SOIL BIOCHEMISTRY
BOOKS IN SOILS, PLANTS, AND THE ENVIRONMENT

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Preface

It has been almost ten years since the publication of the last volume in this series. This interim has been unfortunate, as there have been many exciting advances, as well as increasing interest, in the broadly termed field of soil biochemistry, which is a continually evolving discipline steadily gaining importance with the recognition of the prominent role of biochemical processes in the maintenance of the soil environment. Mounting evidence about the potential applications of these processes in environmental biotechnology has resulted in a strong desire for more knowledge about soil biochemistry and, specifically, the activity of microorganisms in soil. The horizons of soil biochemistry have been vastly broadened in the past few years from their origins in agronomy.

Soil microorganisms have a variety of beneficial roles in soil. For example, they are essential agents in soil formation and in the promotion of soil fertility, as they are the major agents in both the degradation of organic matter and the synthesis of humus as well as in the cycling of such essential elements as carbon, nitrogen, phosphorus, and sulfur. In addition, soil microorganisms and their enzymes are becoming prime candidates as alternative methods of pollution control as recent studies indicate their utility in the detoxification of pesticides, oil spills, nitrates, and heavy metals in soil and water. Moreover, the use of microorganisms and their products as control agents of both insects and pathogenic microorganisms is increasingly being considered as an alternative to chemical control agents.
In addition to the environmental applications of data obtained, it is obvious that the principles established by studies in soil biochemistry can also be applied to the treatment of soil to improve agricultural productivity. For these reasons alone, soil biochemistry is of great interest to scientists from many fields including, but not limited to, agronomy, forestry, horticulture, plant pathology, pedology, and environmental science.

Soil biochemistry is interdisciplinary and, as any legitimate field of inquiry, transcends national boundaries, as indicated by the contributors to this volume. Following the pattern established in past volumes of *Soil Biochemistry*, the topics in this volume are diverse and emphasize again the multidisciplinary nature of soil biochemistry. This diversity has been purposely maintained, and we hope that this multidisciplinary approach will continue to result in new insights into the basic principles of soil biochemistry. We intend to continue this valuable forum of scientific exchange with future volumes to be published at regular intervals and to include relevant contributions from such areas as soil and environmental sciences, microbiology, pedology, and plant pathology, especially as they relate to the application of biotechnological approaches.

We hope that this series will continue to serve as a basic reference for students, teachers, and researchers by providing a source of up-to-date information about soil and soil processes, new developments in soil biochemistry that will have an impact on agriculture and crop production, and the current status of alternative methodologies for the improvement of soil and crop quality. The large number of references included in each chapter of this volume should provide a good beginning for those wishing to gain in-depth knowledge of the current state of the art in a particular area of soil biochemistry.

Research in soil biochemistry will continue to provide information useful for improving crop production and soil quality, and for developing the biotechnology necessary to solve problems of environmental concern.

We thank the contributors to this volume and encourage other colleagues in the field to submit contributions to future volumes.

Jean-Marc Bollag  
G. Stotzky
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SOIL BIOCHEMISTRY
I. GENERAL ASPECTS OF CLAY-PROTEIN INTERACTIONS

Essentially all of the noncellular protein present in soil is associated with the clay mineral and humus fractions. More than 90% of the nitrogen (N) in the surface layers of soil is present in organic forms. As much as 50% of this nitrogen can be identified as amino acid-N derived from proteins. Obviously, there is a significant component of noncellular proteinaceous material in soil, and its very presence is evidence that it is resistant to microbial decomposition. This resistance is further demonstrated by the fact that only a small portion (<5%) of the soil organic N is mineralized annually [1]. Soils are also known to contain a wide variety of active cell-free enzymes [2], which display remarkable stability [3,4].

The persistence and stability of proteins, including enzymes, in soils are generally attributed to their association with clays and humus. As the result of the complex nature of soil and the association of soil clays and humus, the isolation of discrete clay-enzyme or humus-enzyme complexes for study has proved difficult. However, such complexes can be prepared in the laboratory using purified clays and proteins, and these complexes have been studied in some detail. Much of the work on clay-protein interactions has been detailed in previous reviews [2,5,6] and will not be covered in detail here, except to summarize the general concepts that have emerged from this work. In doing so, representative studies will be cited, but these citations will not be inclusive.
Proteins contain a variety of polar (e.g., C=O, O–H) and ionizable (e.g., NH₂, COOH) functional groups that can interact with clay mineral surfaces. Clay minerals possess a net negative electrical charge that is compensated by exchange cations, such as Ca²⁺ and Mg²⁺, on their surfaces. Proteins possess positive (as well as negative) charges and may become electrostatically bound to clays via cation exchange reactions. The amount of positive charge on the protein increases as the pH decreases, so that higher adsorption vis-à-vis ion exchange would be expected as the pH decreases below the isoelectric point of the protein. However, results from numerous studies have shown that protein adsorption by smectite clays is often greatest at or near the isoelectric point of the protein [7-10]. A decrease in positive charges associated with the protein as the pH increases toward the isoelectric point may require higher uptake to satisfy the negative charges on the clay. Formation of a more compact structure has also been suggested as a possible factor contributing to the increased adsorption of protein as the pH approaches the isoelectric point [5]. Other types of binding mechanisms that may be involved include van der Waals interactions, hydrogen bonding, and ion-dipole interactions with metal exchange ions on the clay surfaces [11].

The earliest investigations into the nature of clay–protein complexes were by Ensminger and Gieseking [12,13]. The reaction of smectite with gelatin and albumin in aqueous media resulted in the intercalation of the proteins, as shown by basal spacings of ~5 nm in these complexes. These studies and others [10,14-16] have provided evidence for the important role of cation exchange in the clay-protein reaction. This evidence included the observation by some workers [12,13] of higher protein adsorption at lower pH values, decreased adsorption resulting from chemical deactivation of the protein amino groups, and more facile exchange of lower-valence exchange cations. Protein uptake has also been shown to be directly related to the amount of exchangeable Na ions released into solution and the cation exchange capacity of the mineral.

Protein uptake by smectite depends on the exchange cation, decreasing in the order H⁺ > Na⁺ > Ca²⁺ > Al³⁺ [9,14,16,17]. Only H- and Na-smectite have been shown to intercalate protein, presumably as a result of two factors: the relative ease of displacement of H⁺ and Na⁺ via cation exchange and the greater accessibility of interlayer surfaces due to higher swelling. When fully hydrated, smectites saturated with polyvalent ions have only 1 nm between the aluminosilicate sheets, whereas Na-smectite expands to the individual crystallites, thus permitting entrance of larger molecules. Proteins may occupy the interlayer spaces of smectite clays in single or multiple layers. This has been demonstrated in the laboratory, primarily using Na-smectite, which undergoes extensive interlayer
Enzyme Interactions with Clays

expansion in water. Basal spacings reported for protein-smectite complexes range from ~1.4 to greater than 7.0 nm.

An exact description of the relation between basal spacing and the interlayer organization of adsorbed protein has proved difficult. However, it is clear that at least double layers of proteins may be intercalated and that adsorption onto clay surfaces does not always lead to alterations in the conformation of the protein. That clay-adsorbed enzymes retain their catalytic activities [6,14,18] is evidence that adsorption does not necessarily result in denaturation via conformation changes (e.g., uncoiling or unfolding) to more random forms. However, some proteins, notably gelatin and pepsin, tend to unfold when adsorbed by smectite [5,15]. Theng [5] concluded that the final conformation of the adsorbed protein depends on the balance between the intramolecular structural forces of the protein and its interaction with the clay surface. It was suggested that proteins with certain tertiary structures, e.g., fibrous proteins, tend to make greater contact with the clay surface and are, therefore, more likely to unfold.

It is important to emphasize that there is no convincing evidence for the presence of interlayer protein in swelling soil clays, although Theng et al. [19] found organic matter within the interlayers of acid soil smectite. The absence of interlayer protein-smectite complexes in soil probably results from several factors, including the predominance of limited-swelling divalent cation (e.g., Ca²⁺)-exchanged clays in soils, the easier accessibility of external surfaces, and the rapid microbial degradation of free protein. Thus, it appears that, in soils, the adsorption of protein by clays is limited to the external surfaces of swelling and nonswelling clays. It is, therefore, unlikely that the stability of protein and enzymes present in soils can be attributed to intercalation in swelling clays.

Proteins are also adsorbed on vermiculite, illite, and kaolinite but in significantly lower amounts than on smectite. For example, the amount of lysozyme bound at a pH near neutrality decreased in the order smectite > vermiculite = illite > kaolinite; the actual amounts of protein adsorbed for these four mineral types were approximately 1.0, 0.2, 0.2, and 0.02 g/g, respectively [10]. Lysozyme appeared to be intercalated by smectite, but adsorption was limited to the external surfaces of vermiculite, illite, and kaolinite. Similar results have been obtained for the adsorption of urease on kaolinite, vermiculite, and smectite [20].

The effect of pH on the adsorption of proteins on kaolinite is different from that on smectite. For kaolinite, the curve relating adsorption to pH typically shows a broad adsorption maximum, which may begin several pH units below the isoelectric point of the protein [7,21,22]. The mechanism of adsorption on kaolinite is primarily ion exchange, and the pH-adsorption curve has been attributed to pH-dependent charges associated with kaolinite and the protein [5].
II. CLAY-ADSORBED ENZYMES

In soils, clays and organic matter can serve as adsorbing surfaces for enzymes in exactly the same fashion as for proteins in general. In this regard, McLaren [23] referred to soil as "a system of humus- and clay-immobilized enzymes." The adsorption of enzymes by clay minerals results in significant changes in enzyme properties, such as pH optimum, stability, activity, and kinetics. These changes are due, in part, to the influence of clay surface properties (e.g., acidity) on enzymes and enzyme substrates and to the fact that catalysis apparently occurs at the mineral-solution interface rather than in the bulk solution.

The apparent pH optimum of clay-adsorbed enzymes is generally displaced one or two pH units to more alkaline values [5,24,25]. This shift in pH optimum to higher values occurs because the Brønsted acidity at the clay surface is significantly greater than in the bulk solution. This effect has been shown clearly by infrared studies of the protonation of ammonia at clay surfaces [26,27]. The difference in acidity between the clay surface and the bulk solution ($\Delta$PH) is generally about one to two pH units [5], although the exact magnitude of this difference depends on such factors as the clay mineral type, exchange cation, and water content [27]. The adsorbed enzyme experiences the acidity of the clay surface, and hence the bulk solution pH needs to be raised above the pH optimum of the enzyme to bring the clay surface to the optimum acidity. As a result, the apparent pH optimum of the adsorbed enzyme (i.e., the bulk solution pH corresponding to maximum activity) increases by an amount equivalent to $\Delta$PH. In contrast to the general observation of a shift in the pH optimum of clay-adsorbed enzymes, Sundaram and Crook [28] found no pH optimum shift for urease adsorbed on kaolinite. Bacteriolytic enzymes immobilized on smectite or unadsorbed showed maximum activity at a similar pH [29].

The stability of enzymes can be affected either positively or negatively by adsorption onto clay surfaces. For example, Garwood et al. [18] observed that glucose oxidase adsorbed on Na-smectite lost significant activity over a 75-hour period at 20°C, whereas little or no denaturation of the free enzyme was observed. Stability increased as the amount of enzyme adsorbed increased. This was attributed to less free mineral surface, which appeared to promote enzyme uncoiling. Coverage of the free mineral surface area by added tetra butylammonium ion also resulted in higher enzyme stability, supporting the concept that contact with the free mineral surface may promote enzyme denaturation.

The resistance of clay-adsorbed enzymes to enzymatic hydrolysis (proteolysis) has also been studied. In these systems, both the enzyme and its substrate (also protein) are likely to be associated with the mineral surface. In early studies, Ensminger and Gieseking [30]
found that smectite reduced the hydrolysis of protein by pepsin, whereas kaolinite had minimal effects. The proteolysis of denatured lysozyme adsorbed on kaolinite by chymotrypsin was approximately two-thirds of the rate obtained in homogeneous solution [31]. Subsequent work by Estermann et al. [32] showed that lysozyme adsorbed on smectite was even more resistant to proteolysis than lysozyme adsorbed on kaolinite. The higher degree of protective effect of smectite was attributed to lower accessibility of interlayer protein, leading to greater difficulty in forming the clay-substrate-enzyme complex [5]. Adsorption on clays may also result in higher rates of proteolysis [33, 34]. In this instance, clays appear to provide a concentrating surface for both enzyme and substrate, leading to enhanced proteolysis as compared with homogeneous solutions. In the former examples, the protection of adsorbed protein from proteolysis may have resulted, in part, from the lower activity of the adsorbed proteolytic enzyme and the lower availability of the adsorbed substrate protein.

The biological stability of enzymes may also be affected by adsorption onto clays. The biodegradation of protein complexed with smectite was evaluated by measuring changes in basal spacings and in the amount of evolved CO$_2$ after a 4-week incubation in soil [35-37]. Protein present in smectite complexes with low basal spacings of about 1.5 nm, corresponding to about 10% (w/w) of protein, was resistant to biodegradation. A relatively small amount of protein carbon was converted to CO$_2$ (~18%), and basal spacings remained constant. Protein present at higher loadings (80 to 90% w/w) on smectite was degraded, as evidenced by about 85% conversion to CO$_2$, with a concomitant decrease in basal spacings from about 3.0 to about 1.2 nm. Estermann et al. [32] observed little difference in the digestion of adsorbed (on kaolinite and smectite) and nonadsorbed protein by a soil inoculum. Again, basal spacings of the protein-smectite complexes decreased from about 4.6 to 1.7 nm, at which point degradation essentially ceased. Apparently, extracellular soil enzymes are unable to penetrate the interlayer regions of smectite-protein complexes with relatively small basal spacings (1.2 to 1.7 nm) but can access protein present in complexes with larger basal spacings of about 3.0 nm or greater. In these studies, it was noted that drying the clay-protein complex resulted in considerably less microbial attack, and the maximum protective effects of smectite were at low protein loadings. The microbial degradation of acid and alkaline phosphatases and urease adsorbed on homolonic clays was greatest with Ca-clays, intermediate with Na-clays, and lowest with Al-clays. The microbial release of CO$_2$ was higher from protein on kaolinite than from that on smectite or vermiculite [38].

Thus, it appears that the protective effects provided by clays to proteins, including enzymes, are most prominent at low loadings of proteins on the clay and when the proteins are adsorbed in the
interlamellar regions of swelling clays. Environmental factors, such as wetting and drying cycles, may also increase the stability of clay-adsorbed enzymes. However, as noted earlier, clear evidence of interlayer protein in soil clays does not exist, and the protective effects afforded by clays may be limited to those occurring on external surfaces. It is likely that other factors, such as association with soil organic matter and physical entrapment in soil aggregates, may also contribute to the remarkable stability of soil enzymes [4].

The activities of clay-adsorbed enzymes are often lower than those of free enzymes in homogeneous solution. This was shown in early studies by Mortland and Gieseking [39], where the presence of clays reduced the hydrolytic activity of phosphatases. The reduction in enzyme activity by clays decreased in the order smectite > illite > kaolinite. This reduction in activity is apparently related to the total surface area of the clay mineral [39,40]. In a similar fashion, the addition of clay to a fixed volume of clay-enzyme suspension resulted in decreased activity [29,39]. A general observation in these and other studies [e.g., 14] was that the activity of the enzyme increased with the amount adsorbed, i.e., that inhibition of enzyme activity was less at higher loadings. At loadings that exceeded monolayer exterior surface coverage, enzymes held by secondary surface adsorption forces appeared to retain high activity, as shown for arylsulfatase bound on kaolinite and smectite [40]. These results may be due, in part, to heterogeneity of binding sites on clay surfaces, where the most energetic sites, which would be occupied preferentially, lead to lower activity [5]. As Garwood et al. [18] suggested, an exposed mineral surface at low enzyme loadings may lead to greater denaturation and, therefore, less activity.

Numerous examples of decreased activities of clay-adsorbed enzymes exist; however, the extent of deactivation may vary widely. We have already referred to several instances in which the activities of clay-adsorbed enzymes were reduced. Several other studies have confirmed the reduction in enzyme activity resulting from adsorption on clays [22,29,41-49]. The general observation is that swelling clays with high surface area (smectites) produce greater effects than nonswelling clays with low surface area, such as the kaolinite group. There are, of course, exceptions to this general observation, and it appears that the adsorption of enzymes on clays does not necessarily lead to decreased activities. For example, Ross and McNeilly [43] observed that kaolinite had no effect on the activity of glucose oxidase, whereas increased activity of urease on kaolinite was reported by Sundaram and Crook [28]. Stotzky [6] found that the activity of catalase bound on various homoionic smectites was at least four times greater than that of free catalase. Similarly, Makhoul and Ottow [46] reported increased activity of alkaline phosphatase in the presence of Ca-smectite. Although Ross [49] observed a reduction
in activity of α-amylase in the presence of clays, kaolinite had a greater effect than smectite or illite, contrary to the general order of clay types. For invertase, the inhibitory effects of clays on activity decreased in the order smectite > illite > kaolinite, as is commonly observed.

Adsorption of enzymes by clays may also affect the kinetics of enzyme-catalyzed reactions. Although most such reactions involving clays follow Michaelis-Menten kinetics, the Michaelis constant, $K_m$, and the maximum enzyme velocity, $V_{max}$, may differ from those for the corresponding clay-free system. The general observation is that $V_{max}$ values either decrease or are unchanged for clay adsorbed enzymes and that $K_m$ values may be greater or smaller than those for the free enzyme, the former case being more common [50]. These effects are usually more pronounced for swelling clays (smectites) than for nonswelling clays, such as kaolinite. Thus, compared with free enzymes in homogeneous solution, clay-adsorbed enzymes often show lower reaction velocities and changed substrate affinities. Whether the $K_m$ of a clay-adsorbed enzyme increases or decreases depends in part on how the clay surface affects the distribution of the enzyme and its substrate [5]. If the charges on the clay-enzyme complex (generally negative) and the substrate are opposite, the concentration of the substrate near the surface will be greater than in the bulk solution, and the $K_m$ will decrease relative to that of the enzyme in free solution. If the charges are of the same sign the $K_m$ will increase, and if the substrate is uncharged the $K_m$ will be unaffected by these processes. However, other factors may affect changes in $K_m$, including steric hindrance to the approach of the substrate, conformational changes in the enzyme, and diffusional effects, especially for large molecules. As Theng [5] has pointed out, these other factors may be of overriding importance in affecting the kinetics of clay-adsorbed enzymes.

In early work on the kinetics of clay-enzyme complexes, Durand [42] found that the urea concentration required for maximum velocity was higher when urease was adsorbed on smectite than when urease was in solution; i.e., the $K_m$ for clay-adsorbed urease was greater than for unadsorbed urease. An increase in the $K_m$ of clay-adsorbed urease was also observed by Makhoul and Ottow [44]. The increase in $K_m$ produced by smectite and kaolinite was greater than that produced by illite. The $V_{max}$ values of urease were always higher in the clay-free system. The lower substrate affinity (higher $K_m$) was explained, in general terms, as resulting from steric hindrance of the adsorbed urease, especially on smectite. The $K_m$ value of glucoamylase acting on soluble starch of different molecular weights increased five to six times in the presence of H-clay for the higher-molecular-weight substrate but was unaffected for lower-molecular-weight starch, showing the importance of diffusional effects [51].
Studies of the kinetics of clay-adsorbed enzymes have focused on the activities of phosphatase enzymes. Inhibition of alkaline phosphatase activity by smectite, illite, and kaolinite have been reported [46]. The degree of inhibition was more significant at low substrate concentrations. These authors concluded that the $K_m$ increased for smectite and kaolinite but decreased in the presence of illite. However, the decrease in the $K_m$ for illite was questioned by Tabatabi [52], whose analysis of these data suggested that the $K_m$ was unchanged by illite (see also response by Makhoul and Ottow [53]).

Dick and Tabatabi [54] examined the effects of Na-smectite, Na-illite, and Na-kaolinite on the kinetics of acid phosphatase and pyrophosphatase derived from corn roots. For kaolinite, the $V_{\text{max}}$ was unchanged, but the $K_m$ values were increased three to five times. This behavior was explained in terms of partial competitive inhibition which occurs when substrate and inhibitor (clay) bind to the enzyme at different sites. The substrate has greater affinity for the free enzyme than it does for the enzyme-clay complex, so the $K_m$ increases in the latter system. Because both the enzyme-substrate and the clay-enzyme-substrate complexes produce product at the same rate, the $V_{\text{max}}$ remains unchanged. For smectite and illite, $V_{\text{max}}$ was reduced by a factor of 0.3 to 0.5, while the $K_m$ values were unchanged. A partial noncompetitive kinetic model was used to describe the effects of smectite and illite. According to this model, the clay-enzyme-substrate complex forms product at a reduced rate but is not unproductive. It was noted that sequential clay additions produced less inhibitory effects, so that the activity of clay-bound phosphatases was reduced but not eliminated. The inhibition of the phosphatases was attributed to enzyme inactivation resulting from adsorption.

### III. ENZYME INTERACTIONS WITH ORGANIC MATTER

In addition to adsorption on clays, enzymes may be associated with other soil constituents including clay-organic matter complexes [55] and soil humus [56-58]. A variety of enzymes have been extracted from soil as "enzyme-humic complexes," including urease [55,59], diphenol oxidases [60,61], proteases [62,63], and hydrolases [64,65]. These soil organic matter extracts all contained enzyme activity, which in some cases was resistant to proteolytic attack and stable at an elevated temperature. The exact nature of the association of enzymes with soil humus remains unclear, and as Ladd and Butler [56] have pointed out, the simultaneous extraction of soil humus and soil enzymes may be coincidental, with no particular association between...
the two. However, the formation of humus-enzyme complexes is plausible, and several mechanisms have been proposed. These include physical entrapment in humus or clay-humus particles, H-bonding, ionic bonding, and covalent attachment of the enzyme to soil humic substances [57].

One approach to the study of humus-enzyme complexes has been to prepare so-called model humic acids in the presence of certain enzymes. The enzyme may become covalently attached to the model humic acid polymer as it is being synthesized in the laboratory. This is accomplished by mixing the enzyme of interest with certain phenolic constituents (polyphenols, e.g., resorcinol, pyrogallol, vanillic acid) and a phenol oxidase enzyme (e.g., peroxidase, laccase). The phenols are enzymatically oxidized by the phenol oxidase to semiquinone-type radicals, which then couple to form a humic acid-like structure [66]. This process is referred to as oxidative coupling and has been implicated in the formation of natural humic materials in soil [67]. The resulting structures contain quinones with which amino groups present in the enzyme may react via nucleophilic addition to yield a humic acid-enzyme copolymer [68,69]. Alternatively, the enzyme may be adsorbed on the preformed model humic acid polymer. Work of this nature has been performed by Rowell et al. [70], who prepared p-benzoquinone copolymers with trypsin and pronase. The complexed enzymes had greatly reduced activities but displayed increased thermal stability. Urease and β-D-glucosidase have also been incorporated into model humic acid-type polymers [50]. Enzyme added during the oxidative coupling reactions was strongly associated with the polymer but displayed very low (<5%) or no activity. Enzyme added after the polymer was formed was easily removed by washing in buffer. The low enzyme activity in the copolymer could be overcome by eliminating certain phenolic constituents of the polymer, which were apparently acting as enzyme inhibitors. Thus, Sarkar and Burns [71,72] reported that copolymers of β-D-glucosidase and resorcinol or tyrosine retained >50% of the original enzyme activity, displayed enhanced thermal stability and resistance to proteolytic attack, and were stable for weeks when incubated in fresh soil. In contrast, enzyme adsorbed on preformed aromatic polymers did not display enhanced stability. It was suggested that the incorporation of enzymes during the synthesis of humic materials in soils may be an important immobilization and stabilization process.

Recently, Boyd and Mortland [34,73,74] presented the hypothesis that enzymes may be associated with natural organic matter via nonpolar interactions, and this is the subject of the following section. Hydrophobic residues on the enzyme would interact with similar regions of soil organic matter. This type of interaction has been shown to be important in the sorption of small organic compounds (e.g.,
pesticides) by soil organic matter \[75,76\] and also seems to be plausible for large organic molecules, such as enzymes. Nonpolar interactions between hydrophobic residues of peptides and reverse-phase liquid chromatography columns have provided the basis for the chromatographic separation of peptides \[77,78\]. Hydrophobic interactions, such as those proposed by Boyd and Mortland for enzyme-humus complexes, have also been shown to be involved in the attachment of baculoviruses to hydrophobic surfaces, such as octyl-Sepharose beads and leaf surfaces \[79\].

IV. ENZYME INTERACTIONS WITH CLAY-ORGANIC MATTER COMPLEXES

Although considerable research has been performed on enzyme-clay and enzyme-organic matter interactions \[5,6,50\], very little information exists on interactions of enzymes with natural clay-organic matter complexes. This is a reflection of the paucity of research on natural clay-organic complexes in general. Relevant questions seem to be: "How does soil organic matter in a free condition compare with that complexed with clays, with respect to composition and chemical and physical properties? Can it be assumed that the data obtained in organic matter studies apply to organic matter complexed with clay?" Studies by E. A. Paul (personal communication) suggest differences between these two categories of organic matter, whose separation for appropriate analysis appears to be extremely difficult.

Though little work has been done on enzyme interactions with natural clay-organic systems, some work has been performed with synthetic clay-organic complexes. Most of this has been with systems in which specific organic cations are adsorbed on the clay surface and that complex is then used as an adsorbent for enzymes \[18,34,73,74\] (T. J. Pinnavaia, M. M. Mortland, and S. A. Boyd, U.S. Patent No. 4,605,621, 1986). When a long-chain alkylammonium cation, such as hexadecyltrimethylammonium (HDTMA), is placed on the cation exchange complex of smectite, it completely changes the surface properties from a hydrophilic nature, due to hydratable metal cations, to a hydrophobic and, thus, organophilic character \[80\]. Depending on the specific organic cation used, very different sorptive properties for enzymes \[34\] and for organic compounds, in general \[81\], may result. The maximum loading levels of urease on four different organic-smectite complexes are shown in Figure 1. These data show tremendous variation in enzyme adsorption capacities, depending on the organic cation on the smectite. In general, the more hydrophobic the organic cation, the greater the adsorption capacity for urease and the lower the enzyme activity in the supernatant. For cations of the form \[(\text{CH}_3)_3\text{NR}\]^+, adsorption increases with the size and hydrophobicity of the hydrocarbon tail (R group).
Enzyme Interactions with Clays

Figure 1 Urease activity in supernatant solution as a function of urease loading on four smectite-organic complexes: TMP$^+$ is trimethylphenylammonium smectite, HDPy$^+$ is hexadecylpyridinium smectite, Fe(bipy)$_3^{2+}$ is tris(2,2'-bipyridine)iron(II) smectite, and HDTMA$^+$ is hexadecyltrimethylammonium smectite. Ammonia production by the supernatant indicates presence of unadsorbed urease. From Boyd and Mortland [34].

This strongly suggests that adsorption is by nonpolar interactions between hydrophobic residues of the enzyme and the hydrophobic surface of the clay-organic complex.

Garwood et al. [18] contrasted the adsorption and activity of glucose oxidase on Na-smectite with those on HDTMA-smectite. As discussed earlier in this chapter, enzyme adsorption on metal ion-saturated clay is affected very much by pH, as pH will determine the extent of the ionic nature of the enzyme and, consequently, its ability to interact with clay surfaces via coulombic binding. Thus, glucose oxidase adsorbed strongly at pH values below and near the isoelectric point of the enzyme, but when the pH was raised above that, some enzyme desorbed. In contrast, glucose oxidase adsorbed strongly on HDTMA-smectite, regardless of pH, and could not be removed by successive washings with water. This was in contrast to results for enzymes added to preformed model humic polymers, which were easily displaced by washing with buffer [50]. The kind of adsorption observed by Garwood et al. [18] for HDTMA-smectite was called
Chapter 1

Figure 2  A model for the hydrophobic binding of enzymes (E) on a clay-organic complex composed of hexadecyltrimethylammonium (HDTMA)-exchanged smectite. The net charge of adsorbed enzymes may be positive (E⁺), or negative (E⁻), or neutral (E), depending on the isoelectric point of the enzyme and the pH. Adsorption results from nonpolar interactions between hydrophobic residues of the enzyme (dark portions of circles) and the hydrophobic alkyl tails of HDTMA. From Garwood et al. [18] and Boyd and Mortland [34].

hydrophobic binding, as it was thought that hydrophobic portions of the enzyme interacted with the projecting alkyl groups of HDTMA. Although the energy involved for one adsorption site is probably quite small, a summation of a large number of these kinds of interactions for a single enzyme molecule could lead to a very large binding energy. Figure 2 shows a schematic representation of this interaction. Another feature of the glucose oxidase adsorbed on HDTMA-smectite was that its activity was 50 to 60% that of the same enzyme in homogeneous solution, whereas its activity when bound on Na-smectite was only about 5 to 10% that of the free enzyme. In addition, although other workers have shown shifts in the pH profile by as much as one to two pH units to higher values for enzymes bound on clays [5], the glucose oxidase bound to HDTMA-smectite had the same profile maximum as the enzyme in homogeneous solution.
Boyd and Mortland [34] studied the activity of jack bean urease immobilized on HDTMA-smectite and found that the immobilized urease was as active as the free enzyme. When the loading was increased from 1 to 4% (w/w), there was no decrease in enzyme efficiency. A similar pH profile was observed for free urease and for urease adsorbed on HDTMA-smectite. The adsorbed and free enzymes also had very similar Michaelis-Menten parameters. However, the adsorbed urease had less thermal stability and less resistance to proteolysis than the free enzyme. In the latter case, it was suggested that the adsorbing surface of alkyl groups provided a concentrating region where both urease and pronase were brought together by adsorption, and proteolysis proceeded at a greater velocity than in homogeneous solution.

Very subtle changes in the nature of the organic exchange ion on the clay surface produced striking differences in the activities of adsorbed enzymes [73,74]. For example, arginase was completely inactive when adsorbed on HDTMA-smectite, yet it was quite active when adsorbed on hexadexylpyridinium (HDPy)-smectite or on smectite whose exchange complex was saturated with tris(2,2'-bipyridine)-iron(II) ([Fe(bipy)₃]²⁺). On the other hand, urease, either jack bean or bacterial, was active on all three clay-organic complexes.
Table 1 Activity (+) or Absence of Activity (−) of Different Enzymes Immobilized on Three Different Smectite–Organic Complexes

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Exchange cation(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[Fe(bipy)(_3)]^{2+}</td>
</tr>
<tr>
<td>Urease</td>
<td>+</td>
</tr>
<tr>
<td>Arginase</td>
<td>+</td>
</tr>
<tr>
<td>Pronase</td>
<td>−</td>
</tr>
<tr>
<td>Glucose oxidase</td>
<td>ND(^c)</td>
</tr>
<tr>
<td>Horseradish peroxidase</td>
<td>ND</td>
</tr>
</tbody>
</table>

\(^a\)From Boyd and Mortland [74].

\(^b\)[Fe(bipy)\(_3\)]^{2+} = \text{tris}(2,2'\text{-bipyridine})\text{iron}(II); HDTMA\(^+\) = \text{hexadecyltrimethylammonium}; HDPy\(^+\) = \text{hexadecylpyridinium}.

\(^c\)ND = not determined.

When a combination of urease and arginase was adsorbed on the latter two complexes, the following two-step reaction occurred:

\[
\text{arginine} \xrightarrow{\text{arginase}} \text{ornithine} + \text{urea} \xrightarrow{\text{urease}} \text{CO}_2 + 2\text{NH}_3
\]

This is illustrated in Figure 3, where arginine was used as a substrate and ammonia production was monitored over time.

A summary of the activity or nonactivity of several enzymes when immobilized on three different clay–organic complexes is shown in Table 1. All the enzymes were presumably bound by similar nonpolar interactions (i.e., hydrophobic bonding), yet some were active and others inactive on the same matrix. This suggests very striking microenvironmental effects on the enzymes. Perhaps these observations reflect the relation of the adsorption site on the enzyme to its active site, wherein there is no activity because of steric problems if these sites are close together, but if they are far removed from each other the active site can function unhindered.

The degree to which the foregoing synthetic clay–organic complexes resemble natural clay–organic complexes is open to question. The synthetic systems, such as HDTMA–smectite, are surely more hydrophobic, as natural organic materials possess some areas of polarity, as the result of carboxyl or hydroxyl groups, as well as the more hydrophobic zones where hydrophobic bonding could occur. This is supported by the observation that the organic matter normalized partition coefficients (\(K_{om}\)) for the sorption of hydrophobic
molecules, such as benzene and trichloroethene, on HDTMA-smectite are larger by an order of magnitude than on natural organic matter [81]. It seems that, in soils, this type of interaction between enzymes and soil organic matter may be important. The partitioning of small organic molecules into soil organic matter apparently occurs by similar forces, and this has been shown to be an important mechanism in the sorption of organic compounds by soils [75,76]. Although HDTMA-smectite complexes did not provide resistance to proteolysis for adsorbed urease, hydrophobic sites in natural soil organic matter may be sufficiently removed from one another that interactions between adsorbed enzymes (e.g., urease) and proteases would be limited. Also, the subtle effects of different organic surfaces on enzyme activities may have an important role in stabilizing enzymes held on hydrophobic sites. If these same sites result in deactivation of adsorbed proteolytic enzymes, while maintaining the activity of other soil enzymes, such as urease, this would result in increased stability. The highly selective effects of different organic surfaces on enzyme activities, as shown in Table 1, where some enzymes were active and others inactive on the same surface, would seem to make this possible.

V. CLAYS AS PROTOENZYMES

A number of scientists have assigned a role to clay systems in chemical evolution and even in the origin of life [82]. This is a natural thought, considering the fact that clays have a number of properties that suggest the possibility of such functions. These clay characteristics are high surface area, cation exchange capacity, and catalytic properties. In addition, it is certain that clays existed on the early Earth as a consequence of weathering processes, and in fact they are ubiquitous in the earliest sedimentary rocks on the Earth's surface. Clays have been given a prominent role in petroleum formation through their catalytic effect on plant and animal residues [83]. Although clays are generally not as specific in their catalytic abilities as are modern proteinaceous enzymes, such properties may, nevertheless, have been important in the evolution of complex organic species on the early Earth. Some of these complex organics formed initially by interaction with clay may, by further change, have evolved into the more specific organic catalysts (enzymes) and ultimately into self-replicating systems.

Reviews of the catalytic properties of clays describe in detail some of these processes [84-86]. No attempt will be made here to recapitulate all these reactions. Rather, general comments about clay-catalyzed reactions, as related to natural systems, will be stressed.
Clays have intrinsic catalytic properties that, as in homogeneous catalysis, may proceed via Brönsted or Lewis acidity. However, the planar and internal surface of some clays provide a unique environment wherein the reaction may be modified, so that the products may be quite different from those found during the course of homogeneous catalysis. In a sense, this property is perhaps analogous to the steric constraints of enzyme structures that result in their selectivities for substrate. The Brönsted acidity on clay surfaces may arise from one of the following: (1) exchangeable H\(^+\) on cation exchange sites, (2) H\(^+\) arising from hydrolysis of water molecules associated with metal cations on exchange sites, and (3) protonated bases (i.e., amines) that can donate their H\(^+\) to stronger bases. The surface acidity is obviously related to the nature of the exchange cation \([27]\). The more electronegative an exchangeable metal cation (Mn\(^+\)) is, the stronger will be its ability to hydrolyze associated water molecules and produce H\(^+\). For example,

\[
\text{M(H}_2\text{O)}_{x}^{n+}\text{-clay} \rightleftharpoons \text{M(H}_2\text{O)}_{x-1}^{n+}\text{-OH-clay} + \text{H}^+ 
\]

Another factor in clay systems is water content. It has been demonstrated that clay surfaces become stronger Brönsted acids as water content decreases \([27]\).

Examples of some reactions catalyzed by Brönsted acidity on clays are the inversion of sugars, ester hydrolysis \([87]\), and the conversion of atrazine to hydroxyatrazine by H-smectite \([88]\). Peptide formation from clay-amino acid complexes via amide linkage through dehydration and surface acidity has been reported \([89]\). Lehav et al. \([90]\) reported peptide formation from glycine on clays in an environment of fluctuating dry and wet conditions. They suggested that this would simulate a lake bed alternately wet and dry through the seasons, resulting in catalysis during the dry regime, because of development of the stronger Brönsted acidity and dehydration, followed by possible extraction of the peptide from the clay during the wet cycle. The initial presence of complex organic molecules, such as amino acids, is inferred, as they have been found in carbonaceous chondrites (meteorites), suggesting they may have been present from primordial times \([91]\). Amino acids and other biomolecules are also presumed to have been formed from simpler molecules present on the prebiological planet \([92]\).

Clays may catalyze various reactions via Lewis acidity, as they may have various sites in their structures that act as electron acceptors. These include structural aluminum and transition metal cations at edges of the mineral and exchange cations, particularly transition metal cations with unfilled d orbitals. Even when iron occupies sites within the octahedral layer of 2:1 layer silicates, it may take part in
redox reactions by electron acceptance from or donation to external reactants. Examples of reactions catalyzed by the Lewis acidity of clays are found in reviews by Solomon and Hawthorne [84], Rupert et al. [86], and Laszlo [85]. When Fe(III) and Cu(II) occupy the exchange sites in smectites, they can act as electron acceptors for aromatic- and alkene-type molecules. The unsaturated organics donate $\pi$ electrons from the double bonds to the metal cation, which is reduced, forming, in turn, radical cations of the organic. These radical cations may then undergo further reaction, i.e., polymerization [93-100]. Although this is not true catalysis, as Cu(I) is a product of the reaction, it acts as a catalyst, as the Cu(I) is readily oxidized back to Cu(II) in air.

Clays may facilitate the polymerization of phenols to form humic-like materials, particularly when the exchange ion is Fe(III). Minerals in soils have, in fact, been assigned a primary role in the abiotic formation of organic matter. In laboratory experiments, in particular, minerals with transition metal ions (e.g., Fe, Mn) on the surface or within the structure (both clay minerals and oxides) have been shown to accelerate greatly polymerization reactions of various phenols, leading to humiclike material [101-105]. Goodman and Siegel [106], however, showed that the presence of even an inert surface, such as cellulose, causes up to a threefold increase in the oxidized products of pyrogallol.

To what degree do clays approach the selectivity of proteinaceous enzymes in catalysis or adsorption of substrate? As the discussion above indicates, there are reactions that are rather generally catalyzed by clays without a high degree of specificity. However, as also mentioned before, swelling clays provide internal surfaces between the layer silicate structures that, by their charge densities and steric constraints on substrate, may drastically modify reactions. An example of this is shown by the work of Weiss [92] on the dimerization of oleic acid using a smectite catalyst. His results indicated that charge density on the mineral surface was very important, in that reactant pair proximity was crucial. An optimum distance between charge sites gave the maximum reaction, whereas more or less distance decreased reaction. Other examples are found in the review by Rupert et al. [86]. It is likely that many specific $\Pi^+$- and metal ion-catalyzed chemical reactions on clay surfaces require pair proximity within the interlayer. In a real sense, this is an approach to a kind of selectivity.

Among the most selective clay catalysts are clays that have been complexed with homogeneous catalysts, which are then able to perform their catalytic function on the mineral surface. Examples of this are the rhodium (diene)(diphos)$^+$ complexes, where diphos is a chiral bidentate phosphine ligand. Asymmetric hydrogenation of certain prochiral olefins and $\alpha$-acylaminoacrylates was accomplished, the
latter producing chiral amino acid derivatives [107,108] (H. M. Chang, Ph.D. thesis, Michigan State University, East Lansing, 1982). The differences between the activities of the catalyst on swelling clays and in homogeneous solution depended on the nature of the substrate and the diphos ligand. In addition, the greater the swelling of the silicate layers, the more closely did the catalytic activity resemble homogeneous catalysis. Yamagishi [109] investigated adsorption of an optically active metal complex, tris(1,10-phenanthroline)metal(II), on smectite and found that when it was added as a pure enantiomer, it was adsorbed in an amount equivalent to the cation exchange capacity. However, when the complex was added as a racemic mixture, it was adsorbed in an amount twice that of the cation exchange capacity. This suggested that when the pure enantiomer was added and the exchange capacity was saturated, there was no further "fitting" of the "like" molecules between the ions on the exchange sites because of shape restrictions. However, in the racemic mixture, the adsorbed enantiomer on the exchange site could "recognize" the other kind of enantiomer, which could fit into a cavity between the complex ions on the exchange sites. Thus, these clay systems exhibited stereoselectivity, and they have been applied to chromatographic separation of optical isomers [110].

Tsvetkov and Mingelgrin [111] indicated that the Cu-L-lysine complexes on smectite provided an adsorption system that displayed a stronger affinity for the L-isomers of α-amino acids than for the D-isomers. They suggested that the mechanism of optical resolution involved ligand exchange and that such stereoselectivity may have relevance to the accumulation of L-isomers of amino acids in the prebiotic era and their ultimate incorporation into peptide or protein-like materials. The above examples emphasize that clay minerals alone and in combination with certain complexes may exhibit considerable selectivity for catalysis and adsorption.

Another example of clays being involved in relatively specific biochemical reactions is the deamination of glutamic acid by a catalyst composed of Cu(II)-smectite and pyridoxal phosphate (PLP) [112]. Pyridoxal phosphate is a cofactor in enzyme systems important in amino acid metabolism, including deamination, transamination, and decarboxylation reactions. Pyridoxyl phosphate can be an active catalyst independent of the protein matrix of the enzyme, and its activity can be promoted or modified by the presence of such metal cations as Cu$^{2+}$, Fe$^{3+}$, and Al$^{3+}$. The combination of Cu(II)-smectite and PLP was very specific in the deamination of amino acids. Of the amino acids tested, only glutamic acid was deaminated by this system. The activity of other types of Cu(II)-clays (i.e., kaolinite, vermiculite, and some zeolites) was essentially in proportion to their external surface areas. The reaction pathway suggested was the formation of a Schiff base between the amino acid and PLP, followed
by complexation with Cu(II) at the mineral surface. At this point, the complex is hydrolyzed to NH₃ and the α-keto acid. The importance of the mineral surface to the reaction was demonstrated when it was shown that Cu(II) salts alone had very little activity. This catalyst system was equally effective for both D- and L-forms of glutamic acid; thus, it was not stereoselective. The reaction was markedly inhibited by the presence of the strong chelating agent orthophenanthroline, probably as a result of the competition of orthophenanthroline with the Schiff base for coordination sites on Cu(II). The concept of enzyme cofactors, or of chemicals that act as cofactors, performing biochemical functions in concert with inorganic matrices would seem to merit further interest. In this system, the PLP-Cu(II)-smectite acted as a pseudoenzyme, where the silicate structure substituted for the apoenzyme.

Siffert and Naidja [113] also demonstrated that clays can act as catalysts (pseudoenzymes) in biochemical reactions. They showed that the decarboxylation of oxaloacetic acid to pyruvic acid was promoted by smectite. It was suggested that the catalytic effect is a function of the nature of the exchange cation (i.e., its ability to form chelate complexes with oxaloacetic acid). They also attributed some function to Al³⁺ at the edge of the mineral layers.

Studies of interactions between other enzyme cofactors and clays have been performed. Riboflavin and its nucleotide, flavomononucleotide (FMN), have been investigated by Mortland and Lawless [114] and Mortland et al. [115]. When Fe(III) or Cu(II) occupied exchange sites on smectite, the riboflavin formed a charge-transfer complex on the clay surface, probably via the isalloxazine moiety. However, FMN did not form a charge-transfer complex with similar clay systems but interacted with Fe(III)-smectite via the phosphate group. Lawless and Edelson [116] found that clay interactions with nucleotides were profoundly influenced by the nature of the exchange cation, in particular by transition metal cations. Zn(II)-smectite was the greatest adsorber of 5'-adenosine monophosphate (AMP), whereas clays with alkali metals as exchange ions did not adsorb any nucleotide. Graf and Lagaly [117] found that certain clay minerals affected the decomposition of adenosine triphosphate (ATP).

Any role that clays may have had in prebiotic chemical evolution and in the development of self-replicating systems will probably never be resolved with certainty. Our present genetic principle is, in general, chemically the same in all known life forms. Information for replication is fixed in the nucleic acids within the double helix, and from this system biological evolution proceeded. However, are there other systems capable of replication—simpler systems, from which the present nucleic acid system could have evolved? Cairns-Smith and Hartman [82] suggested the possibility of inorganic matrices capable of replication, which ultimately, through "genetic takeover," may
have resulted in the modern system. Clays were included among inorganic matrices that might be candidates for such a process. Weiss [91] has, in fact, performed experiments and has found a smectite that was able to transmit the same charge density as the original parent through 22 generations of new crystallites.

VI. CONCLUSIONS

The nature of enzyme-clay interactions will continue to interest scientists, with respect to natural systems and in relation to immobilization of enzymes for industrial, medical, and scientific purposes. The different environmental conditions provided by specific kinds of clay-organic systems to adsorbed enzymes would seem to be an area for future investigation. Such clay-organic systems may also provide equally interesting environments for living entities, such as viruses and microorganisms. The adsorption of enzymes on synthetic clay-organic complexes via nonpolar (hydrophobic) interactions, as suggested by studies cited here, would seem to provide a model for enzyme interactions with natural soil organic matter. Enzymes adsorbed on synthetic clay-organic complexes exhibit activities quite different from those of enzymes adsorbed on purely inorganic clays. The effects exerted by organic surfaces may be important in determining the activities and stabilities of enzymes associated with organic matter and clay-organic matter complexes in soils.

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Anaerobic Microbial Transformation of Nonoxygenated Aromatic and Alicyclic Compounds in Soil, Subsurface, and Freshwater Sediments


**Microbial Production of Cytokinins**


Pseudomonads as Antagonists of Soilborne Plant Pathogens: Modes of Action and Genetic Analysis


incorporation.


Ecological Significance of the Biological Activity in Soil


50:706-709.


The Significance of Soil Microbial Biomass Estimations


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Soil Lipids: Origin, Nature, Content, Decomposition, and Effect on Soil Physical Properties


**Interactions Between Soil Microbial Communities and Organometallic Compounds**


Effects of Microorganisms on the Environmental Mobility of Radionuclides


"metallothionein and other low molecular weight metal-binding proteins." Birkhauser, Basel.


Viruses in the Soil Environment


