Ryszard J. Chróst
Editor

Microbial Enzymes in Aquatic Environments

With 122 Figures and 46 Tables

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This volume is dedicated to

Professor Jürgen Overbeck

who, in the early 1960s, was one of the first scientists to examine the ecological importance of microbial enzyme activities in aquatic ecosystems.

Preface

Most organic matter in nature consists of molecules that cannot directly enter cells, because of their polymeric structure, high molecular weights, and large size. The hydrolysis of such polymers is a rate-limiting step in the microbial utilization of organic matter in aquatic environments. Before they can be incorporated into microbial cells, polymeric materials must undergo step-wise degradation by a variety of enzymes, which are located either on the cell surface, or in the periplasmic space (ectoenzymes), or may be particle-bound, or released from the microorganisms into the environment as free, dissolved enzymes (extracellular enzymes). Low-molecular-weight compounds, the products of enzymatic action, can then be taken up by microbial cells to meet energy requirements and to build up biomass. Thus, the enzymatic mobilization and transformation of organic matter is the key process regulating the turnover of organic and inorganic compounds in aquatic environments.

Studies of the enzymatic degradation processes of organic matter are being performed by workers in disciplines as diverse as water and sediment analysis, bacterial and algal aquatic ecophysiology, eutrophication, nutrient cycling, and biogeochemistry, and in both freshwater and marine ecosystems. This diversity has resulted in inadequate communication among the scientists working in this field. Therefore, Professor Jürgen Overbeck, Dr. Uwe Münster, and I felt it was very important to bring workers together for an exchange of ideas and experimental data. Therefore, we organized the First Workshop on Enzymes in Aquatic Environments. This meeting was held from 23 to 27 July 1989, under the auspices of the Max-Planck Gesellschaft zur Förderung der Wissenschaften and the Deutsche Forschungsgemeinschaft, at Ringberg Castle, situated above Lake Tegernsee in the Bavarian Alps (Germany). The aim of the workshop was to discuss problems relevant to the study of microbial enzymes in aquatic ecosystems, such as methods for measuring their activity, regulation of synthesis, distribution patterns in waters and sediments, their role in microbial metabolism, and their ecological significance. The contributions of scientists from 12 different countries yielded much useful information on

recent approaches and developments in this field. The workshop also facilitated closer contacts between specialists from different areas of aquatic research, improved our knowledge of environmental processes mediated by microorganisms in aquatic ecosystems, and also contributed to a better understanding among scientists from different parts of the world.

The large number of enzymes considered in this volume indicates the richness of microbial metabolism and its leading role in the decomposition and transformation processes of organic matter in the aquatic environments. The study of microbial enzymes is a relatively new but rapidly developing field of aquatic microbial ecology; it is important from both a practical and an ecological point of view. On one hand, microbial enzymes now are widely used in water biotechnology; on the other hand, their study contributes significantly to our knowledge of the principles of aquatic ecology and microbial processes that affect the whole ecosystem. Aquatic microbial enzymology also represents a border area between different scientific disciplines, such as microbiology, biochemistry, physiology, and ecology, and demonstrates that their integration enhances our understanding of many ecological processes.

The chapters in this volume are based on papers presented at the workshop and represent the state of the art in this field of enzyme studies in both freshwater and marine ecosystems. The first part of the book (Chapters 1 to 6) covers general aspects of extracellular enzymes and ectoenzymes in aquatic environments, such as their storage and distribution, regulation of their synthesis and activity, methodology of enzyme activity measurements in water and sediment, and the enzyme-substrate relationship. The next section (Chapters 7 to 9) deals with the activities of enzymes responsible for degradation of proteinaceous materials. The activity and significance of algal and bacterial phosphohydrolytic enzymes is described in Chapters 10 to 15. The last part of the volume discusses the activity of hydrolytic enzymes (e.g., chitinase, glucosidase, and esterases) in various aquatic habitats.

The purpose of this volume is to provide a range of examples of areas in which "ecological enzymology" are being employed in aquatic research. It is addressed to the students and scientists who are interested in using an enzyme approach to study the ecophysiology of microorganisms, organic matter degradation and transformation processes, and biogeochemical cycles in aquatic ecosystems. In microbial ecology many fascinating and important questions urgently need to be answered and I believe that the enzyme approach presented throughout this volume will be extremely useful for solving them. I hope that this work will draw the attention of microbial ecologists and biochemists to the major role of the enzymatic activity of the microorganisms in aquatic ecosystems. To my knowledge, this is the first book to discuss both cell-bound and extracellular microbial enzymes and their ecological significance in aquatic ecosystems.

I want to express my gratitude to Dr. Uwe Münster for his valuable help in mounting the workshop, and to Karin Schmidt and Cordula Stielau for technical and administrative assistance during the meeting. The financial and technical support of the Max-Planck Institute for Limnology, Department of Microbial Ecology (Plön), during the preparation and editing of this book is also acknowledged. Special thanks go to the contributors for coming in from the field long enough to prepare their manuscripts.

Ryszard J. Chróst

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1

Early Studies on Ecto- and Extracellular Enzymes in Aquatic Environments

Jürgen Overbeck

The idea that free enzymes could act as "catalysts" in freshwater and marine environments is not new. Vallentyne (1957) in his review on the molecular nature of organic matter in lakes and oceans pointed out "some provocative and controversial evidence" that free enzymes may be important for chemical transformations in both lakes and oceans, and he listed some data from early studies. Fermi (1906) was the first to observe proteolytic enzymes in stagnant waters of pools. Harvey (1925) called attention to the possible presence of catalases and oxidases in sea water. Kreps (1934) assumed that extracellular enzymes originating from bacteria and from marine plants and animals may be present in sea water. In his filtration experiments he reported that bacteria were retained on a Seitz "ultrafilter" and that "the enzymes liberated could pass through and were able to continue in the filtered water their specific catalytic action of nitrate reduction, ammonia oxidation, etc." In 1938 the biochemist Maximilian Steiner reported that "intrabiocönotischer Phosphatkreislauf" (Elster and Einsele, 1937; Ohle, 1952), i.e., repeated incorporation of molecules of phosphorus by epilimnetic phytoplankton during one production period, would not be possible without the active participation of phosphatases. He showed in filtration experiments that phosphatases were excreted by zooplankton, and the evidence for enzyme activity was demonstrated by the cleavage of organic phosphorus compounds.

Thirty years ago, during the fourteenth International Association of Theoretical and Applied Limnology (Societas Internationalis Limnologiae; SIL) Congress in Vienna in 1959, I presented data on free dissolved enzymes in lake water for the first time. At that time I was interested in the phosphate metabolism of phytoplankton. The object of the study was a small artificial pond in the park of Sanssouci near Berlin. The pond was filled once a week with water from the highly eutrophicated Havel river; therefore the content of phosphorus

was high, about 600 µg of phosphorus (P) per liter. However, only a very small part of the total P was present as inorganic phosphate (on average 13 µg $P\text{-}PO_4^{3-}$ l^{-1} during the 4-year study). From this observation arose the question of how the dense phytoplankton communities could use different phosphorus compounds other than inorganic phosphate.

Scenedesmus quadricauda was the main alga of summer phytoplankton in the experimental pond (10,000–80,000 coenobium per ml). For the study of phosphate metabolism, an axenic culture of *Scenedesmus quadricauda*, strain 276-4E from the Pringsheim algal collection in Göttingen (originally isolated by W. Rodhe from Lake Erken, Sweden), was used (Overbeck, 1961). Measurements of uptake kinetics under sterile conditions showed very clearly, that *Scenedesmus* used only the inorganic phosphate. Various organic phosphorus compounds (Na-glycerophosphate, Na-nucleinate, Ca-phytate, and Na-pyrophosphate) were not taken up by *Scenedesmus* in an axenic culture. Similar results were also obtained with samples of lake water which were sterilized by filtration. However, when a natural water sample without filtration was used, the uptake rates amounted for 70–100 fg P $cell^{-1}$ and agreed well with the results of Rodhe's studies on the physiology of phytoplankton (Rodhe, 1948).

For a better understanding of these results, which indicated the possible participation of free phosphohydrolytic enzymes outside the *Scenedesmus* cells, I began to study the phosphatases in this alga. Homogenized cells from axenic batch cultures of *Scenedesmus* were extracted with 0.25 M saccharose solution (16 hours, 4°C) and the crude enzyme preparation was precipitated with acetone (Overbeck, 1962). This enzyme powder, after freeze drying, was used for experiments. It soon became clear that the alga possessed two active types of phosphatases, an acid and an alkaline phosphatase, that instantaneously hydrolyzed organic P-compounds. The question then arose of how *Scenedesmus* in the pond could utilize the organic phosphorus compounds whereas in axenic cultures and in bacteria-free lake water, this alga was not able to do so.

I then added an *Escherichia coli* suspension to an axenic culture of *Scenedesmus* grown on medium supplied with sodium glycerophosphate. The results were surprising: the organic P-compound was hydrolyzed immediately by bacterial phosphatases and the phosphate was taken up by algae. Apparently phosphatases had been excreted by *E. coli*. To prove this assumption I filtered *E. coli* suspension and measured the phosphatase activity. I found that free phosphatases were present in the culture solution of *E. coli* as well as in the water of the experimental pond. Measurements of free phosphatases in lake water in that time applied the following assay (Overbeck and Babenzien, 1963, 1964): 1 ml 0.5% Na-glycerophosphate, 1 ml buffer (pH 6.4, 8.2, and 9.6), and 1 ml lake water. The samples were incubated for 16 h at 32°C.

The activity of free enzymes in the experimental pond (besides phosphatases, I studied amylase and saccharase) was closely related to the standing crop of phytoplankton, i.e., high abundance of phytoplankton was proportional to increased enzyme activity. High enzyme activity was observed in winter (December-January) because probably the enzyme protein remained stable

for a longer time at low water temperatures. Much higher activity of phosphatases was found in the sediments of the pond where an enrichment of the enzyme by precipitation with ammonium sulfate was possible. On the basis of these studies the following model of phosphorus cycling within the epilimnetic water zone, mediated by free enzymes, was proposed (Figure 1.1; Overbeck 1967): Bacteria generally take up inorganic and organic phosphorus using their active transport systems. A variety of bacterial species can store polyphosphates inside their cells. Free, extracellular phosphatases excreted by bacteria hydrolyze dissolved organic phosphorus compounds which cannot be directly incorporated into the algal cell in the absence of cell-bound phosphatases, as in *Scenedesmus*. Otherwise, organic phosphorus compounds, e.g., phosphate esters such as glycero-6-phosphate, and fructose-1,6-diphosphate, have to be hydrolyzed by cell-surface phosphatases, which are widely distributed in various bacteria, algae, and fungi.

A great improvement in the accuracy of the measurement of phosphatase activity was the application of p-nitrophenyl phosphate as substrate instead of the previously used Na-glycerophosphate. W. Reichardt demonstrated in my laboratory that the distribution pattern of phosphatase activity in Plußsee was well correlated with the abundance of phytoplankton and bacteria. Very high activities of lake water phosphatases were detected in the extremely stratified lake at the end of the summer stratification. Also ecological studies on pronounced diurnal activity and on the regulation of intracellular and extracellular

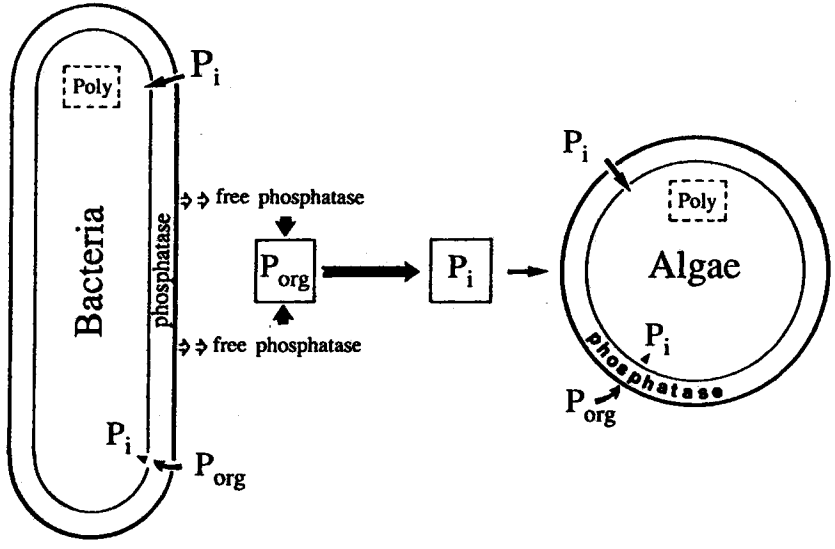

Figure 1.1 Model for phosphorus cycling within epilimnetic phytoplankton and bacteria mediated by free phosphatases (P_i, soluble inorganic P; P_{org}, soluble organic P; Poly, polyphosphate; modified from Overbeck, 1967).

phosphatases in the lake were conducted (Reichardt et al., 1967; Reichardt and Overbeck, 1969; Reichardt, 1969).

These above mentioned studies were the first steps in the exciting field of study of ecto- and extracellular enzymes in aquatic ecosystems. Today, it is a matter of fact that ecosystem functioning cannot be understood without the active participation of enzyme processes. But one important point must be stressed: we should be very careful and cautious in the interpretation of data from measurements in the field. Because of the complexity of metabolic pathways and the possible interference of manifold uncontrolled conditions, it is extremely difficult to understand enzyme regulation only from field data. All data must be complemented by biochemical studies with purified enzymes. I believe that the rapidly expanding field of biochemical and molecular ecology will develop methods in the near future for an adequate coupling of field and laboratory work.

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2

Extracellular Enzymatic Interactions: Storage, Redistribution, and Interspecific Communication

Robert G. Wetzel

2.1 Extracellular Enzymes: Their Position Relative to the Cell in the Environment

I begin with the naive question: Where are the enzymes located in relation to the cells that are attempting to acquire a needed exogenous substrate or element? This question is important because many scientists speak rather casually about endo- and exoenzymatic activity in relation to cellular metabolism. Obviously most of the enzymatic activity is intracellular, associated with biosynthetic reactions driven by energy liberated from the hydrolysis of ATP.

An extracellular enzyme is one that hydrolyzes substrates external to cells, and is either bound to the cell membrane or free in the water. Many important enzymes are associated with cell membranes (e.g., carbonic anhydrase, alkaline phosphatase). Presumably these membrane-bound enzymes functionally bridge the finite distance between substrates in the surrounding milieu, their acquisition, and the transport of substrates and/or nutrients to and across the membrane for subsequent intracellular metabolism. Such membrane-bound enzymes are functionally exoenzymes, even though they are chemically bound to the cell surfaces. Important to subsequent discussion in this chapter, these "exposed" membrane-bound enzymes also are susceptible to inhibitory influences similar to the situation among "free" exoenzymes, as delineated below.

First, I emphasize that the separation of free from bound enzymes is quite variable with changes in the phases of growth and growth conditions. For example, in *Bacillus*, both membrane-bound and soluble alkaline phosphatase (APase, orthophosphoric monoester phosphohydrolase, E.C. 3.1.3.1.) forms occur (Hulett, 1986). A peripherally bound APase (salt extractable) is found on the inner leaflet of the cytoplasmic membrane (Figure 2.1). An integrally bound form, requiring detergent extraction, occurs on the outer leaflet. Soluble APase is secreted through the periplasmic membrane to form a truly extracellular enzyme. No discernible physico-chemical differences were found in the enzymes from these different locations. Many investigators have evidence to suggest that some enzymes, particularly phosphatases, are secreted or released by algae into the ambient medium and that they function extracellularly (e.g., Mills and Campbell, 1974; Aaronson and Patni, 1976; Walther and Fries, 1976; Yamane and Maruo, 1978; Cembella et al., 1984, 1985). Bacteriological evidence indicates that Gram-negative bacteria with multi-layered cell walls release little of the periplasmic enzymes, whereas Gram-positive and other bacteria lacking one or more cell-wall layers, tend to release enzymes extracellularly (cf. review in Cembella et al., 1984).

In both soil and aqueous ecosystems, it is commonly assumed that enzymes are released from cells into the environment (Figure 2.2). Affiliation of these compounds with inorganic and organic particulate surfaces can vary greatly with characteristics of both the enzymes and the particles (see discussion below). Numerous workers have specifically sought, in ecological studies, to differentiate between free enzymatic activities and those associated with particles (see Chapter 3). Separation methods vary, of course, with the questions being addressed. Physiological methods use various bond disruptors (salts, detergents) or cell wall disruptors (e.g., lysozyme) and membrane-impermeable enzyme-specific inhibitors to separate surface-bound and intracellular enzymes and their activities. Separation methods, such as centrifugation and filtration, that address ecological questions, such as the enzyme distributions in the en-

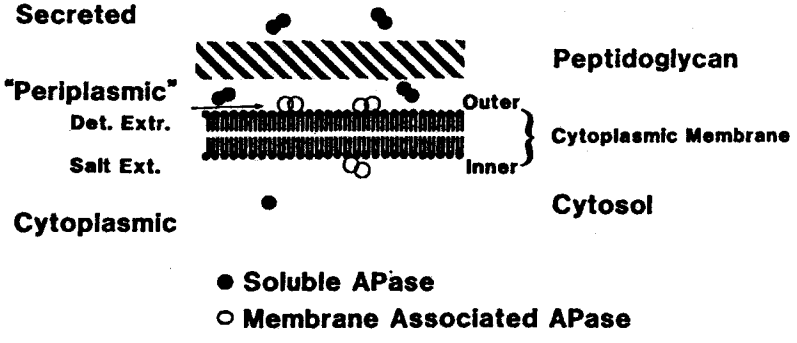

Figure 2.1 Distribution of alkaline phosphatase (APase) in *Bacillus licheniformis*. Single circles = inactive monomers; double circles = active dimers; solid circles = soluble enzyme protein; open circles = membrane-associated APase (modified from Hulett, 1986).

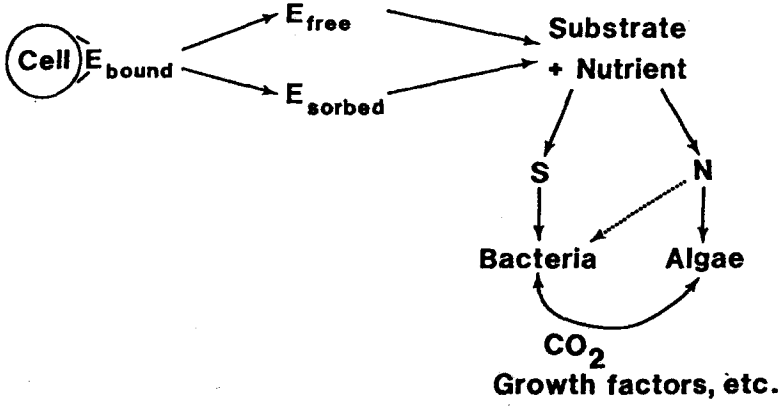

Figure 2.2 Generalized functional pathways of released extracellular enzymes. E = enzyme; S = substrate; N = nutrient.

vironment, allow distinction between enzyme activities of free and particulate phases. Although some workers suggest that certain exoproteolytic activity in filtrates may result from cell fragments due to the lysis of bacteria (Rego et al., 1985; Sharp, 1977), many careful studies have demonstrated very appreciable amounts of total enzyme activities in dissolved phases, particularly among the phosphatases (Fogg, 1977; Aaronson, 1978; Stewart and Wetzel, 1982b).

Among the first to point out the potential occurrence of "free" enzymatic activities in fresh waters were Overbeck and Babenzien (1963, 1964; cf. also Reichardt et al., 1967). Because of the central importance of phosphorus as a commonly regulating nutrient of primary productivity in fresh waters, much attention was given to the phosphatase activities of algae. Wetzel (1981) demonstrated, for example, over numerous annual periods that 30 to 50% of the pelagic alkaline phosphatase was in a dissolved "free" form (less than 0.6-μm-pore-size filtration). These findings were subsequently verified and extended by centrifugation separation methods and additionally showed that much (15 to 73%) of the particulate alkaline phosphatase activity was associated with bacterioplankton (Table 2.1; cf. Stewart and Wetzel, 1982b; also Chróst and Overbeck, 1987). A number of other investigations have also shown that a significant portion of the phosphatase activity in the pelagic zone is "soluble" or "free" (Aaronson and Patni, 1976; Pettersson, 1980; Chróst et al., 1989; Münster et al., 1989). In contrast, relatively little (<10%) of the peptidase, galactosidase, and glucosidase activity was found in dissolved form, perhaps related to differences in enzyme structure, binding characteristics, and recalcitrance to degradation.

2.2 Strategy of External vs. "Free" Enzymes

Because it has been clearly established that a considerable amount of extracellular enzyme activity is free, I would like to address the biochemical and eco-

Table 2.1 Percentage of total alkaline phosphatase activity contributed by algae, non-algal particulates, and "dissolved" enzyme in four lakes in southwestern Michigan

		Percentage of total APase activity		
Sample source	Depth (m)	Algae	Non-algal particulates	"Dissolved" enzyme
Lawrence Lake				
	2	5.3	42.9	51.8
	4	6.1	44.7	49.2
	6	12.3	39.6	48.1
Lefebre Lake				
	2	9.6	46.1	44.4
	4	4.7	53.7	41.6
Little Mill Lake				
	2	25.1	60.5	14.4
	4	13.8	72.5	13.7
Gull Lake				
	4	29.4	31.0	39.6
	9	33.2	14.8	52.0
	15	32.0	6.8	61.2

Modified from Stewart and Wetzel, 1982b. Separations were made by differential centrifugation; "dissolved" APase activities were measured in the supernatant (20,000 x g; 5 min).

logical significance of the existence of both free and bound enzymes. Enzymes in solution are susceptible both to degradation and to chemical alteration. Through these processes, the functional capacities of the enzymes are reduced. From the standpoint of the individual cell or organism, it would appear energetically inefficient to release enzymes from cells and to increase the distance between sites of enzymatic reactivity and cellular utilization. The probability of a cell being able to utilize the hydrolytic products decreases precipitously as the distance of the enzyme from the cell increases (Figure 2.3). Nonetheless, if the hydrolyzed molecule is within circa 500 μm of the cell surface, the probability of assimilation is sufficiently high to warrant enzymatic release.

Surface-bound enzymes exhibit boundary-layer diffusional problems. Diffusion of a substrate to bound enzymes is relatively slow, and the substrate concentration is lower in the microenvironment at the cell boundary surface than in the free macroenvironment (Engasser and Horvath, 1974a; 1974b). Although the concentration gradients can enhance diffusional rates, flux rates are inadequate and the diffusional resistances can cause both substrate depletion and product accumulation in the microenvironment of bound enzymes. Thus, diffusional inhibition could be reduced by releasing enzymes into the immediate medium surrounding the cells (Figure 2.3). Although some losses would result from products diffusing away from the cell, these losses may be compensated for by improved availability of substrates. For example, Ammerman

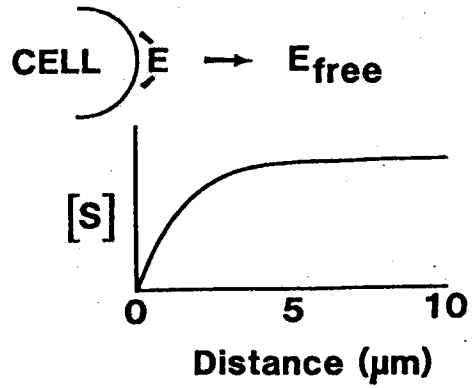

Figure 2.3 Hypothetical potential advantage of the release of extracellular enzymes from cells into the immediate ambient environment of high substrate concentration [S] (based on models of Engasser and Horvath, 1974a; 1974b).

and Azam (1985) found that from 15 to 100% of the hydrolyzed dissolved organic phosphorus was assimilated. Moreover, from the standpoint of the community and ecosystem, it is possible for multiple interactions among many cells and different species to be operational in which, for example, the mobilization of nutrients from organic compounds could be increased by exoenzymatic activities of microfloral metabolism while simultaneously increasing nutrient and carbon availability to algae cohabiting the same water parcel (Figure 2.2). Thus both bacteria and algae could benefit from the interactions.

2.3 Immobilization of Enzyme Activity

The hydrolytic enzymes catalyze the cleavage of covalent bonds such as C-O (esters and glycosides), C-N (proteins and peptides), and O-P (phosphates). Often these same enzymes can also affect synthesis by condensation reactions. Hydrolytic cleavage of C-C bonds is rare and hydrolytic enzymes do not react with aromatic nuclei of phenolic compounds but do react with phenolic hydroxyl and other substituted functional groups that often occur in phenolic compounds.

Conversely polyphenolic compounds, largely of plant origin, form a major component of dissolved organic acids of fresh waters. The dissolved organic acids frequently constitute some 80% of the total dissolved organic matter (ca. 5–10 mg dissolved organic carbon per liter; Wetzel, 1983; 1984). Hence, concentrations of 4–8 mg polyphenolic organic acids per liter or more are common (Perdue and Gjessing, 1990). Polyphenolic organic acids induce precipitation of proteins by binding to one or more sites on the protein surface (Figure 2.4a)

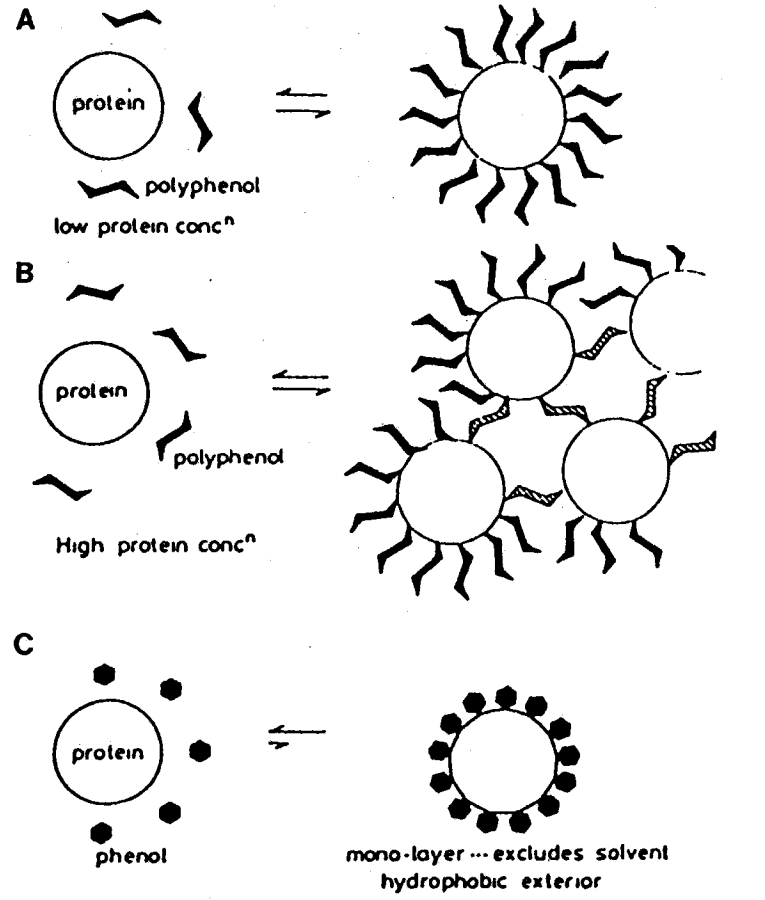

Figure 2.4 Protein complex formation and precipitation by phenols and polyphenols (modified from Haslam, 1988). See text for explanation. Hatched = cross linkages among phenol-protein molecules.

to give a monolayer that is less hydrophilic than the protein itself (Figure 2.4c; Haslam, 1988; Haslam and Lilley, 1988; Spencer et al., 1988).

Aggregation and precipitation can ensue, but clearly enzymatic activity is inhibited (e.g., Ladd, 1985). At higher concentrations of enzymes, cross-linking can occur among phenol-protein molecules (e.g., Haslam, 1988); the result is that less polyphenol is required to precipitate proteins from concentrated enzyme solutions (Figure 2.4b). More aromatic and condensed humic acid molecules are more rigid and can distort bound enzymes to a greater extent than is the case with simpler compounds, such as fulvic acid (e.g., Ladd and Butler, 1975).

The effectiveness of plant polyphenols to complex protein molecules derives from their polydentate ligands. These ligands have many potential hydrogen binding sites provided by numerous phenolic groups and aryl rings on the periphery of the molecule (Beart et al., 1985). Polyphenolic compounds have the proper molecular size and structure to form stable cross-linked structures with a number of different protein molecules (Figure 2.5). The inhibition of enzymes occurs in a classical noncompetitive manner, in which the inhibitor, polyphenol, and substrate bind simultaneously to the enzyme (Figure 2.5).

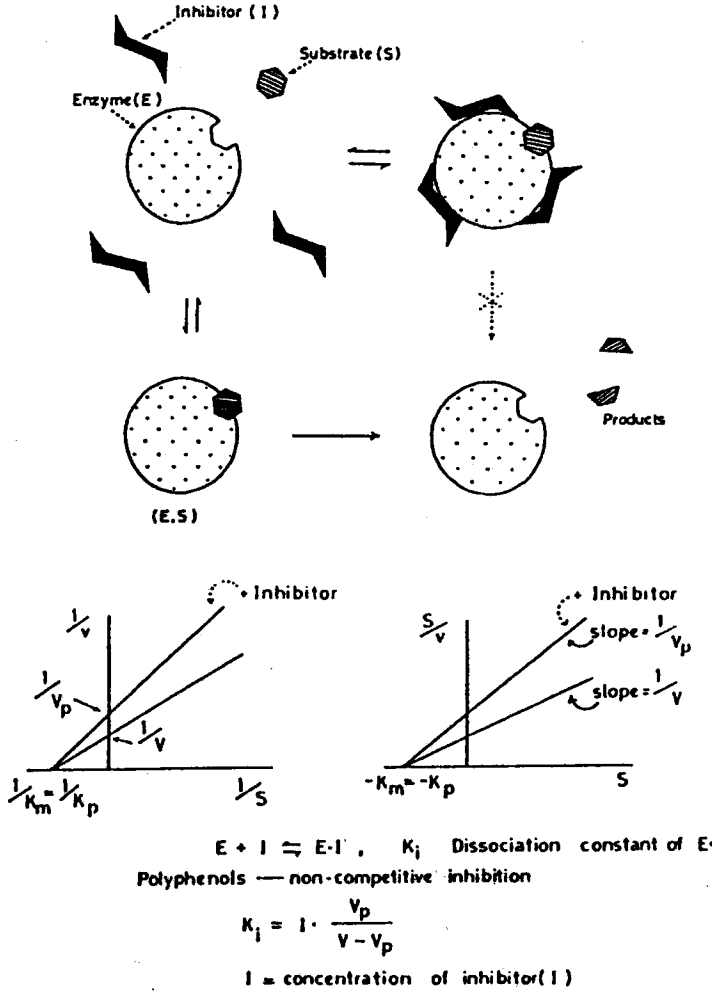

Figure 2.5 Mechanism and kinetics of enzyme inhibition by polyphenols (modified from Haslam and Lilley, 1988).

Let me digress briefly with some examples of the interactions of humic compounds with enzymes in fresh waters. This subject has been evaluated to a much greater extent in terrestrial soil systems. For example, proteolytic enzymes can be reversibly bound to humic compounds in soils by ionic linkages (Ladd and Butler, 1975). The extent of binding by covalent, ionic, or hydrogen bonding to humic acids in soils is unclear. Covalent bonding appears to dominate; the rate of extraction of enzymes by buffers is low, and thus the amount of ionic binding of enzymes to humic compounds must also be low.

Earlier we examined the effects of dissolved humic compounds upon the phosphatase activities of planktonic bacteria and algae of hardwater, phosphate-limited lakes (e.g., Stewart and Wetzel, 1981; 1982a). Strong interactions were determined experimentally between dissolved humic compounds, phosphorus availability, photosynthetic rates, and dissimilation rates of recent photosynthate released from the algae. It was apparent that high-molecular-weight humic compounds of emergent macrophyte origins suppressed photosynthetic carbon fixation and enhanced APase synthesis as presumably greater amounts were inactivated by means of humic-enzyme complexation. Abiotic mechanisms associated with iron and dissolved humic materials can also be involved with the availability of phosphorus under certain conditions (e.g. Francko, 1986; Jones et al., 1988). However, my recent analyses demonstrate an additional mechanism by which the dissolved humic compounds regulate phosphorus availability.

The formation of polyphenolic-enzyme complexes can partially or entirely inactivate the enzyme. Often the reaction is reversible, for example, by means of ultraviolet photolytic degradation of the phenolic compounds or autodigestion by other enzymes. As inorganic phosphorus availability commonly becomes limiting spatially and temporarily in fresh waters, the microflora depend increasingly upon the hydrolysis of dissolved organic phosphorus (DOP) compounds. Loading of polyphenolic and related dissolved organic compounds to an aquatic environment can result in the formation of enzyme complexes and the inhibition of enzyme activities and can become a dominant regulatory mechanism of phosphorus and hence photosynthetic and respiratory metabolism.

Much of the dissolved organic matter in fresh waters, as already noted, emanates from organic compounds of plant origin. The more recalcitrant portions of these compounds have relatively slow turnover rates and persist in the dissolved phase for considerable time periods (average decomposition rates of 0.5 to 1% per day). Phenolic organic acids usually dominate this pool. Particularly important for complex formation mechanisms are those phenolic compounds which include an array of plant substances that possess an aromatic ring with one or more hydroxyl substituents. In regard to this discussion, we are here more concerned with phenolic compounds of structural tissue origin (phenols, phenolic acids, phenylpropanoids, and polymers) because of the high quantitative loadings of these organic materials to fresh waters from wetland and littoral sources. Other phenolic compounds (flavonoid and other pigments,

flavonols, flavones) can have important regulatory functions but are quantitatively less significant.

Certain phenolic compounds are more reactive with proteins than others. For example, certain simple phenols and phenolic acids (e.g., *p*-hydroxybenzoic acid) are less reactive with proteins than phenylpropanoids (e.g., the hydroxycinnamic, *p*-coumaric, caffeic, and ferulic acids). In contrast, hydrolyzable tannin polymers (gallo- and ellagitannins), esters of gallic and diphenic acids with glucose, strongly complex with proteins and enzymes. Positively charged groups, such as protonated amino groups of proteins, could bind electrostatically with the negatively charged ionized organic acids. Adsorption of organic acids increases as their charge decreases and their molecular weight and hydrophobic properties increase (Perdue and Gjessing, 1990). At the pH of most natural waters (pH 5–8), a large percentage of carboxylic acid groups will be ionized. Major cations (e.g., Ca^{2+}) will react with organic acids and can reduce complex formation with enzymes and their inactivation.

Several representative purified and generic compounds were assayed for interactions with enzyme activities, and alkaline phosphatase was selected as a model enzyme because of its ubiquitous importance in many fresh waters. Simply evaluating the effects of organic acids upon alkaline phosphatase activity (APase) in buffer demonstrated a marked suppression of activity, using simple low-molecular-weight phenolic acids, phenylpropanoids, or larger polymers (Figures 2.6 and 2.7). The enzyme is more stable in the enzyme-humic acid complex, in part because of stearic effects that interfere with proteolytic activity. When the same assays were performed in lake water or a synthetic medium that simulated the ionic composition of hardwater lakes of the gla-

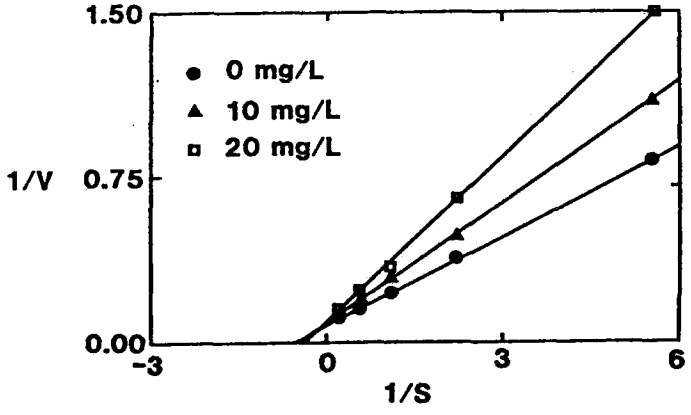

Figure 2.6 Effect on alkaline phosphatase activities of increasing concentrations of a humic acid mixture. Methylumbelliferyl phosphate concentrations were measured at 0.2, 0.5, 1.0, 2.0, and 5.0 x K_m.

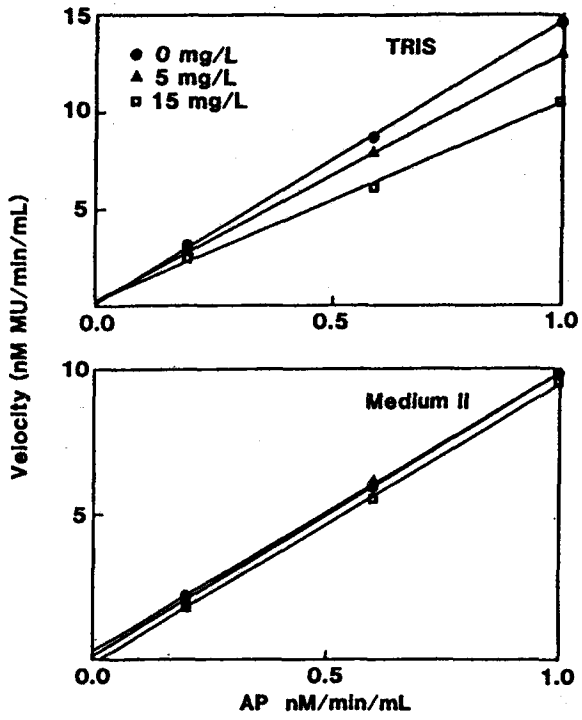

Figure 2.7 Effects of increasing concentrations of a humic acid mixture on the reaction velocities of alkaline phosphatase in Tris (pH 8.0) and in a synthetic hard lakewater medium (Wetzel Medium II, pH 8.0).

ciated midwestern USA, the extent of inhibition was still highly significant but less than in organically buffered water (Tris, pH 8.0) (Figure 2.8). Further assays in which the divalent cation concentrations were varied showed a reduced inhibition of dissolved humic compounds upon enzyme activity with increasing cation concentrations (Figure 2.9). In both soils and fresh waters, humic acids are known to reversibly bind enzymes by a cation exchange mechanism (e.g., Scheffer et al., 1962; Ladd, 1972; Ladd and Butler, 1970; Tipping et al., 1988). At high concentrations, inorganic cations, particularly divalent cations, can displace enzymes from complexes by binding at the humic acid carboxyl group where the enzymes bind.

The rationale underlying these experiments emanates from a complex series of potential interactions in natural waters among humic compounds, cation concentrations, and enzyme activities. The following hypotheses are congruous with manifold observations that have been made along lake gradients (Figure 2.10):

1. Organic acids complex chemically with enzymes. Their enzymatic ac-