂ and ammonia dissolves in a distilled water menstruum acted as a surface catalyst, enabling coliform bacteria to subsist on laboratory air as a source of carbon and nitrogen. ZoBell (1943) concluded that solid surfaces concentrated adsorbed nutrients from very dilute solutions, provided a resting place for sessile bacteria, and adsorbed extracellular enzymes and their hydrolysates, bringing organism, enzymes and substrates into close contact. He inferred that surface adsorption reduced the probability that exoenzymes would diffuse away from the producer or nutrients from the prospective consumer during hydrolysis.
ZoBell's conclusions seem reasonable in view of the available data concerning adsorption of organic materials by clay minerals. Because the conditions under which clay minerals interact with simple and complex organic molecules are of such importance to agriculture and waste water treatment, they have been studied extensively.

Silicate clays, the characteristic soil colloids of temperate regions, consist mainly of montmorillonites, e.g. bentonite; the hydrous micas, of which illite is an example; and the kaolinites. These colloidal - hence biologically important - portions of the soil and the silt loads of rivers, lakes and oceans, represent three stages in the weathering of feldspars and micas. All are ionic lattices with loosely associated, hydrated, exchangeable cations (Buckman and Brady, 1960).

Montmorillonite consists of an alumina layer tightly bound between 2 hydrated silica layers by mutually shared oxygen atoms. These 3-layered units are loosely associated with each other by O-O bonds which easily allow expansion of the lattice. Montmorillonite crystals fracture easily into individual particles.
(micelles) having diameters ranging from 0.02 \( \mu m \) and thicknesses ranging to 0.002 \( \mu m \).

Illite, also a 3-layered silicate clay mineral, has about 15\% of the silicon ions of the middle layer replaced by aluminum. Potassium ions are generally associated with the aluminum ions, balancing the charge deficit created by the ion substitution. This type of lattice is less expansive than that of montmorillonite. The individual particles are generally larger, with 0.1 \( \mu m \) to 0.2 \( \mu m \) diameters.

Kaolinite, formed from montmorillonite, or directly from parent rock by weathering which removes alkali and alkaline-earth metals, is a 2-layered structure with 1 alumina and 1 hydrated silica layer in a non-expanding lattice. The dimensions of flake surfaces range from 0.3 \( \mu m \) to 4.0 \( \mu m \) and thicknesses from 0.05 \( \mu m \) to 2.0 \( \mu m \).

On paper, the structures described for these 3 clay minerals are ionically balanced. But experimental evidence points overwhelmingly to the fact that the mineral particles carry not only net charge, but point charges, both positive and negative. Three mechanisms have been advanced as possible sources of these charges: isomorphous replacement, ionization of
water of hydration and lattice defects. Isomorphous replacement of Si by Al, or of Al by Mg would produce a net negative charge on the crystal faces and could account for the high surface charge and ion exchange capacity of montmorillonite (ca. 100 meq/100 g) and illite (10 - 4 meq/100 g) relative to kaolinite (2 - 4 meq/100 g) (Wayman, 1967). Ionization of water molecules bonded to aluminum along the edges of micelles would create sites of positive charge in an acid environment, negative charge in alkaline solutions. This could explain why the cation exchange capacity of clay minerals increases with increase in pH. Under acid conditions, water of the alumina layer could adsorb $\text{H}^+$ or other cations and neutralize the site, creating a positive surface charge. Lattice defects, due to leaching of aluminum hydroxide in acid solutions, would result in a net negative charge. Cashen (1966) remarked that the argument is not over the existence of positive edge charges on clays, rather it is over their source and permanence.

Whatever their origin, unbalanced surface charges can be satisfied by adsorption of other charged particles. Clays are good adsorbents because of their large surface area (hence high total surface charge) per unit weight. The expanding lattice of montmorillonite,
by presenting interlamellar as well as micellar surfaces to solutions, makes this type of clay a much more efficient adsorbent than non-expanding kaolinite (Talibudeen, 1951). Adsorption of organic molecules to clay minerals at pH's thought to render both species anionic may be due to cation bridging.

Some authors have adduced data indicating that forces other than ionic bonding are responsible for sorption of organic materials to clays (Mattson, 1932; Lynch, Wright, and Cotnoir, 1956; Bader, Hood, and Smith, 1960). But Evans and Russel (1959) found that adsorption of fulvic and humic acids to kaolinite and bentonite was independent of temperature, and related to the cation of homoionic clays. These data indicated that Coulombic forces rather than hydrogen bonding or Van der Waal's forces were involved.

Ensminger and Gieseking (1939) suggested that at pH's below their isoelectric points (pI's), proteins adsorb to bentonite as cations. In 1942, these same authors related the ability of kaolinite and bentonite to adsorb protein with the base exchange capacities of the two minerals. Hendricks (1941) stated that large organic cations were held to montmorillonite not only by Coulombic forces between ions, but also by Van der Waal's attractions between surfaces.
McLaren (1954a) studied the relationship between the pI's of 9 proteins and their adsorption to kaolinite as a function of pH. Since kaolinite acts as a weak acid above ca. pH 4, he used proteins with pI's above and below pH 4. For 7 of the proteins, maximum adsorption occurred at pH's 1-3 units lower than the pI. He observed that decrease in binding of proteins to kaolinite would be expected above the pI if net surface charges were solely responsible for binding. But even above the pI, some surface regions of the protein would bear positive charges and unless the molecule as a whole were highly negative, some binding to kaolinite would occur. His data bear this out; although binding dropped off below or at the pI, it did not fall abruptly to zero. In this, and in a later paper (1957), McLaren pointed out that adsorption on a solid surface alters the apparent pI of a protein. The pH for optimal activity of chymotrypsin on kaolinite was 2 units higher than the optimal pH in solution because the surface of the clay colloid was more acid than the solution proper.

Further elucidation of the nature of the clay-protein relationship at various pH's was reported by McLaren, Peterson, and Barshad (1958). In this paper, Peterson's interpretation of curves plotted for adsorption of lysozyme on kaolinite and bentonite
indicated maximum adsorption of lysozyme on both minerals occurred at pH's above the pI of the protein. As pH increases above 2, the net negative charge on clays increases and the net positive charge on protein decreases, becomes zero, then reverses to a negative charge above the pI. Thus the increasing tendency for a negative clay to adsorb a positive protein is counteracted by the protein's tendency to become less positive, and eventually negative. The maximum adsorption of protein to clay near the protein's pI may be accentuated by the lessening of self-repulsion by the protein as its net charge approaches zero. But the adsorption maximum would be shifted away from the pI by the increased hydrogen ion activity on the clay mineral surface.

Investigations by soil scientists of clay mineral - organic associations were prompted by their need to understand the nature and extent of the resistance to microbial degradation afforded organic matter by soil particles. Ensminger and Gieseking (1942) found that kaolinite had no significant effect on hydrolysis of albumin or hemoglobin, but bentonite interfered with hydrolysis of these proteins by both pepsin in acid solution and pancreatin in alkaline solution. They assumed that either the protein was oriented in such
a way that enzyme could not attack it, or the enzyme itself had been adsorbed and denatured. Pinck and Allison (1951) reported that when gelatin was reacted with montmorillonite, the protein adhering to the outside of the micelles was digested. Gelatin occupying interlamellar spaces was not. Esterman and McLaren (1959) observed that kaolinite acted as a concentrating surface for adsorbed organic substrates and microbial exoenzymes, thereby accelerating enzymatic breakdown of substrate. Esterman, Peterson and McLaren (1959) contrasted the rate of hydrolysis of protein freshly adsorbed to montmorillonite from solution with the rate for dried and rewetted protein-clay complexes. The former hydrolyzed at rates nearly equal to those for non-adsorbed protein in solution, but the dried, rewetted complex was somewhat resistant to attack. It should be noted here that Ensminger and Gieseking's data were obtained with dried protein-montmorillonite complexes. Barbaro and Hunter (1965), studying the effect of kaolinite and bentonite on the biodegradability of surfactants, found that both clays inhibited degradation, bentonite much more so than kaolinite. They concluded that the adsorption of surfactant to clay minerals rendered inaccessible sites of enzymatic hydrolysis. Ambroz (1966) reported that in complexes
of bentonite containing 15% protein, microbial degradation of the protein was retarded. At higher percentages of protein, proteolysis was stimulated. Weber and Coble (1968) measured microbial decomposition of the pesticide diquat adsorbed to kaolinite and montmorillonite. Kaolinite-adsorbed diquat was oxidized at CO$_2$, but montmorillonite-adsorbed pesticide resisted oxidation to an extent comparable to the calculated degree of interlamellar penetration.

These data clearly indicate that protein adsorbed to clay minerals is subject to enzymatic attack if the appropriate enzymes can contact active sites on the adsorbed substrate. The next obvious question is whether adsorption of proteins which themselves have enzymatic activity, interferes with that activity. McLaren (1954b) found that adsorption of enzyme led to reduction or loss of activity of lysozyme, pepsin, trypsin and chymotrypsin. Some activity of lysozyme and trypsin could be recovered on elution of the enzyme with ethylamine. Fraser (1957) concluded that dense packing of enzyme on an immobile surface led to less denaturation than would occur at a liquid:air interface. As before, the nature of the clay mineral, whether kaolinite or montmorillonite, was very important. Talibudeen (1955) found from X-ray diffraction data that
trypsin adsorbed to montmorillonite had unfolded and lost activity.

The previous studies were made with a view to explaining soil-organic matter interactions. But in another context, clay minerals were being used to purify enzyme preparations of impurities (Dixon, 1926; Adams and Hudson, 1943). "Success" varied with enzymes, adsorbents and authors' points of view. One writer reported "elution of enzyme in active form" but another author, citing the same data, says, "addition of an adsorbent to the system markedly reduced enzyme activity."

Since the publication of ZoBell's hypotheses about the effects of mineral surfaces on microbial metabolism (1943), several microbiologists have reported either enhancement or inhibition of microbial growth by various minerals, chiefly kaolinite and montmorillonite. Meadows and Anderson (1966) postulated that sand grain surfaces provided distinct and characteristic micro-habitats for clones of marine microorganisms. Lee and Hoadley (1967) stated, "In open water, bacteria may exist freely as single cells or associated with particulate matter...The surfaces of particulate matter, where organic substances are adsorbed and excreted products
of other community members are available afford the associated bacteria an opportunity to function in an enriched situation relative to the surrounding open water. Colonization of surfaces may afford an opportunity to create special localized conditions, permitting metabolism of certain organic materials by altering the pH, redox potential or by preventing dispersal of extracellular enzymes."

Cralley (1968) found that both kaolinite and bentonite stimulated growth of actinomycetes in liquid cultures containing either soluble or particulate carbon sources; kaolinite produced a greater effect. Nováková (1968) reported that Na⁺ and Ca⁴⁺ forms of bentonite shortened the lag phase of *Escherichia coli* cultures by about 75%, but lowered total cell yield. Kaolinite lengthened the lag phase and also diminished total cell yield. With both minerals, an increase in concentration produced an increase in effects. Pfister, Dugan, and Freia (1968), using particulates of various sizes removed from Lake Erie water by differential centrifugation, found that stimulation or inhibition of microbial growth varied with particle size and with the type of microorganism. Button (1969) added bentonite to single-phase continuous cultures of yeasts and *E. coli*
to measure adsorption of the limiting nutrients, glucose and thiamine. He concluded that no significant adsorption occurred, attributing a temporary reduction of cell number in one culture in unbuffered medium to perturbation caused by an increase in free heavy metal concentration.

Other authors have offered other explanations for effects produced by minerals in microbial cultures. Stotzky, in a series of papers (Stotzky and Rem, 1966; Stotzky, 1966a; Stotzky, 1966b; Stotzky and Rem, 1967) reported that montmorillonite in soils favored growth of bacteria over fungi by exchanging clay-associated cations for the hydrogen ions produced by microbial metabolism; thus maintaining soil pH at a level more favorable for bacteria.

There is a real dearth of data concerning the effects of clay minerals on the growth of aquatic microorganisms (Wayman, 1967). An example of the kind of oversight prevalent in the literature is Stotzky's statement: "Presence of mineral particulates is a feature which distinguishes soil from other habitats." (Stotzky and Rem, 1966).
Another aspect of protein-to-mineral adsorption which has begun to receive attention is flocculation. Bader and Jeffrey (1958) studied the efficiency of kaolinite, illite and montmorillonite in removing radioactive organic matter from sea water. Much literature describes the behaviour of polyelectrolyte polymers as mineral flocculants or dispersants (Ruehrwein and Ward, 1952; Heller and Pugh, 1960; Ulinska and Huppenthal, 1966; Black, Birkner, and Morgan, 1966; Mukherjee and Chakravarti, 1968; Kitchener, 1969). Busch and Stumm (1968) considered bacterial cell-surface material as a natural polyvalent polymer effecting flocculation. Ulinska and Huppenthal (1966) and Narkis, Rebhun, and Sperber (1968) warned that "model systems" involving purified minerals react very differently from natural systems in which clays are covered with soil salts and polymers. Although clay minerals are readily recognized as adsorbents in natural water purification processes, their possible use in conjunction with activated sludge seems to have been largely ignored.

The actinomycetes have been extensively studied for their antibiotic producing qualities. Less attention has been paid to their ability to degrade many resistant organic materials such as collagen, chitin and cellulose. Erikson (1941) studied the cellulolytic
and chitinolytic capacity of many strains of *Micromonospora*. Reese, Siu, and Levinson (1950); Reese, Smakula, and Perlin (1959); Hagen, Reese, and Stamm (1966); Waksman (1967); Enger and Sleeper (1965); Mandel (1969); and Jagnow (1969) have reported on the cellulases of *Streptomyces*. Reynolds (1954); Berger and Reynolds (1958); Skujins, Potgieter, and Alexander (1965) and Jagnow (1969) have studied the chitinases of *Streptomyces*. Hagihara (1960) in a review of proteases, mentions the work of Nomoto and Narahashi (1959) who found that a protease of *Streptomyces griseus* was as active as the *B. subtilis* enzymes generally used commercially. Cralley (1968) studied the effect the clay minerals kaolinite and bentonite had on the growth of *Streptomyces* and *Micromonospora* cultured in defined media with cellulose as a carbon source or chitin as a carbon and nitrogen source.

McCabe and Frea (1971) reported that addition of kaolinite to a cell-free solution of extracellular enzymes produced by a streptomycete, concentrated the enzyme from dilute solutions and brought it into intimate contact with particulate substrate. These data seem to confirm the hypothesis that mineral particulates influence the metabolism of microorganisms by serving as adsorbent surfaces for nutrients and enzymes.
MATERIALS AND METHODS

Organisms

Cultures of *Streptomyces fradiae* 5063 (Waksman), and *Micromonospora chalcea* ATCC#12452, from stocks maintained by the Ohio State University Culture Collection, were used for preliminary studies. Three strains of *Streptomyces* and 4 of *Micromonospora*, isolated from Lake Erie, were screened for substrate utilization and ability to grow well in chemically defined broth medium. Of these, one streptomycete, here designated S-1, was used for all studies of interactions among cells, extracellular enzymes, substrates and clay minerals.

Media

*Yeast Extract Agar (YME)* (Pridham et al., 1956/1957)

Slants of YME at pH 7.6 were used for maintaining all stock cultures. YME plates were used to check purity of broth cultures and to culture actinomycetes isolated on other media, to determine color and appearance of substrate and aerial mycelia.
Tryptone, Yeast Extract Broth (TYE) (Pridham and Gottlieb, 1948)

TYE, at pH 7.6, was used for routine culture of S. fradiae and M. chalcea, to obtain cells for subsequent inoculation into chemically defined media. TYE cultures of the 7 organisms isolated from Lake Erie were both lyopholized and frozen in liquid nitrogen to preserve the original strains.

Mineral Salts Medium (MSM)

I modified the carbon utilization medium of Pridham and Gottlieb (1948), to eliminate precipitates which interfered with dry weight determination and optical density estimation of cell mass. MSM contained per liter:

Trizma [Tris(hydroxymethyl)aminomethane and hydrochloride] (Sigma) 0.1210 g
Maleic anhydride 0.0980 g
K$_2$HPO$_4$ 1.1300 g
MgCl$_2$ 0.4130 g
Trace salts:

CuSO$_4$·5H$_2$O 0.0064 g
FeSO$_4$·7H$_2$O 0.0011 g
MnCl$_2$·4H$_2$O 0.0079 g
ZnSO$_4$·7H$_2$O 0.0015 g
The pH was adjusted with NaOH to 8.2-8.3. The final pH after autoclaving was about 7.6. MSM was used with substrates containing both carbon and nitrogen to test organisms' ability to utilize the substrate as a source of those elements.

Mineral Salts Medium with $(NH_4)_2SO_4$ (MSM-SO$_4$)

Ammonium sulfate, 2.64 g/liter, was added to MSM for use with substrates containing carbon, but no nitrogen, to test organisms' ability to utilize that substrate as a carbon source.

Mineral Salts Medium with 0.01M CaCl$_2$ (MSM-CaCl$_2$)

(MSM-CaCl$_2$) was used with native collagen to test for specific collagenase activity. MSM was used also with autoclaved collagen to determine whether Ca$^{++}$ influenced utilization of denatured collagen by Streptomyces sp. S-1.

Glucose, Asparagine, Ammonium Acetate Medium (GM)

MSM containing per liter, glucose, 4.0 g; and L-asparagine•H$_2$O, 0.5 g, was autoclaved and cooled. Filter sterilized CH$_3$COONH$_4$ in distilled water was added aseptically to give a concentration of 4.93 g/liter. This medium was used for routine culture of S-1, and for culture of washed S. fradiae or M. chalcea cells.
harvested from TYE cultures. In some experiments, Trizma concentration was doubled (0.2420 g/liter) to increase buffering capacity. Maleic acid, which may have inhibited growth, was omitted from GM or MSM. These media are designated GM-2TB, MSM-2TB, or MSM-SO₄-2TB.

**GM Agar**

Agar, 20 g/liter, was added to the autoclavable components of GM and sterilized. Filter sterilized ammonium acetate solution was added aseptically to the medium cooled to 47°C. Plates of this agar were used to test organisms' ability to grow on chemically defined solid medium.

**Cellulose Agar**

Coil cotton (Kendall Co., Walpole, Mass.) was hydrolyzed by Hungate's (38) method, but not milled. The pH was adjusted to 7.0 with NaOH and the slurry washed until the AgNO₃ test for chloride was negative. Cellulose at 1 mg/ml was added to MSM-SO₄-2TB containing 2% agar, pH 8.2.
Starch, Casein Agar and Actinomycete Isolation Agar

Starch, casein agar (JEN) (Jensen, 1930) and Actinomycete isolation agar (AIA) (Difco Supplementary Literature, September, 1966) were used to isolate Streptomyces and Micromonospora from Lake Erie water and bottom mud. To both media, I added Rose Bengal (Matheson, Coleman, and Bell, C. I. 45440) at concentrations of 1:20,000; 1:30,000; or 1:40,000 to curtail bacterial and fungal growth (Smith and Dawson, 1944) without completely inhibiting actinomycete growth.

Substrates Tested

Collagen (Sigma); chitin, blood fibrin and keratin (Nutritional Biochemicals); cellulose (Whatman, CF11, W & R Balston, Ltd., England) and hydrolyzed cotton (Kendall Co., Walpole, Mass.); cellobiose (Pfansteil); casein (Bacto-Tryptone, Difco); Azocoll, Hide Powder Azure (HPA), and Azocasein (Calbiochem) were used in substrate utilization tests.

Mineral Particulates

Kaolinite (Peerless Coating Clay) and illite (Fithian #35) kindly supplied by Dr. Robert H. Miller, Department of Agronomy, The Ohio State University; and
bentonite (American Colloid Co., Skokie, Ill.) were used, as supplied.

**Isolation of Organisms**

A sample of bottom mud and water was collected at a depth of 15M from the Western Basin of Lake Erie. Four 10 ml portions of this sample were pipetted into sterile test tubes. The tubes were incubated in a 70 C waterbath with agitation, one tube per time period, for 0, 10, 20 or 30 min. The samples were used to streak plates of GM agar, Jensen's agar, or AIA, all containing various concentrations of Rose Bengal. The plates were incubated at 27 C. As colonies, apparently of Streptomyces or Micromonospora, were detected by stereoscopic microscopy at 250X, the colonies were picked off and each aseptically dispersed in a drop of distilled water. Some of the material was examined microscopically, with phase contrast, or stained with crystal violet, to determine cell morphology. If the cells appeared to be mycelial, the rest of the colony was used to streak fresh plates of the medium on which the colony had developed. When pure cultures were obtained on solid medium, the organism was cultured on YME, Jensen's agar, GM agar and AIA, all without Rose Bengal, to determine pigmentation. Appearance of substrate mycelium; of aerial mycelium, if any; spore morphology; pigmentation of
the mycelia and medium as the organism developed on various media, and ability to utilize the substrates tested, were used as criteria to distinguish strains. No further attempt was made to identify the strains isolated. Their behavior under my culture conditions was recorded, and they were assigned arbitrary OSU numbers. A second sample of Lake Erie water, collected from the same location, but at the surface on a calm day, was used for further isolation of actinomycetes. This sample was not heated prior to use; otherwise the same procedure was used.

**Preparation of Standard Inocula**

Cells cultured in TYE were centrifuged and washed twice in Tris-maleate buffer (0.01 M, pH 7.6) (26). Cells cultured in GM were washed once. Washed cells were resuspended in about 10 ml of buffer and homogenized with a TRI-R Stir-R (TRI-R Instruments, Jamaica, N. Y.) until the suspension contained no macroscopically visible clusters of cells. The cell suspension was diluted with buffer until its absorbance at 660 nm was 0.3. The diluted suspension was agitated continuously as samples were pipetted into flasks of test media. Dry weight determinations showed that separate
filling of the pipette for each inoculation was necessary. If 1 ml or 2 ml inocula were dispensed sequentially from a 5 ml or 10 ml pipette, the inocula contained sequentially increasing amounts of cell material. Dry weight determinations of triplicate samples of inoculum were made in experiments in which the absolute increase of cell mass during incubation was to be determined.

**Tests of Substrate Utilization**

Weighed amounts of substrates were added to MSM or MSM-SO₄, at concentrations calculated to provide the same carbon and nitrogen ratio as that of one type of control: MSM-SO₄ with 4.0 g/liter of glucose. Another control, MSM with no added carbon or nitrogen source, was included in these experiments. When particulate substrates were used, increase in cell mass was judged visually, sometimes recorded photographically. Microscopic examination of the cell morphology was sufficient to determine whether normal growth or autolysis were occurring. When soluble substrates were tested, cultures were centrifuged and dry weights of the pellets determined.

**Spectrophotometry**

Optical densities were determined with a Spectronic-20 (Bausch and Lomb) spectrophotometer. Matched
Pyrex tubes were used for reading absorbance at wavelengths below 625 nm. There was no significant difference between disposable soft glass 13 x 100 mm culture tubes at wavelengths above 625 nm. These were used in sedimentation tests where suspensions could not be transferred to a cuvette during the settling period.

**Estimation of Protein**

The total protein content of Fluids was estimated by the method of Lowry, et al., (1951) as described in *Manual of Microbiological Methods* (46). Absorbance of 3 replicates of each sample were read at 660 nm. Triplicate samples of freshly prepared solutions of Bovine serum albumin (BSA) in 0.01 M phosphate buffer at pH 7.0, at concentrations of 0, 30, 60, 90, 120 and 150 µg of protein per 0.5 ml sample, were included in each determination. Protein concentrations of experimental solutions were calculated from standard curves computed from BSA data.

**Assay of Enzyme Activity**

Rate of dye release from substrates conjugated with azo chromophores was used to assay for enzyme activity (Oakley, Warrack, and Van Heyningen, 1946;
Nelson, Ciacco, and Hess, 1961; Rinderknecht, et al., 1968). For substrates suspended in buffer, samples were centrifuged to remove suspended particles, and absorbance by dye in the supernatant fluid was measured spectrophotometrically at an appropriate wavelength. Dye concentration was calculated from a standard curve of known amounts of hydrolyzed substrate. Dye release from substrate particles included in agar pour plates was estimated visually and recorded photographically. Sufficient quantity of dyed substrate was used in each test to insure that at least 100 macroscopically visible particles were included in each plate. Decolorization of 90% of the substrate particles in a plate was used as an endpoint, since the rate of dye release from individual particles varied with the surface-to-volume ratio of the particles.

Density of Gradient Centrifugation

Sucrose

Discontinuous gradients of water and 65% (w/v) sucrose in water, were prepared in 12 ml conical glass centrifuge tubes. These were centrifuged at 2,000 x g for 15 min. Material was removed from the interface and
from the bottom of the gradient with a Pasteur pipette.

Renografin

Renografin-76 (Squibb). To form each gradient, 1 ml of Renografin-76 (N-N'-diacetyl-3,5-diamino-2,4,6-triiodobenzoate; density 1.45-1.46) was pipetted into a 20 ml cellulose nitrate tube. Fourteen ml of a linear Renografin and water mixture was added from a linear gradient maker, and 1 ml of the sample was layered over the gradient. Tubes were centrifuged at 13,200 x g for 1 hour. Temperature was maintained at 2-4 C throughout formation and centrifugation of the gradient. Gradients were photographed as soon as possible after removal from the centrifuge, and were kept in an ice bath until fractionated. Fractions of 1 ml or 0.4 ml were collected upward by using Renografin as a propellant. The linearity of gradients was checked by measuring the refractive index of the Renografin solution from each fraction of a gradient, and comparing the values obtained with those of a standard curve based on the refractive indices of known concentrations of Renografin. Densities of individual fractions were estimated from the standard curve.
Estimation of Kaolinite-Cell Aggregation by Determination of Sedimentation Velocity

S-1 cells were homogenized or agitated in a Waring Blendor until mycelial clumps were dispersed. Samples of cell suspension were added to 13 x 100 mm culture tubes containing appropriate concentrations of kaolinite and of buffer adjusted to give a particular ionic strength and pH, at a final dilution of 6 ml. Triplicate samples of each level of the variables were used. The tubes were vortexed vigorously before a zero time absorbance was measured at 660 nm. Absorbance was measured at suitable time intervals, with care taken not to shake or jar the tubes. Changes in absorbance with time were plotted graphically and analyzed statistically.

Statistical Analyses

Programs written for the Smith-Corona Marchant Cogito Model 1016PR calculator were used to compute mean, standard deviation and standard error of mean; slope and intercept of linear portions of standard curves; and analysis of variance for one-way classification. Programs written for the Ohio State University CPS-360
computer were used for single variable, linear regression analyses, one-way analyses of variance and two-way analyses of variance.
RESULTS

Preliminary Studies of Streptomyces Fradiae and Micromonospora Chalcea

Laboratory strains of S. fradiae and M. chalcea were used to make preliminary tests of (1) substrate utilization; (2) adherence of mineral particulates to mycelium; (3) adherence of proteins in general, and enzymes in particular to mineral particulates, and (4) degradation of dye-conjugated collagen substrates by cell-free enzyme preparations. The preliminary tests indicated that (1) actinomycetes were suitable organisms for studying enzymatic degradation of complex organic materials; (2) mineral particulates adhered to mycelia and protein substrates; (3) active enzyme was absorbed to kaolinite and bentonite, and (4) the collagen preparations, Azocoll and HPA, used in conjunction with mineral particulates, permitted qualitatively sensitive assays of enzymatic activity.
Substrate Utilization by Streptomyces Fradiae and Micromonospora Chalcea

Standard inocula of *S. fradiae* or *M. chalcea* were added to 250 ml shaker flasks containing 50 ml of MSM with 1 g/liter of total substrate supplied in the following forms: GM; MSM + CH$_3$COONH$_4$ + cellulose; collagen; chitin and casein.

I judged growth by the macroscopic appearance of the cell mass and by microscopic examination of the mycelia. Both organisms utilized all 4 complex substrates. *M. chalcea*'s growth was poor in the cellulose medium. Both organisms grew much more extensively in complex media than in GM, a chemically defined, adequate medium. Both organisms cultured especially well in medium containing chitin, a polymer of N-acetylglucosamine. Growth was less rapid in media containing collagen or casein. The reason for this was not obvious, unless the chitin, a rather crude preparation, contained growth factors not present in the other media. Table 1 summarizes the results. Growth was rated relative to the cell mass produced by the organism cultured in GM, which was arbitrarily set at 1+. The observations concerning pigment were included for those media in which suspended substrate could have been mistaken for ...
<table>
<thead>
<tr>
<th>Substrate</th>
<th>S. fradiae</th>
<th>M. chalcea</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSM + glucose + asparagine + CH₃COONH₄</td>
<td>1+</td>
<td>1+</td>
</tr>
<tr>
<td>MSM + CH₃COONH₄</td>
<td>2+</td>
<td>2+</td>
</tr>
<tr>
<td>MSM + cellulose</td>
<td>1+</td>
<td>2+</td>
</tr>
<tr>
<td>MSM + collagen</td>
<td>2+</td>
<td>0</td>
</tr>
<tr>
<td>MSM + chitin</td>
<td>3+</td>
<td>1+</td>
</tr>
<tr>
<td>MSM + casein</td>
<td>2+</td>
<td>2+</td>
</tr>
</tbody>
</table>

aMinimal salts medium.

bCollagen was autoclaved; therefore denatured.

cGrowth was judged relative to the increase in cell mass of the organism cultured in MSM + glucose asparagine + CH₃COONH₄, rated as +1.

dThe observations concerning pigmentation were included for media in which suspended substrate could have been mistaken for cells were it not for the pigmentation; greenish yellow for the S. fradiae, bright orange for the M. chalcea.
cells were it not for the pigmentation; bright orange for the *M. chalcea*, greenish yellow for *S. fradiae*.

**Adherence of Kaolinite to Mycelia of**

**S. Fradiae and M. Chalcea**

I wished to test whether adherence of kaolinite to the mycelia of *S. fradiae* and *M. chalcea* were stable enough, or too stable, to be used in conjunction with density gradient centrifugation to effect the following separations: (1) the organisms from each other in mixed culture; (2) organisms from particulate substrates, and (3) mineral adsorbed enzyme from cell surfaces.

Kaolinite was added to cultures of each organism in MSM + gelatin (1 g/liter); MSM + blood fibrin (1 g/liter), and TYE. Particulate materials in each culture were sedimented and a sample layered over a discontinuous water:65% (w/v) sucrose gradient. Kaolinite adhered to some extent to all organic materials, but especially to *M. chalcea* mycelium and to blood fibrin. Table 2 describes the materials found at the water:sucrose interface and in the pellet of each gradient. Little kaolinite was found in the interface layer but much kaolinite and organic matter was aggregated in pellets. The density of a given suspended
<table>
<thead>
<tr>
<th>ORGANISM</th>
<th>MEDIUM</th>
<th>MATERIAL AT INTERFACE</th>
<th>MATERIAL IN PELLET</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. fradiae</td>
<td>TYE&lt;sup&gt;b&lt;/sup&gt;</td>
<td>cells with some adherent kaolinite</td>
<td>cells much more dense, with large masses of adherent kaolinite</td>
</tr>
<tr>
<td>S. fradiae</td>
<td>MSM + gelatin</td>
<td>large dense colonies, not much associated kaolinite except small crystals adhering to the periphery of mycelial masses</td>
<td>large cell masses with large kaolinite crystals adhering to them; much dispersed or slightly aggregated kaolinite</td>
</tr>
<tr>
<td>S. fradiae</td>
<td>MSM + blood fibrin</td>
<td>2 layers: upper, of cells; lower of blood fibrin, both associated with kaolinite; very little free kaolinite</td>
<td>2 layers: upper, cells and mats of blood fibrin, but not much kaolinite associated with cells; lower, large flakes of fibrin and clusters of cells associated with mineral</td>
</tr>
<tr>
<td>M. chalcea</td>
<td>TYE</td>
<td>no interface layer; 6 clumps of cells hung in upper portion of sucrose layer; some kaolinite associated with these.</td>
<td>2 layers: upper, mycelium heavily loaded with kaolinite but much free kaolinite also; lower, cell masses even more heavily loaded with kaolinite, large crystals of free mineral</td>
</tr>
<tr>
<td>M. chalcea</td>
<td>MSM + gelatin</td>
<td>dispersed cell masses heavily loaded with small kaolinite crystals</td>
<td>cells heavily loaded with kaolinite; little free mineral</td>
</tr>
<tr>
<td>M. chalcea</td>
<td>MSM + blood fibrin</td>
<td>continuous dense mesh of blood fibrin and small crystals of kaolinite; cells if present were obscured by fibrin</td>
<td>large mats of fibrin and mycelium with adherent kaolinite, very little free kaolinite</td>
</tr>
</tbody>
</table>

<sup>a</sup>2-layer, water: 65% (w/v) sucrose.
<sup>b</sup>Tryptone, yeast extract broth.
<sup>c</sup>Minimal salts medium.
particulate seemed to depend more on the organic material-to-kaolinite ratio than on the nature of the organic material. A more discriminating gradient would be required to separate these organic materials. Kaolinite was not removed from particulate substrate or from cells by this type of centrifugation, which would be inappropriate for removing enzyme from cell surfaces by kaolinite adsorption.

**Adsorption of Protein in General**

*and Protease in Particular*

to Kaolinite and Bentonite

My first approach to this was to take samples of *S. fradiae* or *M. chalcea* culture medium, filtered to remove all cells and spores, and add kaolinite or bentonite to one sample, mix the particulate well and remove it by centrifugation. I then tested the two samples for protein content, assuming the difference to be due to adsorption by the mineral particulate. The results obtained for kaolinite were reasonable. Fig. 1 contains a graph of the estimated protein content of dilutions of culture medium filtrate (CMF). Undiluted CMF contained about 65 µg of protein per ml. Addition of 1 mg/ml of kaolinite reduced the protein content to
Fig. 1. Estimation by the Lowry method of protein in cell-free filtrates of chemically defined medium in which Micromonospora chalcea had been cultured. (a) Concentration of protein in medium diluted with buffer (*). (b) Concentration of protein remaining in medium after adsorption with kaolinite (o) or bentonite (•) at concentrations of 1 or 2 mg/ml.
about 64 \( \mu g/ml \) and 2 \( mg/ml \) of kaolinite reduced it to about 55 \( \mu g/ml \). Estimated protein concentrations were the average of three determinations. When bentonite was used as the adsorbent, the apparent protein content was frequently higher than that of the original CMF and results were neither consistent from experiment to experiment, nor related linearly to protein or bentonite concentration. Fig. 1 includes a graph of one such determination, Fig. 3, another. Bentonite seemed to react in unpredictable ways with the reagents used for protein estimation. At higher concentrations, kaolinite seemed to increase the apparent protein content also.

In Fig. 2 and Fig. 3, the reduction of apparent protein content upon addition of 0.1 \( mg/ml \) of particulate is obvious, but so is the apparent increase of protein over the level of the control as increasing amounts of particulate were added. The sharp inflections of the curve in Fig. 3 at 40\% CMF and 0.1 \( mg/ml \) of bentonite and at 20\% CMF and 0.5 \( mg/ml \) of mineral are typical of the aberrant results obtained with bentonite. It is interesting to note in Fig. 3 that at concentrations of 1.0 \( mg/ml \) and 2.0 \( mg/ml \) of bentonite, the apparent increase of protein is essentially linear with increasing concentration of CMF and has the same slope as the control.
Fig. 2. Adsorption of protein by kaolinite. Dilutions of filtered medium in which Micromonospora chalcea had been cultured, were assayed after adsorption with 0, 0.1, 0.5, 1.0 and 2.0 mg/ml of kaolinite. Protein concentration was estimated by the Folin procedure (Lowry modification).
Fig. 3. Adsorption of protein by bentonite. Dilutions of filtered medium in which Micromonospora chalcea had been cultured, were assayed after adsorption with 0, 0.1, 0.5, 1.0 and 2.0 mg/ml of bentonite. Protein concentration was estimated by the Folin procedure (Lowry modification).
Although precise quantitation of protein adsorption to mineral particulates was not possible by this method, removal of protein from CMF and its presence on the mineral particulates could be demonstrated by assaying for enzymatic activity. The assay was done on the same culture which provided the data for Figs. 1-3. I resuspended the cells in some of the CMF and homogenized them, then removed them by filtration.

I resuspended the cells in buffer, diluting them to an absorbance at 520 nm of 0.035. I added 4 ml of cell suspension aseptically to matched cuvettes containing (o) no additions; (•) 3 mg of sterile Azocoll, or (+) 3 mg of sterile Azocoll and 10 mg of sterile kaolinite. Prior to each reading of absorbance, I centrifuged the cuvettes to sediment the suspended particulates. Fig. 4 is a graph of absorbance due to release of chromophore from the Azocoll by the proteolytic activity of the cells in buffer. In 17 hr, the cell control (o) changed only slightly. By contrast, hydrolysis of Azocoll (•) increased the absorbance from 0.035 to 0.7. Addition of kaolinite may or may not have influenced enzyme activity. In cuvettes containing kaolinite (+), released chromophore adhered to the mineral, making spectrophotometric measurement of released dye imprecise.
Fig. 4. Azocoll chromophore released by proteolytic activity of Micromonospora chalcea. Cell homogenate (absorbance at 520 nm = 0.035) was added to sterile matched cuvettes containing: (○) no additions; (●) 3 mg of sterile Azocoll; (+) 3 mg of sterile Azocoll and 10 mg of sterile kaolinite. All cuvettes were centrifuged to remove suspended particulates before absorbances were read at 520 nm.
The proteolytic activity of CMF is illustrated in Fig. 5. The absorbance at 520 nm of CMF (o) was low and remained virtually constant throughout the 17 hr period. That CMF contained proteolytic enzymes is obvious from the curve of absorbance due to dye release (●). Even in the presence of kaolinite, which adsorbed some of the chromophore, absorbance by released dye was considerable (+). To determine whether enzyme could be removed from CMF by kaolinite, I added the mineral (10 mg/ml) to CMF, agitated the tubes for 5 min, then centrifuged the suspension to remove the kaolinite. I decanted the supernatant and assayed it, resuspended the pellet in buffer and assayed it for enzymatic activity. Fig. 6 shows the extent of dye release by the supernatant and a control sample of untreated CMF. The supernatant, with no dye, had a low, constant level of absorbance (o). Dye release by the supernatant fluid (●) was considerable (reaching an absorbance of 0.5, after 17 hr) but was not so great as the activity of the intact CMF (+). The last, and most critical question to be asked in this experiment was whether enzyme adsorbed by kaolinite from CMF were active. I resuspended the kaolinite pellet removed from CMF, in buffer and measured absorbance due to the dye released (Fig. 7) by adsorbed enzyme. There was a marked release of chromo-
Fig. 5. Azocoll chromophore released by proteolytic activity of medium in which Micromonospora chalceca had been cultured and homogenized. Cells were removed by filtration. The filtrate was added to sterile, matched cuvettes containing: (o) no additions; (●) 3 mg of sterile Azocoll; and (+) 3 mg of sterile Azocoll and 10 mg of sterile kaolinite. All cuvettes were centrifuged to sediment suspended particles before absorbances were read at 520 nm.
Fig. 6. Adsorption of enzyme by kaolinite. Medium in which Micromonospora chalcea had been cultured and homogenized, was filtered to remove cells. Kaolinite (10 mg/ml) was added to the cell-free filtrate, tubes were shaken for 5 minutes, then centrifuged to remove kaolinite. The supernatant fluid was added to sterile, matched cuvettes containing: (o) no additions; and (●) 3 mg of Azocoll. As a control, cell-free filtrate to which kaolinite had not been added, was incubated with 3 mg of Azocoll (+). Absorbance at 520 nm of Azocoll chromophore released by residual enzymatic activity was measured after cuvettes were centrifuged to remove suspended particles.
Fig. 7. Azocoll chromophore released by proteolytic enzyme adsorbed to kaolinite. Kaolinite was shaken with and removed by centrifugation from cell-free filtrate of medium in which Micromonospora chalcea had been cultured and homogenized. The kaolinite was resuspended in 0.01M Tris buffer at pH 7.6, and added to sterile, matched cuvettes containing: (o) no additions; (●) 3 mg of sterile Azocoll.
phore, but its absorbance was diminished by the fact that kaolinite adsorbed the chromophore. The adsorbed chromophore could not be eluted from kaolinite (or bentonite) with 8M urea. No other elution procedures were attempted because a different experimental approach was used.

Degradation of Dye-Conjugated Collagen Substrates

by Cell-Free Enzyme Preparations

I used the same procedure to prepare (1) control CMF; (2) CMF to which kaolinite or bentonite had been added, mixed, then removed by centrifugation; (3) and particulates removed from CMF and resuspended in buffer. To each of these solutions or suspensions, I added 0.25 mg/ml of HPA or Azocoll, mixed the suspensions well, pipetted 1 ml of 2% agar at 47-50 C, agitated the mixture well, and poured the agar mixture into 15 mm x 55 mm petri plates.

My original intention was simply to assay chromophore release macroscopically and microscopically by measuring the area of diffused dye surrounding the collagen particles. I found that mineral particulates exposed to CMF and resuspended in buffer adhered to the HPA or Azocoll particles and effected decolorization and dissolution of the collagen at a rate much greater than
that of the original CMF. But dye diffusion could not be measured accurately in preparations containing mineral particulates. Again, the released chromophore adhered to the mineral particulates and was retained in the area of the collagen particle. However, I found that dissolution of the collagen and release of dye could be followed by time-lapse photomicrography of selected collagen particles. Even after a mineral-coated collagen particle had been completely dissolved, its site was delineated by the mineral particles still trapped in the surrounding agar.

Table 3 lists the estimated total protein content (the same data as those graphed in Fig. 1) and enzymatic activity of the 9 preparations. Active enzyme was obviously located on the resuspended particulates; not in the CMF from which the particulates had been removed. More enzyme activity was associated with bentonite than with kaolinite. This could be due either to bentonite's smaller particle size, hence more surface area per mg of material, or to its greater surface charge and exchange capacity.
Enzyme activity, estimated by rate and extent of chromophore release from Azocoll, (1) in culture medium filtrate (CMF₁) from Micromonospora chalcea cultured 3 days in chemically defined medium; (2) on particulates added to CMF₁, removed by centrifugation and resuspended in Tris buffer; and (3) in supernatant fluid (CMF₂) after addition and removal of mineral particulates. Azocoll + sample were poured in agar.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mineral Particulate</th>
<th>Concentration of Mineral</th>
<th>Total Protein Concentration of Fluid$^a$</th>
<th>48 hours</th>
<th>72 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMF₁</td>
<td>None</td>
<td>0</td>
<td>65 ± 2 μg</td>
<td>NC$^b$</td>
<td>Slight diffusion of dye</td>
</tr>
<tr>
<td>Buffer</td>
<td>Kaolinite</td>
<td>2 mg/ml</td>
<td>ND$^c$</td>
<td>Partial decolorization of some particles</td>
<td>Partial decolorization of most particles</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 mg/ml</td>
<td>ND</td>
<td>Considerable dye release</td>
<td>≥ 50% of particles completely decolorized</td>
</tr>
<tr>
<td></td>
<td>Bentonite</td>
<td>2 mg/ml</td>
<td>ND</td>
<td>≥ 50% of particles completely decolorized</td>
<td>Azocoll completely dissolved; only mineral outlines remained$^d$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 mg/ml</td>
<td>ND</td>
<td>≥ 50% of particles completely decolorized</td>
<td>Azocoll completely dissolved; only mineral outlines remained</td>
</tr>
<tr>
<td>CMF₂</td>
<td>Kaolinite</td>
<td>2 mg/ml</td>
<td>63 ± 1 μg</td>
<td>NC</td>
<td>Some diffusion of dye</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 mg/ml</td>
<td>79 ± 2 μg</td>
<td>NC</td>
<td>Some diffusion of dye</td>
</tr>
<tr>
<td></td>
<td>Bentonite</td>
<td>2 mg/ml</td>
<td>55 ± 2 μg</td>
<td>NC</td>
<td>Little, if any, change</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 mg/ml</td>
<td>64 ± 2 μg</td>
<td>NC</td>
<td>Little, if any, change</td>
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</table>

$^a$Estimated by Lowry method.  $^b$No change.  $^c$Not determined.  $^d$delineating sites of Azocoll in the agar.
Isolation of Streptomyces and Micromonospora Strains from Lake Erie

To compare reactions with mineral particulates and enzyme activity of recently isolated actinomycetes with the results obtained with laboratory strains, I used samples of surface or bottom water and mud from Lake Erie to isolate streptomycetes and micromonosporas. To discourage vegetative cells of bacteria and select for the more heat resistant mycelia and spores of actinomycetes, I incubated water samples at 70°C for various periods of time. Table 4 lists the strains isolated; the media (mostly selective for actinomycetes) used; and the concentrations of Rose Bengal included in media to inhibit fungi and bacteria. All individual actinomycete colonies were picked for isolation. In no case were any two colonies apparently of the same strain of organism. Because of an error in the collecting procedure, trichloroacetic acid was added to the water sample collected on 7/2/71. On the 24 plates streaked from that water sample, only one colony developed, the isolate designated S-3. (Naturally, this aroused my suspicions, and I inquired about the history of the sample.) This incident seems to say something about the durability of this strain of Streptomyces, or about the effectiveness of trichloroacetic acid in inhibiting
<table>
<thead>
<tr>
<th>Organism designated:</th>
<th>OSU#</th>
<th>Date of sample collection</th>
<th>Location</th>
<th>Incubation time (min) at 70°C</th>
<th>Medium on which colony developed</th>
<th>Concentration of Rose Bengal</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Streptomyces sp.</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>S-1</td>
<td>823</td>
<td>6/19/70</td>
<td>bottom mud 15 M depth</td>
<td>0</td>
<td>AIA^a</td>
<td>1:20,000</td>
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<tr>
<td>S-2</td>
<td>824</td>
<td>6/19/70</td>
<td>&quot;</td>
<td>10</td>
<td>JEN^b</td>
<td>1:30,000</td>
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<tr>
<td>S-3</td>
<td>827</td>
<td>7/2/70</td>
<td>surface water</td>
<td>0</td>
<td>YME^c</td>
<td>0</td>
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<tr>
<td><strong>Micromonospora sp.</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>M-1</td>
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<td>bottom mud 15 M depth</td>
<td>10</td>
<td>AIA</td>
<td>1:20,000</td>
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<tr>
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<td>826</td>
<td>6/19/70</td>
<td>&quot;</td>
<td>20</td>
<td>AIA</td>
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<tr>
<td>M-3</td>
<td>804</td>
<td>6/19/70</td>
<td>&quot;</td>
<td>20</td>
<td>JEN</td>
<td>1:30,000</td>
</tr>
<tr>
<td>M-4</td>
<td>805</td>
<td>6/19/70</td>
<td>&quot;</td>
<td>20</td>
<td>JEN</td>
<td>1:30,000</td>
</tr>
</tbody>
</table>

^aActinomycete isolation agar
^bJensen's starch, casein agar
^cYeast extract agar
microbial metabolism. The precursor of the S-3 colony had been in contact with the trichloroacetic acid solution for more than 3 hours before I streaked the water sample on the YME plate.

Studies of Organisms Isolated from Lake Erie

Screening of Isolated Actinomycetes for Culture Characteristics and Substrate Utilization

I wished to work with an organism which could utilize a wide spectrum of substrates, and which would culture rapidly in chemically defined medium, with or without addition of amino acids. I screened 3 strains of Streptomyces and 4 of Micromonospora for their ability to grow on a variety of media. Table 5 indicates the substrate utilization by all strains isolated. Fig. 8 elaborates on the growth in GM of the 3 most active strains relative to their growth in TYE. One streptomycete and 2 micromonosporas developed rapidly in all complex media. I chose to work with the isolate of streptomycete designated S-1, because it could be transferred directly from YME to chemically defined media. The micromonosporas required culture in TYE prior to transfer to GM.
<table>
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<th>Substrates</th>
<th>Organisms</th>
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<td></td>
<td>Streptomyces strains</td>
<td>Micromonospora strains</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>S-1</td>
<td>S-2</td>
<td>S-3</td>
<td>M-1</td>
<td>M-2</td>
<td>M-3</td>
<td>M-4</td>
</tr>
<tr>
<td>Actinomycete isolation agar</td>
<td>+(^a)</td>
<td>+</td>
<td>+</td>
<td>+(^a)</td>
<td>+(^a)</td>
<td>NT(^b)</td>
<td>NT(^b)</td>
</tr>
<tr>
<td>Jensen's agar</td>
<td>NT(^b)</td>
<td>+(^a)</td>
<td>+</td>
<td>NT(^b)</td>
<td>NT(^b)</td>
<td>+</td>
<td>+(^a)</td>
</tr>
<tr>
<td>Yeast extract, malt extract agar (YME)</td>
<td>+</td>
<td>+</td>
<td>+(^a)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Tryptone, yeast extract agar</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>GM(^c) inoculated from washed cells</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>NT(^d)</td>
</tr>
<tr>
<td>GM inoculated from YME slants</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+(^e)</td>
</tr>
<tr>
<td>MSM(^f) + chitin</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MSM + denatured collagen</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MSM + casein</td>
<td>+</td>
<td>NT(^b)</td>
<td>NT(^b)</td>
<td>+</td>
<td>+</td>
<td>NT(^b)</td>
<td>NT(^b)</td>
</tr>
<tr>
<td>MSM + cellulose + CH(_3)COONH(_4)</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>NT(^d)</td>
</tr>
</tbody>
</table>

\(^a\) Medium on which organism was isolated.
\(^b\) Not tested because ability to use that substrate had been demonstrated on another medium.
\(^c\) Minimal salts medium + glucose + asparagine + CH\(_3\)COONH\(_4\).
\(^d\) Not tested because organism was difficult to culture.
\(^e\) Very slight growth.
\(^f\) Mineral salts medium.
Fig. 8. Comparison of growth rates of 3 Actinomyces isolated from Lake Erie. The three strains, Streptomyces sp. S-1, Micromonospora sp. M-1, and Micromonospora sp. M-2, were tested for their ability to culture in a chemically defined medium containing minimal salts medium, glucose, asparagine and CH₃COONH₄.
Further Studies of Substrate Utilization
by Streptomyces sp. S-1

Utilization of Collagen

Because there has been some controversy (Mandel, 1961) as to whether streptomycetes produce a true collagenase enzyme, I tested the collagen preparation which I was using for trypsin sensitivity before and after autoclaving the collagen. Before autoclaving, a sample of collagen resisted tryptic digestion for 5 days at 27°C. A replicate sample, autoclaved, was completely dissolved in 3 hours. Because it has been reported that Ca^{++} is necessary for Clostridium histolyticum collagenase activity (26), I added 0.01M CaCl\textsubscript{2} to MSM (designated MSM-CaCl\textsubscript{2}). I added collagen to flasks of both MSM and MSM-CaCl\textsubscript{2}, and autoclaved all the flasks of the media. After flasks of MSM and MSM-CaCl\textsubscript{2} without collagen had cooled, I added unautoclaved collagen, and inoculated all flasks with S-1. One flask each of MSM and MSM-CaCl\textsubscript{2}, contained pure cultures of S-1 (determined by subcultures streaked on YME plates). Whether collagen had been autoclaved or not, growth of S-1 was better in media containing CaCl\textsubscript{2}. Figs. 9b, 15a and b, and Fig. 30 illustrate growth of culture S-1 in MSM containing collagen.
Fig. 9. Comparison of Streptomyces sp. S-1 cultured in (a) chemically defined medium containing glucose, asparagine and CH$_3$COONH$_4$; and (b) minimal salts medium containing 0.01M CaCl$_2$ and native collagen.
Utilization of Chitin

The growth of S-l in MSM + chitin was extremely rapid. A 24 hr culture of S-l with chitin as sole carbon and nitrogen source was equivalent to a 48 hr culture of S-l in the complex organic medium, TYE. Fig. 10 shows flasks of 26 hr cultures of S-l in MSM-2TB; MSM-S0_4-2TB + glucose (4 mg/ml); MSM + chitin (4 mg/ml); and MSM + chitin (4 mg/ml) + kaolinite (1 mg/ml). Fig. 11 is a composite of micrographs of mycelia from the 4 cultures. Because the chitin preparation was not necessarily free of other types of organic material, it was possible that some growth promoting material was present as a contaminant. Cralley (1968) had tested samples from the same chitin preparation for soluble carbohydrates (anthrone procedure) and amino compounds (ninhydrin procedure). She had found <0.14 μg of soluble carbohydrate and 0.5 μg of soluble amino compounds per gram of chitin. Since such low levels of nutrients would not account for the tremendous growth of S-l in chitin media, I decided to test for a trace material which might serve as a growth promoting substance.
Fig. 10. Macroscopic appearance of Streptomyces sp. S-1 cultured in minimal salts medium (MSM); MSM + glucose + NH₄SO₄; MSM + chitin, and MSM + chitin + kaolinite.

All flasks contained 26 hr cultures of standard inoculum. (a) S-1 cells in minimal salts medium with 0.002M Tris buffer; no carbon or nitrogen source was added. The cloudy appearance of the medium was due to precipitation of mineral salts in the absence of cell metabolism. (b) S-1 cells in minimal salts medium with 0.002M Tris buffer; (NH₄)₂SO₄, 2.64 mg/ml; and glucose, 4 mg/ml. Little growth had occurred, but cells had not autolysed. Slight frothing of the medium apparently was due to production of extracellular polymers by the streptomycete. Note the absence of mineral precipitate. (c) S-1 cells in mineral salts medium with 0.002M Tris buffer and 4 mg/ml of chitin. Growth was very heavy. (d) S-1 cells with medium as in (c) to which kaolinite (1 mg/ml) was added. Note the ring of cells-chitin-kaolinite-aggregate adhering to the flask wall (arrow) at the fluid level reached by the medium while the flask was on the rotary shaker.
Fig. 11. Microscopic appearance of Streptomyces sp. S-l cultured in minimal salts medium (MSM); MSM + glucose + NH₄SO₄; MSM + chitin + kaolinite.

All flasks contained 26 hr cultures of a standard inoculum. (a) S-l cells in minimal salts medium with 0.002M Tris buffer, with no carbon or nitrogen source added. The rounded appearance of the cells is typical of autolysis. (b) S-l cells in minimal salts medium with 0.002M Tris buffer; (NH₄)₂SO₄, 2.64 mg/ml; and glucose, 4 mg/ml. Little growth had occurred, but cells had not autolyzed as in (a). (c) S-l cells in mineral salts medium with 0.002M Tris buffer and 4 mg/ml of chitin. Growth was very heavy. (d) S-l cells with medium as in (c) to which kaolinite (1 mg/ml) was added.
Test for growth promoting factor associated with chitin:

To determine whether there were a growth promoting factor in MSM + chitin medium, which was (1) soluble in the medium, or (2) retained on the chitin through several days of incubation on the rotary shaker at 27 °C; and if soluble, if it were (3) able to promote growth in adequate media, or (4) able to substitute for various nutrients in deficient media, the following experiment was conducted.

Chitin (0.2 g) was placed in each of two flasks (A and B) each containing 100 ml of MSM. These were autoclaved, cooled, and placed on the rotary shaker at 27 °C for 7 days. Then the chitin was removed from flask A by centrifugation of the contents for 15 min at 12,000 x g. The supernatant fluid was filtered through a Millipore filter (pore size, 100 μm) and 5 ml of filtrate was added to 1 each of flasks containing 100 ml of one of the following media: (a) MSM-2TB; (b) MSM-2TB-SO₄; (c) MSM-2TB + glucose (4 g/liter); (d) MSM-2TB-SO₄ + glucose (4 g/liter) and (e) GM. From each of the flasks to which filtrate from flask A had been added, a 5 ml sample was removed aseptically and placed in a separate sterile culture tube. From each of the flasks containing media (a) - (e) to which
filtrate from flask A had not been added, a 5 ml sample was removed in the same way. Washed S-1 cells from a GM culture were homogenized. A standard inoculum of 1 ml was added to each of the flasks containing media (a) - (e), with and without filtrate from flask A. Flask B and a flask (C) of MSM to which the chitin removed from flask A was added, were also inoculated. The 10 culture tubes were each inoculated with a loop of Escherichia coli (strain 430) from a 19 hr GM culture. These tubes were incubated for 7 days at 37 C, with daily checks on turbidity.

There was no grossly observable difference of rate or of total amount of growth, of either S-1 or E. coli in media with and without the filtrate from flask A. There was no difference between the rate or amount of growth of S-1 cells in flasks B and C. If a growth promoting factor were present in the crude chitin, either it was not soluble, or greater quantities would be required to substitute for carbon or nitrogen in the deficient media. The fact that E. coli did not respond at all to addition of the filtrate to its media proves only that no metabolite useful to it was present.
Utilization of Cellulose

In cultures of S-1 in MSM-SO\textsubscript{4}-2TB + 4 gm/liter of cellulose, cell mass increased very slowly, but autolysis did not occur. However, S-1 inoculated into MSM-SO\textsubscript{4}-2TB containing kaolinite (1 mg/ml) did not autolyze either. In both cases, S-1 could be seen aggregated with the particulate. To insure that S-1 was utilizing cellulose as a carbon source, not merely surviving in its presence, I tested utilization of cellobiose by S-1, and the ability of S-1 to hydrolyze cellulose in agar.

Triplicate flasks of MSM-SO\textsubscript{4}-2TB + glucose (1 g/liter) and MSM-SO\textsubscript{4}-2TB + cellobiose (1 g/liter) were inoculated with a standard inoculum of S-1. After 14 days, cells were removed from the cultures by centrifugation and dry weights of cell pellets were determined. Statistical analysis of dry weights indicated no significant difference at the 1% level between cell masses of glucose and cellobiose cultures.

I prepared cellulose agar plates with agar about 5 mm deep. As the agar cooled, the cellulose settled to the bottom. The agar appeared opaque due to the layer of white cellulose particles.
S-l was streaked onto the plates from the following media: TYE; MSM-2TB; MSM-SO\textsubscript{4}-2TB + glucose, and MSM-SO\textsubscript{4}-2TB + cellobiose. The plates were placed over moist cotton in containers covered with parafilm and incubated at 27 C. Water was added to the cotton when necessary. The plates were examined daily for 2 weeks. There was no hydrolysis of cellulose visible macroscopically or microscopically, but surface colonies of S-l grew and spread, even on the plate streaked with S-l in MSM. After 6 weeks, plates were examined again; all 4 plates showed hydrolysis of cellulose. Plates streaked with S-l cultured in MSM-SO\textsubscript{4}-2TB + cellobiose and in MSM-SO\textsubscript{4}-2TB + glucose showed the most pronounced cellulose degradation. Macroscopically, the agar in those two plates appeared mottled, with patches of clear and opaque agar. The other two plates still appeared opaque, but microscopic examination revealed changes in the cellulose. Fig. 12a shows undegraded cellulose from an uninoculated area of a plate. Figs. 12b, 13, and 14 show progressive growth of S-l in the area originally occupied by cellulose. Figs. 13 and 14 were photographed from areas in which the agar appeared clear; Fig. 12b from the boundary of clear (c) and opaque (o) areas. Figs. 12 to 14 are of S-l streaked from the cellobiose culture. The plate streaked with S-l from TYE showed
Fig. 12. Hydrolysis of cellulose by *Streptomyces* sp. S-1. Microscopic appearance of cellulose agar plates: (a) shows an area where hydrolysis had not occurred. Macroscopically this area of the plate was opaque due to the suspended cellulose fibers. (b) is an area of the plate where partial hydrolysis had occurred; mycelia had infiltrated the area, some within the fibrils. Macroscopically this area of the plate appeared mottled. The area designated (c) was clear; the area (o) was opaque. (440X)
Fig. 13. Dissolution of cellulose by Streptomyces sp. S-l. (a) Mycelia had infiltrated the area originally occupied by cellulose in the agar. No fibrillar cellulose structure remained, but the streptomycete seemed to have been confined to intra-fibrillar areas. (b) Mycelia were not confined to the area originally occupied by the cellulose. Macroscopically, both areas of the plate appeared clear. (440X)
Fig. 14. Growth of *Streptomyces* sp. S-1 in cellulose agar after complete dissolution of the cellulose. (a) and (b) illustrate progressive growth of the mycelia in areas originally occupied by cellulose fibers. (440X)
only slight penetration of mycelia into the base of the agar, where the cellulose was. Degradation of cellulose had advanced to an intermediate degree in the plate streaked with S-1 in MSM.

Comparisons of the appearance of S-1 mycelia cultured in various media:

The mycelium of S-1 cultures had different appearances in different media. I did not investigate the causes of these differences but have recorded them photographically. Fig 9 (p. 58) compares (a) the mycelium of S-1 cultured in GM with (b) that of S-1 cultured in MSM-CaCl₂ containing autoclaved collagen (0.05 g/100 ml). The unidentified material clustered over the mycelium could be seen in collagen cultures of S-1 in MSM with or without CaCl₂, and with native or autoclaved collagen (see Fig. 15). But the material was most dense in cultures of S-1 in MSM-CaCl₂ + native collagen. Fig. 15 contrasts the appearance of (a) a 5 day culture of S-1 in MSM-CaCl₂ + native collagen; (b) a 3 day culture of S-1 in MSM + autoclaved collagen; about 10% of the colonies in that culture had assumed the ringed form pictured; and (c) a 3 day culture of S-1 in MSM + chitin. The fluffy, reflective appearance of the colony pictured was typical of the whole culture.
Fig. 15. Microscopic appearance of Streptomyces sp. S-l cultured in media containing chitin or native collagen. (a) Five-day culture of S-l in minimal salts medium with 0.002M Tris buffer; 0.01M CaCl$_2$ and 0.5 mg/ml of native collagen. (b) Three-day culture of S-l cells in minimal salts medium with 0.002M Tris buffer, and 0.5 mg/ml of autoclaved collagen. No CaCl$_2$ was added to this medium. About 10% of the mycelial masses had this ringed appearance. See also Fig. 31 for other mycelial clusters from the same culture. (c) Three-day culture of S-l cells in mineral salts medium with 0.002M Tris buffer and 0.5 mg/ml of autoclaved chitin. The fluffy appearance of colonies viewed with darkfield was typical.
Adherence of Mineral Particulates to Cells

Three procedures were used to demonstrate qualitatively and quantitatively that mineral particulates adhere to S-1 mycelium: photography, comparison of sedimentation velocities of cells, particulates, and mixtures of the two; and density gradient centrifugation on gradients which would separate mycelia from particulates unless a strong binding force were responsible for their adherence.

Photography

Effect of pH at constant ionic strength:

A culture of S-1 cells was homogenized and divided into 6 samples. The pH of one sample (8.2) was recorded, those of the other five samples were adjusted to 6.0, 7.0, 8.0, 9.0 and 10.0. Each of these samples was divided into 3 portions and a mineral particulate, kaolinite, illite or bentonite was added to one tube at each pH. No particulate was added to the sample at pH 8.2. The tubes were agitated well to mix the contents, pH's were rechecked and adjusted again where necessary, and distilled water was added to equalize total volumes. Samples were withdrawn by Pasteur
pipette for preparation of wet mounts, which were photographed. Fig. 16 shows the appearance of S-1 mycelia at various pH's after addition of kaolinite. Figs. 17 and 18 show S-1 mycelia under the same conditions with addition of illite and bentonite respectively.

In all cases, mineral particulates adhered to mycelia at all pH's tested. Optimal adsorption of kaolinite to cells seemed to occur at pH's 6 and 7. At higher pH's there was more free kaolinite present in the culture medium. This could be expected since kaolinite carries a net negative charge, and as pH increases, the cell surfaces would become progressively more anionic. That attraction between mineral and cells occurs at higher pH's is obvious from the photographs. This may be explained by McLaren's (51) observation that the actual surface pH of kaolinite is 2 pH units lower than the pH measured in the surrounding solution. Thus, the effective pH's of the cell-kaolinite interfaces in the preparations photographed may have ranged from 4 to 8, not 6 to 10.

Optimal adherence of illite to streptomycete mycelia seemed to occur at pH 8, but at all pH's tested the mycelial clusters were heavily coated with mineral. By contrast, at all pH's, much bentonite was free in the
Fig. 16. Adherence of kaolinite to Streptomyces sp. S-1 at various pH's. S-1 cells from a 10 day culture in glucose, asparagine, \( \text{CH}_3\text{COONH}_4 \) medium were homogenized in the culture medium. (a) control cells, pH 8.2 unadjusted, with no kaolinite added.

(b)-(f): kaolinite added (0.75 mg/ml).

(b) pH 6; (c) pH 7; (d) pH 8; (e) pH 9; (f) pH 10. (All photographs are 220X.)
Fig. 17. Adherence of illite to *Streptomyces* sp. S-1 at various pH's. S-1 cells from 10 day culture in glucose, asparagine, CH$_3$COONH$_4$ medium were homogenized in the culture medium. (a) control cells, pH 8.2 unadjusted.

(b)-(f): illite added (0.75 mg/ml).

(b) pH 6; (c) pH 7; (d) pH 8; (e) pH 9; (f) pH 10. (All photographs are 220X.)
Fig. 18. Adherence of bentonite to *Streptomyces* sp. S-1 at various pH's. S-1 cells from 10 day culture in glucose, asparagine, CH₃COONH₄ medium were homogenized in the culture medium. (a) control cells, pH 8.2 unadjusted.

(b)-(f): bentonite added (0.75 mg/ml).

(b) pH 6; (c) pH 7; (d) pH 8; (e) pH 9; (f) pH 10. (All photographs are 220X.)
culture fluid and only at pH 9 did the mycelium seem to be densely coated with mineral. Some of the difference in appearance of cell-bentonite aggregates may be due to the difference in particle sizes of the mineral. Individual particles of bentonite are so small as to be almost indistinguishable from mycelium at the magnification used. Density gradient centrifugation of cells cultured with bentonite in the medium (see p. 104) demonstrated unequivocally that bentonite, although scarcely visible, was adsorbed to cell surfaces and altered cell density.

Also, due to the small size of particles, bentonite had much higher charge-to-mass ratio. At the arbitrarily chosen particulate concentration, there may well have been more bentonite particles than available adherence sites on the cells.

Effect of time at which mineral was introduced to culture:

Flasks of GM, one containing illite (1 mg/ml) were inoculated with *Streptomyces* sp. S-1. After 3 days, illite (1 mg/ml) was added to a second culture and mixed briefly before samples were withdrawn from each flask for photography. Fig. 19a shows the characteristic appearance of S-1 colonies incubated with mineral
particulate. The illite adhered immediately to the inoculum. Subsequent growth of mycelium produced cell masses in which mycelium + illite formed a central core surrounded by mineral-free mycelium. When illite was added to a 3 day culture of S-1, particles of mineral adhered to peripheral mycelia (Fig. 19b). Because of the increase in cell mass during the 3 day incubation period, illite-to-cell ratio appears to be much lower. In neither case was free illite present in the culture fluid (pH 8.0 - 8.2). All mineral had adhered to mycelial surfaces.
Fig. 19. Effect of the time at which illite was introduced in cultures of Streptomyces sp. S-1. One mg/ml of illite was added to duplicate cultures of S-1 in glucose, asparagine, CH$_3$COONH$_4$ medium: (a) prior to inoculation with S-1 cells; (b) after 3 days inoculation of S-1 cells. Photographs were taken just after illite was added to culture (b). (550X)
Comparison of Sedimentation Velocities of Cells, Particulates and Mixtures of the Two

Effect of Cell-to-Particulate Ratio at Various pH's, at Constant Ionic Strength of Buffer

S-l cells from a 4 day GM culture were washed once with 0.01M Tris buffer, pH 7.6, and homogenized in fresh buffer. The pH of 5 samples was adjusted to 6.5, 7.0, 7.5, 8.0 and 8.5. To 4 ml cell samples in matched cuvettes, at a constant absorbance at 660 nm of 0.3, 1 ml samples of kaolinite in 0.01M Tris buffer at appropriate concentrations and pH's were added to give final kaolinite concentrations of 0, 0.2, 0.4, 0.6, and 0.8 mg/ml. Absorbance of the samples at 660 nm was read at 0, 10, 20, and 30 min.

The results are presented graphically in Figs. 20-24. The differences in sedimentation velocities of samples at different pH's were so obvious that no statistical analyses were made.

Effect of Cell Age at 5 pH's, at Constant Kaolinite and Cell Concentrations

In several experiments designed to determine optimal pH's and cell-kaolinite ratios for maximum cell-
Fig. 20. Sedimentation velocities of homogenized suspensions of Streptomyces sp. S-1 at pH 6.5, 7.0, 7.5, 8.0, and 8.5. Absorbance at 660 nm of the suspensions was read at 0, 10, 20 and 30 min.
Fig. 21. Sedimentation velocities of homogenized suspensions of Streptomyces sp. S-1 and kaolinite (0.2 mg/ml) at pH 6.5, 7.0, 7.5, 8.0 and 8.5. Absorbance of the suspensions at 660 nm was read at 0, 10, 20 and 30 min.
Fig. 22. Sedimentation velocities of homogenized suspensions of Streptomyces sp. S-1 and kaolinite (0.4 mg/ml) at pH 6.5, 7.0, 7.5, 8.0 and 8.5. Absorbance of the suspensions at 660 nm was read at 0, 10, 20 and 30 min.
Fig. 23. Sedimentation velocities of homogenized suspensions of Streptomyces sp. S-1 and kaolinite (0.6 mg/ml) at pH 6.5, 7.0, 7.5, 8.0, and 8.5. Absorbance of the suspensions at 660 nm was read at 0, 10, 20, and 30 min.
Fig. 24. Sedimentation velocities of homogenized suspensions of Streptomyces sp. S-1 and kaolinite (0.8 mg/ml) at pH 6.5, 7.0, 7.5, 8.0, and 8.5. Absorbance of the suspensions at 660 nm was read at 0, 10, 20 and 30 min.
kaolinite aggregation, I noticed that results differed when the cells used had been cultured for different lengths of time. In GM cultures inoculated from YME, S-1 usually began to form large numbers of conidia after 96 hours. To determine whether the age of a culture (and perhaps inception of sporulation) significantly influenced the sedimentation behavior of cell-kaolinite suspensions, I homogenized S-1 cells from 4 day and 5 day GM cultures and diluted them with distilled water to an absorbance of 0.3 at 660 nm. I added 4 ml of kaolinite (1 mg/ml) in Tris buffer (0.01M) at pH's 7.0, 7.5, 8.0, 8.5 and 9.0, to 1 ml samples of the 4 day and 5 day cells. Control tubes contained kaolinite at the various pH's and 1 ml of distilled water. I measured absorbances at 660 nm of triplicate samples of each combination of variables at 0 and 30 min. The difference in absorbance of each sample at the two times was used as an indication of the degree of sedimentation.

The data are graphed in Fig. 25. Sedimentation of aggregates of kaolinite and 4 day cells was greatest at pH 7.5, and decreased as pH increased above 7.5. At all pH's, aggregates of kaolinite and 5 day cells sedimented to a greater extent than kaolinite-4-day-cell aggregates, with maximum settling occurring at pH's from 7.0 - 8.5.
Fig. 25. Sedimentation of suspensions of 1 ml of (a) 4-day and (b) 5-day homogenized Streptomyces sp. S-1 added to 4 ml of kaolinite (1 mg/ml) in Tris buffer (0.01M) at pH's 7.0, 7.5, 8.0, 8.5, 9.0. Absorbance of the suspensions at 660 nm was read at 0 and 30 min. Cells and kaolinite (---); kaolinite (——).
A 2-way ANOVA of the decrease in absorbance of the suspensions showed that age was a significant factor (at the 5% level). Differences in pH significantly affected sedimentation of kaolinite and cells of either age (at the 1% level) but the interaction between age and pH was not significant. A 1-way ANOVA of the kaolinite control showed that the pH factor was highly significant at the 1% level.

**Effect of Buffer Concentration, at 5 pH's, at a Constant Cell-to-Kaolinite Ratio**

To determine whether the concentration of buffer significantly affected aggregation of S-1 cells and kaolinite, I mixed samples of 3 day S-1 cells, homogenized in distilled water (absorbance of cell suspensions: 0.3 at 660 nm), with equal volumes of 0.02M and 0.04M Tris buffers at pH's of 7.0, 7.5, 8.0, 8.5 and 9.0. Samples of kaolinite in Tris buffers at appropriate concentrations and pH's were added to the cell samples so that final concentrations of buffers were 0.01M and 0.02M; of kaolinite, 1 mg/ml. Control samples of cells only, or kaolinite only, at each buffer concentration and pH were included in the test. The absorbance of triplicate samples at each combination of
variables was measured at 660 nm at 0 and 30 min. The data are presented graphically in Fig. 26. Each point plotted is the average of 3 determinations. The differences in absorbance of tubes at 0 and 30 min. were analyzed by 2-way ANOVA. Table 6 lists the significance of buffer concentrations, pH, and the interaction of the 2 factors in the control and cell-kaolinite samples. The pH, buffer concentration, and their interaction all significantly influenced the rate at which kaolinite sedimented. Of the three effects, buffer concentration had the most pronounced influence on kaolinite sedimentation.

Kaolinite was the component of the cell-kaolinite samples which was influenced by buffer concentration and pH. Neither buffer concentration nor pH significantly influenced sedimentation of S-1 cells in the absence of kaolinite, but in the mixture of cells and mineral, both buffer concentration and pH significantly influenced the decrease in absorbance of the suspensions.

**Density Gradient Centrifugation**

To demonstrate that the stability of the adherence of S-1 mycelium to mineral particulates, I layered cell-mineral aggregates on linear density gradients of
Fig. 26. Sedimentation of suspensions of 1 ml of homogenized Streptomyces sp. S-1 added to 4 ml of kaolinite (1 mg/ml) in (a) 0.01M and (b) 0.02M Tris buffer at pH 7.0, 7.5, 8.0, 8.5 and 9.0. Absorbance at 660 nm was read at 0 and 30 min. Cells only (---); kaolinite only (-- --); cells and kaolinite (---).
Significance of buffer concentration, pH, and their interaction in the sedimentation of S-l cells and kaolinite.

Two-way analysis of variance.

<table>
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</tr>
<tr>
<td></td>
<td>pH</td>
<td>*</td>
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<tr>
<td></td>
<td>interaction</td>
<td>*</td>
</tr>
<tr>
<td>Kaolin + cells</td>
<td>buffer concentration</td>
<td>**</td>
</tr>
<tr>
<td></td>
<td>pH</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>interaction</td>
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<td>NS</td>
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</tbody>
</table>

$^a$ * : significant at the 5% level  
** : significant at the 1% level  
NS: effect not significant
Renografin and water (density range 1.0 - 1.46). The cells, if not attached to particulates, would remain in the upper portion of the gradient in the range of 1.12. The particulates, however, having a mean density of 2.7, would be expected to pellet below the pure Renografin. Cells to which no kaolinite was added, did indeed remain in the upper 2 ml of the gradient, but clumps of cell-mineral aggregates were distributed throughout the gradient tubes. Very little mineral pelleted. Fig. 27 is an interesting comparison among cells which had been incubated with the mineral in the medium; those to which particulate had been added after incubation, just prior to centrifugation; and cells to which no particulate had been added. Cells incubated with kaolinite or bentonite formed bands at a lower level in the tube (hence at higher density) than those to which kaolinite had been added just before centrifugation. The gradient tubes were fractionated and photographs made of cell clusters removed from the various fractions. Cells incubated with mineral had adhered to the mineral and formed clusters of cell and mineral which formed a core surrounded by subsequent growth. The cell clusters were large and dense. In the sample to which kaolinite had been added just prior to centrifugation, the mineral was found adhering to the periphery of cell clusters in
loose array. Micrographs of cell-mineral aggregates removed from different fractions show that as the density of the fraction increased, the kaolinite-to-cell ratio also increased (Fig. 28). Even at the forces exerted on the aggregates by the centrifugation through a gradient, there was virtually no free mineral at the bottom of the tubes and no free cells at the top of the gradient. Fig. 27 is a print made from a reversal slide; the dark areas appearing in the picture at the bottoms of the tubes are due to reflected light.

Centrifugation on a density gradient was also used to try to separate kaolinite from HPA. A linear Renografin gradient was layered with various kaolinite-to-HPA ratios. The results were the same as those for kaolinite-cell aggregates. HPA particles to which more kaolinite adhered, were lower in the gradient. Collagen, with a density of about 1.12 would not have penetrated the dense Renografin solution to lodge at a level where the density exceeded 1.45, had not the kaolinite remained attached to increase the density of the aggregate. Since the force of this centrifugation was 13,200 x g., it is obvious that kaolinite is not merely resting on the surface of the HPA particle in Fig. 29.
Fig. 27. Formation of cell-mineral aggregates of Streptomyces sp. S-1, demonstrated by density gradient centrifugation on a linear water:Renografin gradient. The 4 gradient tubes are pictured: (a) Sample of S-1 culture to which kaolinite was added just prior to formation of the gradient; cell-mineral aggregates were dispersed through a wide density range. (b) Sample of S-1 cells cultured with bentonite; cell-mineral aggregates formed a sharp band, with high density. (c) Sample of S-1 cells cultured with kaolinite; cell-mineral aggregates formed a diffused band in an area of greater mean density than the band in tube (a). (d) Sample of S-1 culture to which no mineral was added; cells remained near the top of the tube where the density of the suspending fluid was lowest. Mineral concentration was 0.5 mg/ml of culture medium. No pellets formed in the tubes. The photographs were printed from a reversal film. The dark areas at the bottoms of the tubes are due to reflected light.
Fig. 28. Adherence of kaolinite to Streptomyces sp. S-l demonstrated by density gradient centrifugation. (a) cells were cultured in chemically defined medium containing 0.5 mg/ml kaolinite; (b) and (c) 1.0 mg/ml of kaolinite was added to control culture after incubation. Samples were layered on linear water:Renografin gradients and centrifuged at 13,200 x g for 1 hr. Densities of fractions of the gradients were determined by measuring the refractive index of the suspending fluid. Densities of the aggregates pictured were: (a) 1.24; (b) 1.23; (c) 1.38. The density of cells was about 1.12; of kaolinite, about 2.7.
Fig. 29. Adherence of kaolinite to Hide Powder Azure demonstrated by density gradient centrifugation. Aggregates of kaolinite and Hide Power Azure (HPA) were layered on linear water:Renografin gradients. The density of HPA is about 1.12; of kaolinite, about 2.7. The density of the aggregate shown was about 1.45. Crystals of kaolinite can be seen adhering to the HPA particles (arrows).
Adherence of S-l Mycelium to Substrates

In considering the hypothesis that microbial substrate utilization is enhanced by intimate contact among organism, enzymes and substrates, it is important to know whether *Streptomyces* does adhere to particulate substrates. Into MSM-2TB containing collagen, MSM-2TB containing chitin, and MSM-SO₄-2TB containing cellulose, I inoculated S-l.

Collagen:

Fig. 30 is a micrograph of S-l mycelia surrounding particles of collagen. In a 3 day culture, not every mass of mycelium enveloped a mass of collagen, but every collagen particle examined had mycelium adhering to it.

Chitin:

Particles of chitin appeared macroscopically to remain free in the medium of a 3 day culture, but microscopic examination showed that colonies of S-l adhered to edges and surfaces (Fig. 31). Some small flakes of chitin were embedded in masses of mycelium.

Cellulose:

By the third day, little free cellulose remained in the culture fluid. Mats of cells and cellulose encircled the "high water line" of the shaker flask. Fig. 32
Fig. 30. Adherence of *Streptomyces* sp. S-1 to a particulate substrate: collagen. Cells were cultured for 3 days in minimal salts medium with 0.002M Tris buffer and 0.5 mg/ml of autoclaved collagen. (a) and (b) are two views of the same mycelial cluster surrounding a collagen fiber (arrow). (c) and (d) are two views of another mycelial mass adhering to a piece of collagen (arrow).
Fig. 31. Adherence of *Streptomyces* sp. S-l to a particulate substrate: chitin. Cells were cultured for 3 days in minimal salts medium with 0.002M Tris buffer and 4 mg/ml of chitin. Colonies can be seen adhering to the edges of a chitin flake surfaces (c) and (d) (arrows).
Fig. 32. Adherence of Streptomyces sp. S-1 to a particulate substrate: cellulose. Cells were cultured for 9 days in minimal salts medium with 0.002M Tris buffer, 1% (w/v) CH$_3$COONH$_4$, and 0.1 mg/ml of cellulose powder. (a) and (b) are two views, at different focal levels, of cells and cellulose stained with crystal violet. Strands of mycelium are wrapped around cellulose fibers and are bridging the area between 2 fibers.
shows a typical view of aggregates, stained with crystal violet, from a 14 day culture. Networks of mycelium enmeshed cellulose fibers, in many cases, binding several together.

Adherence of Active Enzyme to Mineral Particulates

Mineral unwashed after exposure to enzyme:

The procedure described for testing the activity of M. chalcea protease was used to test the proteolytic proficiency of S-1. CMF from a 4 day culture of S-1 was added to sterile kaolinite to give a final concentration of 0.4 mg/ml. The low concentration of kaolinite was chosen to increase the probability that all possible kaolinite surfaces would contact enzyme molecules. The kaolinite was removed by centrifugation and added to a sterile suspension of HPA (0.25 mg/ml) in 1 ml of Tris buffer at pH 7.6. The suspension was shaken and kaolinite-HPA aggregates allowed to settle. These were removed by Pasteur pipette and added to 5 ml of 1% agar at 46 C. One HPA particle (0.25 mm x 0.65 mm) was photographed at intervals for 135 min. Fig. 33 is a series of these pictures taken at 12, 32, 62, 90, 105, 120 and 135 min. (A similar preparation from CMF of M. chalcea required more than 48 hrs to accomplish the same amount of degradation and dye release.) Not only dye release was
seen by the microscopist and recorded by the camera. Loss of structure occurred at a rate which was readily recorded by 30 min. photographic intervals, and was visible to an observer during the latter half of the dissolution process. As I watched the HPA particle, strands of fiber seemed to break loose - sometimes with a snapping motion - and float in what appeared to be a liquid matrix within the area originally occupied by the collagen particle. Structural disintegration preceded dye release in some areas of the particle. A careful scrutiny of the photographs of Fig. 33 will reveal structures which have changed orientation between photographic exposures. Dye release can also be detected in the pictures not only by loss of density in the particle area, but by the increased density of the surrounding area. The mottled appearance of the background area of agar is due to diffusion of dye. One detail which can be seen very well in color films is the adherence of dye to the kaolinite which originally clung to the collagen particle. As the HPA particle decolorized, the surrounding kaolinite became progressively more blue.

Minerals washed after exposure to enzyme solution:

Pooled culture media from 4 day and 11 day GM cultures of S-1 were filtered to remove cells and spores.
Fig. 33. Dissolution of Hide Powder Azure (HPA) by Streptomyces sp. S-1 enzyme adsorbed to kaolinite. Kaolinite at 0.4 mg/ml was added to a cell free filtrate of glucose, asparagine, CH₃COONH₄ medium in which the streptomycete had been cultured for 4 days. The kaolinite was removed by centrifugation and added to 1 ml of 0.01M Tris buffer, pH 7.6 containing 0.25 mg of HPA. The suspension was shaken and kaolinite-dye aggregates allowed to settle. These were removed by Pasteur pipette and added to 5 ml of 1% agar at 46°C. One HPA particle 0.25 mm x 0.65 mm was photographed at intervals for 135 min. Times given are the number of minutes after first contact of HPA with kaolinite-adsorbed enzyme preparation: (a) 12 min; (b) 32 min. The arrow indicates an area where loss of structure has begun to occur. (c) 62 min. Dissolution of fiber is evident in several locations (arrows). Strands have reoriented at R. (d) 90 min. Strands in the area of the arrow could be seen moving in an apparently liquid matrix. (e) 105 min. The mottled appearance of the background agar is due to diffusion of dye from the site of the HPA particle. Movement within the particle was visible. (f) 120 min. Note the change in orientation of the strand indicated by the arrow. (g) 135 min. Dissolution was apparently complete when the particle was viewed microscopically, but the micrograph shows some material remaining (at arrows). The outline of the original particle has been preserved by the kaolinite which adhered to its surface. (All photographs are 230X.)
Sterile particulates (0.5 mg/ml) were added to samples of the CMF. One CMF sample was retained as a control, but was centrifuged with the other preparations each time. Samples were centrifuged at 40,000 x g for 1 hr. A sample of each supernatant fluid and the control was removed for protein estimation. The rest was decanted to another sterile centrifuge tube and another 0.5 mg/ml sample of the same particulate was added to the three supernatant fluids (designated KS₁, IS₁, BS₁) which had contained particulate. These were centrifuged again at the same force, for the same time. The mineral pellets (designated K₁, B₁, and I₁) removed by the first centrifugation, were washed once with sterile distilled water, and each was resuspended in sterile distilled water (2.0 mg/ml) for use in tests of enzyme activity. When the second samples were removed from the centrifuge, the supernatant fluids, (designated KS₂, IS₂, BS₂) were decanted, samples were removed for protein estimation and tests of enzyme activity. The particulates (K₂, I₂, B₂) were not washed, but were resuspended in distilled water (2 mg/ml) for enzyme assay. One ml of each sample, resuspended particulate, supernatant fluid or control CMF was added to 0.25 mg of sterile Azocoll; mixed well, and poured with 5 ml of 2% agar at 50 C. Plates were incubated at room temperature (23-25 C).
Table 7 lists the mean and standard error of the mean of the triplicate determinations of protein in each sample fluid. Fig. 34 represents graphically the rate of enzymatic release of Azocoll chromophore by the various particulate fractions and fluids tested. Inspection of Table 7 shows that apparent protein content decreased with the first addition of particulate, but increased with addition of the second sample of particulate. This confirms the statement which I made earlier, that the Folin procedure (Lowry method) is not too reliable when samples have contained samples of particulate material. Control tubes containing no enzyme, but an equivalent amount of particulate; or of enzyme preparations, particulate but no Folin reagents; both have shown that the increase in absorbance is not caused by turbidity of residual mineral. I have concluded that it must be due to reaction between the Folin reagents and some component of the minerals. The effect is less serious with kaolinite, but is of sufficient magnitude with illite and bentonite to render the Folin estimation worthless.

Presence of enzymatic activity on the mineral particulates is, in itself, an indication that protein has been removed from the CMF solution. In this test, I purposely kept the level of particulate below that
previously determined as sufficient to remove essentially all protein from solution. This was to "saturate" the mineral with protein, and a proportionately high level of available enzyme, to give optimal conditions for testing the residual enzyme adhering to the particulates after washing. Fig. 34 shows, as expected, that the level of enzyme in KS₂, IS₂, and BS₂ was still high; nearly that of the untreated CMF. Fig. 34 also shows clearly that washed kaolinite retains much more enzyme than washed illite or bentonite. This is probably the reason why kaolinite has been more effective as an "enzyme carrier" than the other minerals, even though bentonite lessened the enzyme activity of CMF to a greater extent, showing that it had removed more enzyme from solution.

Effect of pH on Enzyme Activity

Four centrifuge tubes, each containing 40 mg of kaolinite were sterilized. To each of the first two, 14 ml of CMF from a 4 day culture of S-1 in GM were added. To the third and fourth tubes, 0.01M Tris buffer, pH 7.3, and distilled water, respectively, were added. All tubes were agitated to suspend the kaolinite, then centrifuged. The supernatant fluids were decanted
Fig. 3. Adsorption of active enzyme to kaolinite, illite and bentonite. Mineral particulates were added to a cell-free filtrate (CMF) of chemically defined medium in which Streptomyces sp. S-1 had been cultured. Enzyme activity was assayed: in CMF (x); on mineral particulates added to and removed from CMF, then washed with distilled water (o); on mineral particulates added to and removed from the supernatant CMF from which the first particulates had been removed (●); in the supernatant CMF after removal of the second particulate sample (+).
Table 7

Estimation of protein (Lowry method) in cell-free filtrate of *Streptomyces* sp. S-1 culture medium (CMF); in supernatant CMF (KS₁, IS₁, and BS₁) after addition and removal of the mineral particulates kaolinite (K), illite (I), and bentonite (B); and in supernatant fluids (KS₂, IS₂, and BS₂) after addition and removal of a second sample of the same mineral particulate.

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>ESTIMATED PROTEIN CONCENTRATION (µg/ml)</th>
<th>STANDARD ERROR OF THE MEAN</th>
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into sterile culture tubes. The supernatant CMF and water, as well as the pellets, from tubes 2 and 4 were frozen and used the next day. The pellets from tubes 1 (CMF) and 3 (Tris buffer) were each washed in 10 ml of buffer. The wash buffers were collected aseptically and tested for enzymatic activity.

The washed kaolinite pellets from tubes 1 (CMF) and 3 (buffer) were each resuspended in 10 ml of buffer. Eight 1 ml samples of each suspension were distributed to centrifuge tubes and the kaolinite resedimented. The buffer washes from each set of eight tubes were pooled and assayed for enzyme activity. No activity was detectable. Despite the fact that one portion of kaolinite had been in contact with CMF, apparently no enzyme was eluted by the buffer wash. (Several other experiments confirmed this result. Once adsorbed, enzyme remained on kaolinite through several washes with Tris buffer in pH range 7.6 - 8.3).

The kaolinite pellets, now distributed in 16 tubes each containing about 4 mg of mineral, were each resuspended in 5 ml of 0.01M buffer containing Tris-maleate, phosphate, and acetate anions, with H⁺, Na⁺ and K⁺. One kaolinite pellet from CMF and a control pellet from Tris buffer were resuspended in 5 ml of
buffer at each of the following pH's: 1.8, 3.0, 4.0, 5.0, 6.0, 7.0, 8.3 and 9.2.

The kaolinite from CMF was difficult to resuspend at the pH's below 7. After the kaolinite had been thoroughly mixed with buffer, the tubes were centrifuged and supernatant buffers decanted for enzyme assays. No enzyme activity was detectable in any supernatant buffer.

The kaolinite pellets from buffers at pH 1.8 - 5.0 were resuspended in Tris buffer and the pH restored to 7.0. The kaolinite was sedimented again. Supernatant buffers were assayed, but evidenced no enzyme activity.

The 16 kaolinite pellets were assayed for enzyme activity. No detectable dye release or dissolution of collagen occurred in plates prepared with CMF-kaolinite exposed to the 3 lowest pH's (1.8, 3.0 and 4.0). There was slight activity in the CMF-kaolinite from buffer at pH 5. In the other preparations, enzyme activity was detectable but was low.

To assay the activity of the original CMF, the supernatant fluids and pellets from tubes 2 and 4 were thawed and the pellets resuspended in Tris buffer.
CMF-kaolinite carried much more activity than had the pellet in the mixed buffers tested the previous day. From the procedure used, it was not possible to determine whether loss of activity was due to failure of enzyme to adsorb to kaolinite, elution of inactive enzyme by the mixed buffer at the lower pH's, or inactivation of adsorbed enzyme. The total concentration of the original CMF (about 350 µg/ml) was too low for detection of protein at the dilutions effected by dividing the kaolinite into so many samples and resuspending each sample in buffer.

This experiment did indicate that freezing and thawing kaolinite-adsorbed enzyme did not destroy enzymatic activity.

To circumvent the possibility that enzyme was inactivated by an effect of the Tris-maleate, phosphate, acetate buffer itself, rather than by the pH factor, I adjusted the pH of samples of CMF to 5, 6, 7, 8, 9, 10, filtered the samples to sterilize them, and added sterile kaolinite (6 mg/ml). I removed a 1 ml portion of each sample to determine the pH after addition of kaolinite. Actual pH's were 5.4, 5.6, 7.0, 7.6, 8.4 and 9.2. Results of assays showed that at all pH's,
the enzyme activity was greater than it had been at corresponding pH's in the mixed buffer. In CMF activity was greatest at pH 9.2. The activity was equivalent at pH's 7.0, 7.6 and 8.4, but somewhat less than at pH 9.2. There was slight activity exhibited by enzyme adsorbed at pH's 5.4 and 5.6.

This procedure did not differentiate between inactivation of adsorbed enzyme in an acid medium and failure of enzyme to adsorb to kaolinite at low pH's, but the former possibility seems more probable. At low pH, proteins are more cationic and bind more strongly to clay minerals. With an increase in number of potential binding sites, there may have been greater deformation of enzyme configuration, resulting in loss of activity.

**Concentration of Enzyme from Dilute Solution**

To a CMF solution containing about 350 µg of total protein per ml, I added 2.5 mg/ml of kaolinite. Previous tests had indicated that this ratio of kaolinite to protein should reduce the protein content of the solution below detectable levels. The kaolinite was removed by centrifugation. Samples of the supernatant CMF and of the kaolinite pellet were assayed for
proteolytic activity. In the remainder of the supernatant CMF, I suspended an additional 1 mg/ml of kaolinite and assayed the suspension for enzyme activity. Fig. 35 is a flow diagram of the procedure, and indicates the results. There was much enzyme activity in the first kaolinite sample, and virtually none in the supernatant CMF. But addition of kaolinite to the supernatant CMF made residual enzyme in that solution detectable. When supernatant CMF was mixed with HPA and poured in agar, what enzyme there was, was distributed in 5 ml of agar. Presumably kaolinite, added to the solution, concentrated the enzyme and as the kaolinite adhered to HPA particles, effected localization of the enzyme on substrate surfaces. This would prevent dispersal of the enzyme throughout the agar.
Fig. 35. Concentration of protease from *Streptomyces* sp. S-1 culture medium filtrate by kaolinite. Kaolinite (2.5 mg/ml) was added to culture medium filtrate (CMF) and removed by centrifugation. The kaolinite and supernatant fluid were assayed for enzyme activity, which was high on the kaolinite but very low in the supernatant fluid. Addition of 1 mg/ml of kaolinite to the supernatant CMF concentrated the enzyme and made its detection possible.
CONCENTRATION OF ENZYME BY KAOLINITE

ENZYME + KAOLINITE

SUPERNATANT

PELLET, HPA, AGAR

ADDITIONAL KAOLINITE

SUSPENSION, HPA, AGAR

HPA + AGAR

+4

+3
DISCUSSION

Interactions among microbial cells and their extracellular enzymes, particulate substrates and clay minerals have been studied from several aspects, but no comprehensive or coherent view of the subject can be synthesized from the literature. Clay minerals have been found to influence microbial metabolism in \textit{in vitro} studies, but the mode of influence has been attributed to different mechanisms by different authors and probably does differ with changes in the microbes, substrates and minerals involved.

The role of clay minerals in the metabolism of soil organisms has been extensively investigated, but in considerations of aquatic microbial activity, the presence of mineral particulates in the milieu has, until recently, been largely ignored.

In attempting to form some picture, based on the available literature, of the array of interdependent interactions which must occur during the degradation of complex insoluble organic materials by lake organisms,
the following conditions could be postulated as a null hypothesis:

1. The mycelia of lake dwelling actinomycetes such as *Streptomyces* and *Micromonospora* (reported to utilize collagen, chitin and cellulose, as well as many other materials such as keratin and lignin) are normally covered with an adherent layer of fine clay particles, and may grow attached to larger particulate surfaces (the way they adhere to laboratory culture flask walls).

2. Fortuitous collisions between organisms and particulate substrates may result in the formation of temporarily stable aggregates, the stability mediated partially by adherent mineral species, and lasting as long as the substrate lasts.

3. Extracellular enzymes produced by organisms do not diffuse into the surrounding water, but are adsorbed to substrate and mineral surfaces.

4. The combination of these interactions has, for aquatic microorganisms, converted a potentially haphazard method of degrading substrates and recovering the components, into an efficient means of recycling organic compounds and acquiring nutrients.
To determine whether there is, in fact, any basis for making these assumptions, I have isolated and studied some aspects of each constituent interaction involved in the network comprising the null hypothesis.

For preliminary studies, to determine appropriate techniques, I worked with laboratory strains of *S. fradiae* and *M. chalcea*. They did utilize collagen and chitin readily, and cellulose slowly. The mineral particulates, kaolinite and bentonite, adhered to the mycelia of both organisms with enough tenacity to withstand separation on a water:65% (w/v) sucrose density gradient. Protein released into chemically defined culture media by *M. chalcea* was adsorbed by kaolinite and bentonite. Although the interference by mineral particulates prevented precise determination, by the Folin method, of the amount of protein removed from solution by the particulates, patent enzymatic activity located on the particulates, even after washings, demonstrated adequately that some protein was adsorbed to the mineral surfaces. Results obtained with HPA and Azocoll proved that dye release from substrate conjugated to a chromophore was a sensitive indicator of enzyme activity. Spectrophotometric quantitation of released dye was
impractical in systems containing mineral particulates because the chromophore adhered to the mineral, but this very situation served to illustrate that small molecules released from the substrate by enzyme activity were adsorbed to the local mineral particulates. The proteolytic activity evidenced by kaolinite and bentonite removed from culture medium containing too little enzyme to effect any decolorization of Azocoll without the mediation of mineral particulates, illustrated the way in which minerals may effect contact between enzymes and substrates occurring in extremely dilute environments.

To determine whether the sorts of results obtained with *S. fradiae* and *M. chalcea* could be achieved with organisms indigenous to a lake, I isolated three strains of *Streptomyces* and four of *Micromonospora* from Lake Erie and tested them for substrate utilization. Of the seven strains, two streptomycetes and one micromonospora cultured well only on media containing casein; starch and casein; tryptone and malt extract, or yeast and malt extract. (This *Micromonospora* grew poorly and was not tested on limited media.) One streptomycete and two micromonosporas far outstripped their laboratory counterparts in the rapidity with which
they proliferated on the entire array of substrates tested. This seems to leave no doubt that actinomycetes capable of recycling plant and animal debris do exist in Lake Erie.

One very significant result of these substrate utilization tests was the unequivocal demonstration that the streptomycete designated S-l was capable of degrading native collagen - a product resistant to tryptic hydrolysis. Since most methods used to sterilize collagen denature it to a trypsin sensitive form, many claims of collagenase activity are dubious; a general protease may have effected the reported results.

The extraordinary growth of several actinomycete strains in a salts medium containing chitin remains unexplained. If a growth promoting factor is present as a contaminant in the crude chitin preparation, it is not soluble, and is not a nitrogen or carbon source per se.

S-l proved to utilize cellobiose and cotton fiber cellulose. On agar plates, S-l not only produced cellulase(s) capable of completely hydrolyzing the fibers within six weeks, but also penetrated the agar to
a depth of 4 to 5 mm and formed colonies completely filling the spaces vacated by dissolution of the cellulose. It may prove disconcerting to taxonomers to learn that while I was examining the extent of cellulose degradation by S-1, I found chains of conidia formed in mycelia embedded in agar 4 to 5 mm below the surface.

Five interactions involving clay minerals occurred. Adherence of kaolinite, illite and bentonite to S-1 mycelia suspended in culture medium at a series of pH's from 6 to 10 was demonstrated photographically. The extent of adherence varied with pH and was not uniform among the minerals used, but at all pH's tested, aggregation of mineral and cells did occur. The most important fact was that cell-mineral interactions were greatest at the pH's encountered under normal lake conditions.

Tests of sedimentation velocity demonstrated the same phenomenon: interaction among cells and kaolinite was greatest at pH 8.0 - 9.0. The pH of the water and mud sample used to isolate organisms from Lake Erie was 8.4. Data from the sedimentation tests show that the amount of settling of cells was
negligible during a 30 min period, and was little affected by change in pH. Sedimentation of kaolinite was rapid at all pH's but the rate varied significantly among those pH's tested. This could be expected since charge sites at kaolinite surfaces influence the edge-to-face attraction among crystals. The sample of kaolinite which I used was self-flocculating at pH 8.0-8.5. The tremendous significance of these data lies in the difference between sedimentation rates of cells and of cell-kaolinite aggregates. All the data I obtained demonstrate overwhelmingly that cells and minerals do aggregate at the pH range normal to Lake Erie mud. These aggregates are stable, withstanding separation by density gradient centrifugation. Kaolinite also formed stable complexes with blood fibrin and collagen, greatly increasing the densities of the proteins. All of these data suggest that in water containing suspended clay minerals, bacterial cells and particulate protein substrates can be coated with mineral particles and would settle at rates largely influenced by the density of the minerals. Cell-substrate interactions, then would occur mainly in the bottom waters and sediments.

All three minerals tested removed enzyme from solution. In all cases, some enzyme was in active form.
Kaolinite proved able to concentrate enzyme from solutions too dilute to exhibit any activity without the mediation of the mineral. Adsorption of enzyme to kaolinite and subsequent adherence of the kaolinite to collagen resulted in enhancement of apparent enzyme activity, presumably by bringing the enzyme and substrate into intimate contact.

One implication of these results is somewhat disquieting. The effects of mineral particulates significantly altered cell densities, enzyme concentrations and enzyme reaction rates. Typical laboratory studies of microbial enzyme activities may hardly describe the actual events which occur in the presence of clay minerals. To assume, for example, that microbial activity in a lake may be measured by studying the activity of organisms sampled from the water column, may be grossly to underestimate the true activity.

Adsorption of organic matter on clay minerals is probably a mechanism with evolutionary advantages. Bernal (8) suggested that it was on the surface of kaolinite that the first dilute organic materials were concentrated sufficiently to allow for the formation of self-replicating units. Aggregation of substrates, cells and enzymes on mineral surfaces optimizes the organism's ability to erode nutrients from the
surfaces of resistant materials, and concomitantly returns the components of organic detritus to the nutrient pool.

Since clay minerals are implicated in the natural processes which recycle organic materials (30), investigation of their use in waste water treatment seems worthwhile. The rapidity with which the protease of S-1 disposed of visible sized particles of Azocoll suggests that mineral-adsorbed enzyme could be used to degrade selected waste materials into usable amino acids or carbohydrates. The use of enzymes linked to cellulose derivatives has proven feasible (6, 27, 28, 36, 37, 55). The enzymes are insolubilized and reusable.

The potency of actinomycete enzymes has received little attention. Nomoto and Narahashi (59) reported that a protease of \textit{S. griseus} was as active as the \textit{Bacillus subtilis} enzymes usually used commercially. Culture media from antibiotic and vitamin producing actinomycetes might prove to be a source of useful enzymes.

In the interactions among cells, enzymes, substrates, and clay minerals points of agreement or disagreement with previous workers are hard to determine.
Because the outcome of any particular interaction seems to vary with slight changes in many factors, comparisons between my results and those of any other worker are almost impossible to make. Since I was not studying the effects of minerals on microbial metabolism as such, my data do not fall into the same categories (metabolic rates, O$_2$ uptake, length of lag period, total cell mass) as those used by previous authors. Rather, my data should help to explain how some of the observations made by microbiologists can be interpreted in the light of concepts developed in the soil sciences.
SUMMARY

I investigated the interactions among actinomycete cells, their extracellular enzymes, substrates and clay minerals to determine what influence mineral particulates might have on microbial degradation of complex organic substrates.

Laboratory strains of Actinomyces and one Lake Erie isolate of Streptomyces could be cultured in a chemically defined salts medium containing any of the following carbon and nitrogen sources: glucose + L-asparagine + ammonium acetate; cellulose + ammonium acetate; collagen, or chitin. Five strains of actinomycetes isolated from Lake Erie rapidly degraded autoclaved collagen, chitin and cellulose. Mycelia enveloped the substrate particles or formed colonies on substrate surfaces.

Mineral particulates adhered to actinomycete mycelia with enough force to resist separation by density gradient centrifugation at 13,200 x g for 1 hr. Adherence of minerals occurred at pH's 6.0-10.0; the extent
varying with mineral species and pH. Cell age, and the concentration of the suspending buffer, significantly influenced the sedimentation rate of cell-kaolinite aggregates.

Density gradient centrifugation demonstrated adherence of kaolinite to Hide Powder Azure (HPA).

Kaolinite, illite and bentonite adsorbed protein from cell-force filtrates (CMF) of chemically defined medium in which actinomycetes had been cultured. Assay of CMF before addition of mineral; and of the supernatant CMF and sedimented mineral after removal by centrifugation, demonstrated adsorption of active enzyme by minerals. Distilled water washing reduced residual enzymatic activity on minerals in the order: illite > bentonite > kaolinite. Enzyme activity on minerals was low at pH's 5-7, and could not be detected at pH's below 5.

Proteolytic enzymes in solution were assayed by spectrophotometric measurement of azo chromophore released from Azocoll or (HPA). Enzyme adsorbed to mineral particulates was assayed by allowing the mineral to adhere to HPA, then pouring the aggregates with agar. Dye release and dissolution of HPA structure could be estimated visually and recorded photographically.
Presence of proteolytic enzyme in solutions too dilute to evidence activity by HPA-agar plate assay, could be detected by adding kaolinite to the solutions, removing the mineral and mixing it with HPA. The kaolinite apparently adsorbed enzyme, and by adhering to HPA, brought the enzyme in contact with the substrate.


Degradation of complex organic materials by microbial enzymes serves both to provide soluble nutrients for the microorganism and to recycle organic detritus. Mineral particulates have been reported both to accelerate and to retard microbial metabolism and enzymatic hydrolysis of mineral-adsorbed organic materials. In order to determine what influence clay minerals might have on microbial decomposition of particulate substrates, I investigated interactions among actinomycetes, their extracellular enzymes; the substrates, collagen, chitin and cellulose; and the clay minerals kaolinite, illite and bentonite.

Lake Erie isolates of Streptomyces and Micromonospora degraded collagen, chitin and cellulose more rapidly than did laboratory strains. Mycelia of
Streptomyces cultured in liquid media containing collagen, chitin or cellulose, enveloped substrate particles, or formed colonies on substrate surfaces.

Density gradient centrifugation at 13,200 x g for 1 hr on linear water:Renografin gradients did not separate cell-mineral or collagen-mineral aggregates. Minerals adhered to mycelia at pH's 6.0-10.0, the extent of adherence varying with mineral and pH. Cell age, pH, and concentration of the suspending buffer significantly influences the sedimentation rate of cell-kaolinite aggregates.

Mineral particulates adsorbed extracellular proteins from cell-free filtrates of chemically defined medium used to culture actinomycetes. Mineral-adsorbed protease hydrolyzed Azocoll and Hide Powder Azure (HPA). Enzyme activity was assayed by spectrophotometric measurement of azo chromophore released from Azocoll and HPA in suspension, or by visual estimation and time lapse photography of dye release and structural disintegration of HPA, effected by kaolinite-adsorbed enzyme. Distilled water washing reduced enzymatic activity on minerals in the order: illite > bentonite > kaolinite. No activity was detectable in Tris buffer used to wash enzyme-coated
kaolinite. Mineral adsorbed protease activity was low at pH < 7 and could not be detected at pH < 5. Presence of protease in solutions too dilute to assay could be detected by addition and removal of kaolinite. Added to HPA suspensions, and poured in agar, the kaolinite apparently concentrated enzyme and by adhering to HPA, brought enzyme in contact with the substrate.

These results indicate that aquatic microorganisms probably are normally associated with adherent clay minerals and may exist attached to particulate substrates. Extracellular enzyme adsorbed to minerals in a cell-mineral-substrate aggregate would be retained in the microenvironment, and allow for efficient substrate degradation and nutrient recovery by the microorganism.