THE USE OF PURIFIED ENZYMES FOR THE EARLY ASSESSMENT OF TOXICITY

by

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Industry now markets 35,000 commercial chemicals on a regular basis, and creates more than 1,000 new compounds each year (1). Since a number of these substances possess toxic characteristics, there exists a growing concern over the presence of toxic chemicals in the environment. Approximately 10 million tons of toxic and hazardous wastes are generated yearly in the United States (2). Because the threat of environmental contamination accompanies the proliferation of chemical industries, additional laboratory methods for the early assessment of toxicity are needed.

The determination of toxic substances in the environment offers the investigator a great challenge because these compounds usually appear at trace levels and are often in the presence of other, more concentrated substances. Therefore, accurate and reliable methods are needed to detect, quantify, and substantiate toxic activity. Some of the most common methods developed and implemented are the Ames Salmonella/mammalian-microsome mutagenicity test (3), the flagellar coordination of Spirillium volutans test (4), the detection of lesions induced in Bacillus subtilis nucleic acid (5), the use of Photobacterium ficheri in the Beckman Microtox System (6), and the respiration rate response of fish (7).

The mode of action of many classes of toxic chemicals on animals is somewhat conjectural, although it has been observed that, in many cases, enzymes are involved. Well-known examples include acetylcholinesterase inhibition by pesticides (8,9), urease inhibition by heavy metal salts (10,11), and carbonic anhydrase inhibition by sulfonamide

drugs (12,13,14). Since these chemicals can inhibit enzymatic reactions in trace amounts, it appears that these and other enzymes offer the potential of serving as analytical tools for signaling the presence of toxic substances and/or detecting toxic activity.

Enzymes offer many advantages in that they are relatively inexpensive, obtainable in purified forms, and often have quick and simple assays. Most enzyme assays require the use of unsophisticated equipment and, therefore, can be applied without having expert knowledge and/or a well-equipped laboratory.

The concept of using enzymes as preliminary monitors for environmental toxicity has only recently been investigated and the limitations are somewhat unknown. Additional research is necessary to determine the type of enzyme that can be used with specific toxicants (selectivity) and the response of an enzyme for detecting toxic activity (sensitivity).

The objectives of this study were to:

1. measure the enzymatic activity in vitro of purified forms of α-chymotrypsin, acid phosphatase, and carbonic anhydrase prior to and following exposure to various inorganic and organic chemical agents.

2. determine the degree of enzyme inhibition induced by the various chemical agents at concentrations which are toxic or environmentally significant.

3. evaluate the potential of the α-chymotrypsin, acid phosphatase, and carbonic anhydrase enzyme systems to reliably signal potential toxicity problems.

LITERATURE REVIEW

Kinetics of Enzyme Catalyzed Reactions

A catalyst is a substance which lowers the energy of activation of the transition state, thus accelerating the rate of a chemical reaction. Enzymes are biologically synthesized proteins specialized to catalyze biological reactions. They have no effect on the free energy or equilibrium of a reaction; they simply speed up the rate at which a reaction approaches equilibrium. Lehninger (15) described the effect of substrate concentrations on the rate of an enzyme catalyzed reaction as follows (see Figure 1). At low concentrations the initial reaction velocity is nearly proportional to the substrate concentration, or first order. As the substrate concentration is increased, the initial rate increases less, becoming more independent of the substrate concentration. The reaction is mixed order when the initial rate is no longer proportional to the substrate concentration. Eventually a maximum velocity is reached and the reaction is essentially independent of the substrate concentration. The reaction is zero order with respect to the substrate and the enzyme is commonly referred to as being saturated with its substrate.

In 1913, a mathematical relationship between the initial rate of an enzyme-catalyzed reaction, the concentration of the substrate, and certain characteristics of an enzyme, was developed by L. Michaelis and M.L. Menten, later extended by G.E. Briggs and J.B.S. Haldane (16). The Michaelis-Menten theory assumes that the enzyme (E) first combines with the substrate (S) to form the enzyme-substrate complex (ES), which

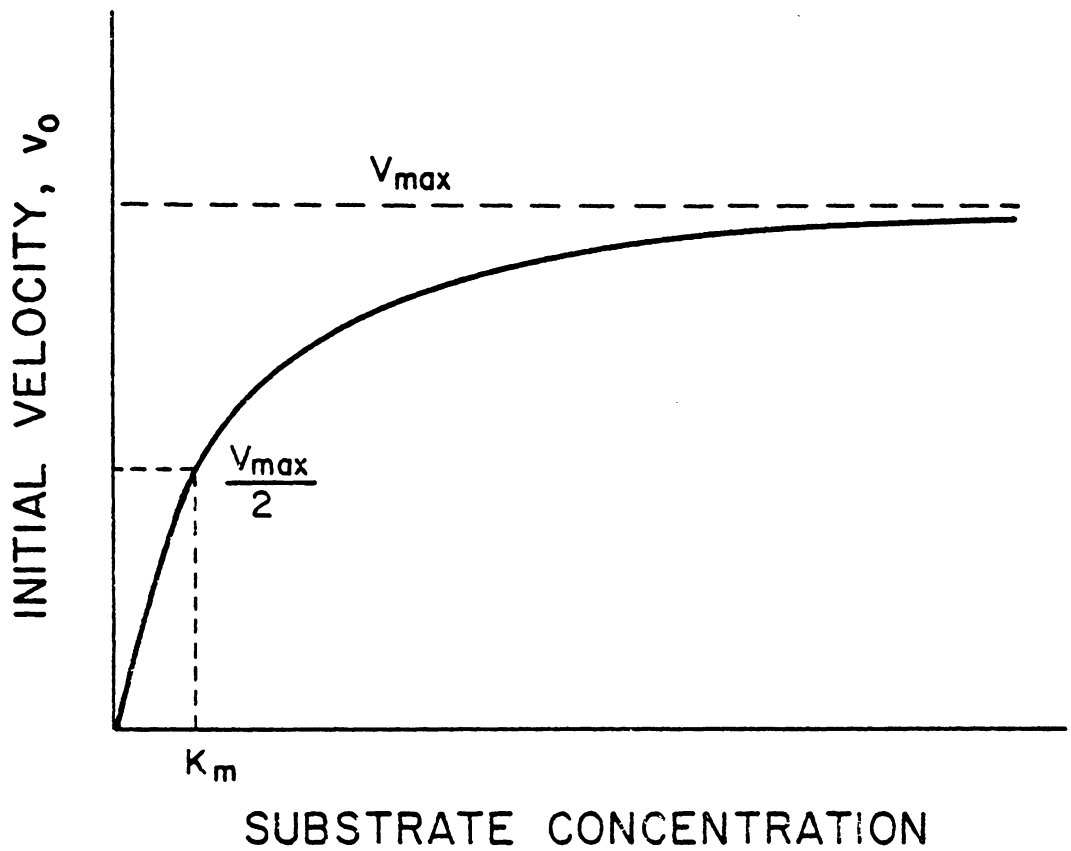


Figure 1. Effect of substrate concentration on the rate of an enzyme-catalyzed reaction [from Lehninger (15)].

then breaks down to form free enzyme and the product P.

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E + P \qquad [1]$$

The Michaelis-Menten equation, as described by Lehninger (15), is derived as follows. The first order equation is expressed as $v_o = k_3[ES]$, where k_3 is the first order rate constant. The second order equation is expressed as:

$$\frac{d[ES]}{dt} = k_1([E_t] - [ES])\ [S] \qquad [2]$$

where k_1 is the second order rate constant and E_t is the total amount of enzyme. The rate of the back reaction k_4 can be neglected because when [S] is very large, P is close to zero. The breakdown of [ES] can be expressed by the sum of the two reactions:

$$-\frac{d[ES]}{dt} = k_2[ES] + k_3[ES] \qquad [3]$$

When [ES] remains constant, that is, the rate of formation of [ES] is equal to its rate of breakdown:

$$k_1([E_t] - [ES])\ [S] = k_2[ES] + k_3[ES] \qquad [4]$$

Rearranging the above equation gives:

$$\frac{[S]\ ([E_t] - [ES])}{[ES]} = \frac{k_2 + k_3}{k_1} = k_m \qquad [5]$$

where k_m is called the Michaelis-Menten constant. Solving for [ES] and substituting the first order equation:

$$v_o = k_3 \frac{[E_t]\ [S]}{k_m + [S]} \qquad [6]$$

When the enzyme is saturated or all the E is essentially in an

ES complex, the maximum initial velocity is reached:

$$V_{max} = k_3[E_t] \qquad [7]$$

Substituting V_{max} for $k_3[E_t]$:

$$v_o = \frac{V_{max}\ [S]}{k_m + [S]} \qquad [8]$$

This is the Michaelis-Menten equation for a one-substrate, enzyme-catalyzed reaction.

The Michaelis-Menten relationship can be algebraically transformed into useful linear expressions. The most common expression is the Lineweaver-Burk equation, which is derived by taking the reciprocal of the entire Michaelis-Menten equation:

$$\frac{1}{v_o} = \frac{k_m + [S]}{V_{max}\ [S]} \qquad [9]$$

Rearranging and reducing gives the Lineweaver-Burk expression:

$$\frac{1}{v_o} = \frac{k_m}{V_{max}} \frac{1}{[S]} + \frac{1}{V_{max}} \qquad [10]$$

The Lineweaver-Burk equation can be plotted as a straight line on double reciprocal axes ($1/v_o$ vs. $1/[S]$), with a slope of k_m/V_{max}, an intercept of $1/V_{max}$ on the $1/v_o$ axis, and an intercept of $-1/k_m$ on the $1/[S]$ axis.

The Lineweaver-Burk graphs are especially useful in analyzing enzyme inhibition. Figure 2 graphically illustrates three major types of simple enzyme inhibition using the Lineweaver-Burk expression (15). Competitive inhibition is easily recognized experimentally because the percent inhibition, at a fixed inhibitor concentration, is decreased

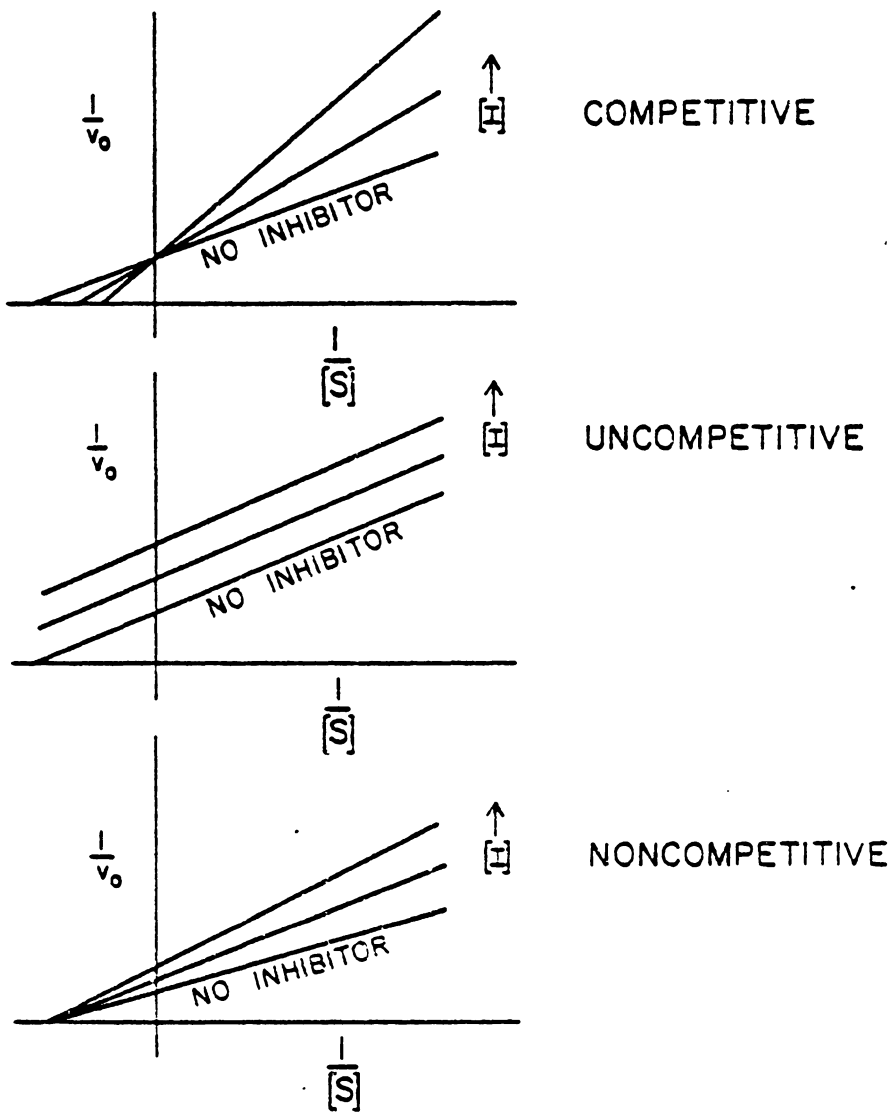


Figure 2. The effect of competitive, uncompetitive, and noncompetitive inhibition of enzymes using the Lineweaver-Burk expression [from Lehninger (15)].

by increasing the substrate concentration. In competitive inhibition, the inhibitor combines with the free enzyme so that it competes with normal substrate binding at the active site. The result is an increase in the apparent k_m of the enzyme for substrate; that is, a higher substrate concentration is required to achieve its maximum velocity.

In uncompetitive inhibition, the inhibitor combines with the enzyme-substrate complex to give an inactive enzyme-substrate-inhibitor complex which cannot undergo formation of the normal product. Subsequently, the degree of inhibition may increase when the substrate concentration is increased. This type of inhibition is rare with one-substrate reactions. An uncompetitive inhibitor decreases the V_{max} and the k_m to the same extent.

In noncompetitive inhibition, the inhibitor often binds to a site on the enzyme other than the active site, deforming the enzyme so that no enzyme-substrate complex is formed. The V_{max} is decreased by the inhibitor and cannot be restored with higher substrate concentrations. Many enzymes possessing an essential sulfhydryl or imidazole group(s) are noncompetitively inhibited by heavy-metal ions which alter the molecule's natural conformation (17).

Enzymes are frequently expressed in terms of activity rather than molar concentration because quite often the latter is unknown. The Commission on Enzymes of the International Union of Biochemistry has defined that one International Unit (IU) of enzyme is that amount that catalyzes the formation of 1 micromole of product per minute under defined conditions (18). "Specific activity" is the number of enzyme units per milligram of protein. Specific activities are usually reported

for optimal assay conditions at a fixed temperature and with all substrates present at saturating conditions. The turnover number or "molecular activity" is the number of substrate molecules transformed per minute by a single enzyme molecule (or by a single active site).

Enzymes Investigated as Potential Toxicity Indicators

Inhibition Methods. Cholinesterases are probably the most investigated enzymes regarding their applicability in the detection of toxic substances in the environment. This is primarily due to the considerable amount of information available concerning the mode of inhibitory action by organophosphorus and carbamate insecticides. Cholinesterases act as highly active catalysts for the hydrolysis of certain esters. The toxicity of insecticides is mainly based on their ability to inactivate acetylcholinesterase by stoichiometric phosphorylation, preventing ester hydrolysis. Hence, the hydrolysis products, alcohol and acid, are not observed. Barendz (19) developed a sensitive tube using butyrylcholinesterase (BuCHE) for detecting cholinesterase inhibiting compounds in air samples. The method involves the use of an orange-red compound, 2,6-dichloro-indophenyl acetate, which is converted enzymatically into a deep blue alcohol. The color transformation will not occur when the enzyme is inactivated. BuCHE is contained as an immobilized gelatin layer inside the tube, along with a gelatin layer of substrate. An air sample is pumped through the tube followed by a buffer solution, and any blue color due to the hydrolysis product is indicated at the bottom. Using this method, Barendz was able to

detect dimethyl-2,2-dichlorovinyl phosphate (DDVP, Vapona) at concentrations as low as 0.4 mg/m^3 (ppb), the MAC value is 1 mg/m^3. Other investigators (9) have used immobilized cholinesterase pads but were only able to detect 0.2 ppm Vapona. Guilbault et al. (20) exposed various types of insect cholinesterases to 15 types of pesticides. Cholinesterase from the house fly was found to be specific for Vapona and carbaryl (Sevin), causing 50 percent inhibition at concentrations of 6×10^{-8} M (14 ppb) and 9×10^{-7} M (180 ppb), respectively.

Townshend (21) has reviewed various pesticides, drugs, and metal ions which effectively inhibit several enzymes. For example, Aldrin and Sevin have been found to inhibit lipase activity at concentrations ranging from 0.1 to 10 ppm, and Lindane in the range of 1 to 100 ppm. Townshend, citing Guilbault and Sadar (22), stated that the herbicide, 2,4-D, could be detected in the range of 10 to 1000 ppm by inhibition of lipase activity. Guilbault and Kramer (23) were able to inhibit lipase activity by as much as 90 percent using 1×10^{-4} M (26 ppm) of the pesticide demeton-O (Systox).

It is well known that even trace amounts of non-essential metals inhibit enzymatic reactions; however, the exact mode in which this occurs is not well understood (17). Many enzymes contain sulfydryl and imidazole groups which are particularly susceptible to metal ions that bind strongly to sulfur and nitrogen. This can result in an alteration of the enzyme configuration, or a blockage of the active site, both of which can suppress enzymatic activity.

Urease is a commonly selected enzyme for monitoring toxic metals because it is known to be highly inhibited by heavy metal salts and

other compounds (8,11). Like acetylcholinesterase, urease is stable, the reaction products can be measured easily, and the technique of immobilization is well established. Ogren and Johansson (11) were able to determine mercury in the range of 0.7 nanomole (nmole), (0.14 µg), using urease immobilized in an enzyme reactor. Mercury inhibited the splitting of urea into bicarbonate and ammonia, the concentration of the latter being measured by an ammonia gas electrode. The amount of inhibition was found to be linear with the amount of mercury added. Urease has been utilized by Toren and Burger (24) to determine mercury and silver in the microgram to nanogram range. Silver is a strong inhibitor of alcohol dehydrogenase activity, and 1 to 20 picogram (pg) per ml of Ag^{+} can linearly inhibit alcohol dehydrogenase (21). Christensen and Reidel (8) have recently developed a system using both cholinesterase and urease immobilized with gelatin onto the surface of glass capillary tubes. Water containing various inhibitors was pumped through the tubes and the differences in enzymatic activities were measured. They were able to achieve inhibition with aqueous solutions of mercuric chloride, cadmium chloride, dicofol, chloropyrifos and pydrin. Limits of sensitivity were found to be 20.1 ppb of carbaryl using acetylcholinesterase and 17.1 ppb of $CuCL_2$ using urease.

The methods described above demonstrate that metals, such as mercury and silver, are especially powerful inhibitors of enzymes containing sulfhydryl groups. Indirect methods of analysis have been devised based on the reversal of heavy metal inhibition. Cyanide ($>10^{-10}$ mole) and sulfide ($> 5 \times 10^{-10}$ mole), for example, may be determined by measuring the degree to which they reverse mercury inhibition of invertase (21).

Phosphatases have been shown to be inhibited by several environmental toxicants. Guilbault et al. (25) observed that alkaline phosphatase was inhibited 50 percent by aldrin (50 ppm) and heptachlor (448 ppm). Sastry and Sharma (26) observed the in vitro inhibition of three phosphatases using mercuric chloride. Glucose-6-phosphatase, acid phosphatase, and alkaline phosphatase were inhibited by 50, 40, and 39 percent, respectively, by 0.54 ppm $HgCL_2$.

Guilbault et al. (27) compared the effects of metals and non-metals on horseradish peroxidase. Ions of interest which caused 50 percent inhibition were S^{2-} (1.7 ppm), Cd^{2+} (470 ppm), and Ni^{2+} (308 ppm). No inhibition was observed using NO_3^-, PO_4^{3-}, and Zn^{2+}.

The main limitations of the studies described above are that the enzyme systems investigated are not always sensitive enough, rarely <1 ppm causes inhibition. In addition, many compounds may inhibit a given enzyme, thereby making the method questionable for use as a selective environmental toxicity test. However, Hudson et al. (28) reported that lead in vivo specifically inhibited δ-amino levulinic acid dehydratase (ALA-D) activity, with essentially no interferences from other metals. The exposure of lead to various fish consistently inhibited ALA-D activity within two weeks at concentrations as low as 10 ppb. Near lethal exposures (ppm level) of cadmium, copper, zinc, and mercury did not significantly inhibit ALA-D activity. There was a linear relationship between ALA-D activity and log of blood lead concentration and lead in water.

Various studies have shown that adenosine triphosphatase (ATPase) is inhibited by trace amounts of organochlorine insecticides (29,30,31) and many organic salts (32,33). Riedel and Christensen (34) studied

the effect of 19 selected chemicals, most of which are common water pollutants, on Na^+/K^+-ATPase activity. Salts such as $AgNO_2$, $HgCL_2$, $CuCL_2$, and $CdCL_2$ were the most inhibitory substances, causing 50 percent inhibition at 12, 68, 64, and 195 ppb, respectively. Cutkomp (35) showed that DDT and related insecticides promote inhibition of mitochondrial Mg^{2+}-ATPase from fish brain when tested in vitro or when fish are continuously exposed to 0.5 to 2.0 ppb DDT. Organophosphate and carbamate insecticides did not produce inhibition in any of the ATPases examined.

Activation Methods. Many enzymes contain tightly bound prosthetic metals such as zinc, iron, molybdenum, and copper, which are essential for activity. Removal of the metal results in a metal-free inactive enzyme called an apoenzyme. The apoenzyme has a high affinity for the missing metal ion and can often be reactivated upon addition of the metal. This technique has been utilized as an extremely sensitive analytical tool for the detection of trace amounts of metals. Lehky and Stein (36) prepared an apoenzyme from the zinc-bound enzyme aminopeptidase (pig kidney), and were able to detect zinc in the range of 5 to 50 pg/ml (part per trillion). A sample containing 5 pg/ml will cause a significant reactivation of aminopeptidase which can easily be monitored. This level is well below the detection limit of metal analysis using an atomic absorption unit, which has an average limit of zinc detection of around 5 µg/ℓ (ppb). Lehky and Stein (36) investigated the action of 18 other cations, such as Mg^{2+}, Cr^{2+}, Mn^{2+}, Fe^{2+}, and Cd^{2+}, all of which failed to bring about any restoration of activity. In contrast, Cu^{2+}, Co^{2+}, and Ni^{2+} could reactivate apoaminopeptidase. Therefore, it was suggested that this enzyme could also be used to

estimate the latter cations. Furthermore, it was possible to distinguish 0.1 ng Zn/ml with 10 percent accuracy in the presence of a 10 to 100-fold excess of most other metals by incorporating EDTA, which progressively removed undesirable cations.

Townshend and Vaughan (37) were able to detect 10 ng of zinc using apoalkaline phosphatase. However, at these levels, the enzyme activity was appreciably lower than at higher concentrations of zinc. Polyphenol oxidase is an enzyme which contains four atoms of copper per molecule (38). The copper in the enzyme can be removed by washing with cyanide, so that the apoenzyme becomes copper-reactive. Stone and Townshend (38) demonstrated that the apoenzyme was able to remove 50 ng of copper from two liters of water; the level of copper being determined by measuring the activity of the regenerated enzyme.

Three Enzymes Potentially Capable of Indicating Toxicity

α-Chymotrypsin. α -Chymotrypsin is a proteolytic enzyme that is synthesized in the acinous cells (glands resembling bunches of grapes) of the pancreas as a catalytically inactive precursor called chymotrypsinogen. Chymotrypsinogen is carried by the pancreatic juice into the duodenum (small intestine) where it is converted into the active enzyme, α-chymotrypsin, through the excision of two dipeptides by the action of trypsin. This type of biosynthesis protects the pancreatic cells against self-destruction due to the proteolytic action of the enzymes (39).

The active α-chymotrypsin molecule has a molecular weight of 24,500 and has 241 amino acid residues. The amino acid sequence was

determined by Hartley (40). The molecule has three polypeptide chains consisting of 13, 131, and 97 residues. The chains are held together by two disulfide bonds. Two specific residues are essential for catalytic activity, histidine number 57 and serine number 195, each being present on different chains. The α, β, γ, δ, and π forms of chymotrypsin are all products derived from α-chymotrypsinogen; however, each differs in details of isolation, crystal habit, specific enzymatic activity, molecular size, and electrochemical characteristics.

α-Chymotrypsin primarily catalyzes the hydrolysis of peptide bonds of proteins adjacent to the carboxyl group of the aromatic amino acids, tryptophan, tyrosine, and phenylalanine. Proteolysis can occur adjacent to other large hydrophobic amino acids, such as leucine and methionine. α-Chymotrypsin also catalyzes the hydrolysis of amides and esters of aromatic amino acids of a large number of hydrophobic compounds. Common substrates used to assay α-chymotrypsin activity are benzoyl-L-tyrosine ethyl ester, N-formyltyrosinamide, N-acetyltyrosinamide, and p-nitrophenyl acetate. α-Chymotrypsin and other proteolytic enzymes increase the rate of hydrolysis of proteins by a factor of about 10^5. Therefore, digestion processes involving these types of proteins take only a few hours, as opposed to the uncatalytic rate which is around 50 years (39).

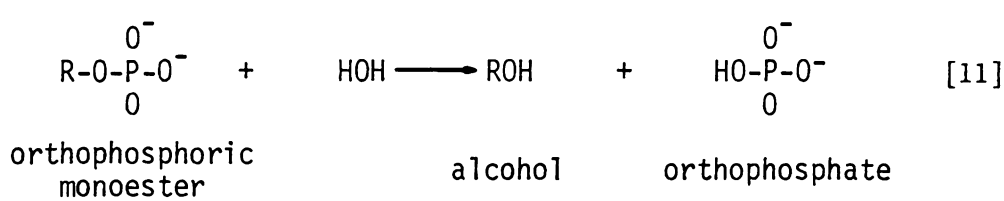
There is an increasing interest in α-chymotrypsin inhibitors due to their possible role in altering the nutritive value of food, and their usefulness in elucidating biochemical interactions. There are a large number of naturally occurring α-chymotrypsin inhibitors. Various α-chymotrypsin inhibitors have been characterized and reported in peanuts (41) and in soybean (42). Johnson and Knowles (43) were able to clarify the characterization of the active site in α-chymotrypsin by

using N-acetyl-D-tryptophan amide, N-acetyl-L-tryptophan, and N-acetyl-D-tryptophan as competitive inhibitors.

Information regarding the action of toxic chemicals on α-chymotrypsin is limited. Some work in this area has been done by Green et al. (44) who studied the effects of cations on α-chymotrypsin. Green observed 50 percent inhibition in enzyme activity resulting from addition of 65 ppm Zn^{2+}, and 100 percent inhibition upon exposure to 64 ppm Cu^{2+} and 200 ppm Hg^{2+}. The α chymotrypsin activity was monitored using the substrate, acetyl-L-tyrosine ethyl ester, at pH 7.8 and 25°C. A slight activation was noted using Ca^{2+} in high concentrations.

The classification number for α-chymotrypsin according to the International Union of Biochemistry is EC 3.4.21.1, where EC is Enzyme Commission, class 3 is hydrolase, subclass 4 is peptide hydrolysis, and sub-subclass 21.1 is serine proteinases (45).

Acid Phosphatase. Acid phosphatase breaks ester linkages liberating inorganic phosphate from a wide variety of phosphomonoesters and phosphoproteins (46). The enzyme-catalyzed reaction is represented according to the following:



[11]

Acid phosphatase is ubiquitous in nature, present in vegetables and grains, and distributed throughout organs and muscles of animals. The enzyme is very active in human prostatic tissue, and is often used as an index of prostatic cancer by clinical chemists. Other areas rich in acid phosphatase include erythrocytes, liver, spleen, and blood (46).

Three acid phosphatases have been isolated by Verjee (47) from wheat germ. Each isoenzyme has a characteristic optimal pH for activity. Verjee (47) observed that Mn^{2+}, Co^{2+}, and Ni^{2+} at 2 mM (around 116 ppm) had no significant effect on the activity of isoenzyme E_1, but inhibited E_2 and E_3 by 10 to 40 percent. All three isoenzymes were strongly inhibited by 0.4 mM (7.6 ppm) of fluoride, 0.4 μM of molybdate, and 2 mM of orthophosphate.

Sastry and Gupta (48) investigated the effect of cadmium on the digestive systems of the Teleost fish. Acid phosphatase activity was found to be inhibited 27 percent in the liver and 20 percent in the intestine by 6.8 mg/ℓ $CdCL_2$. Jachim et al. (49) studied the in vitro and in vivo addition of metal salts to five liver enzymes. In vitro activity of acid phosphatase was almost completely abolished by Pb^{2+} (2070 mg/ℓ), Cu^{2+} (636 mg/ℓ), Ag^{+} (1080 mg/ℓ), and Hg^{2+} (2000 mg/ℓ). However, Cd^{2+} (1124 mg/ℓ) inhibited enzymatic activity by 11.5 percent. Acid phosphatase activity decreased slightly from in vivo exposure (enzyme activity from surviving fish) to the following metals: Pb^{2+} (188 mg/ℓ), Cu^{2+} (3.2 mg/ℓ), Ag^{+} (0.04 mg/ℓ), Hg^{2+} (0.23 mg/ℓ), and Cd^{2+} (27 mg/ℓ). It was concluded that the differences in enzyme reponses from in vitro and in vivo exposure was due to molecular involvement, such as cellular or tissue barriers, and/or interactions with cofactors and regulators.

Guilbault et al. (25) studied the effect of various pesticides and herbicides on acid phosphatase activity. No effect on enzyme activity was observed upon exposure to malathion, Sevin, heptachlor, DDT, aldrin, lindane, and 2,4-D. However, methyl parathion at 5 to 60 ppm caused a slight decrease in acid phosphatase activity.

As previously stated, fluoride has been shown to strongly inhibit acid phosphatase activity (47). Reiner et al. (50) demonstrated that 2 mg/ℓ fluoride can substantially inhibit acid phosphatase activity. Reiner et al. (50) showed that fluoride (60 to 300 mg/ℓ) inhibited prostatic acid phosphatase by 100 percent, and as the fluoride level was increased, the degree of enzyme inhibition declined. However, the inhibition increased again to 100 percent when the level of fluoride was increased to around 600 mg/ℓ. It was postulated that two forms of fluoride became present in the reaction mixture, an ion form and a complex polymer form, which is consistent with the established behavior of fluoride in aqueous solutions (46).

Other anions, such as chloride, bromide, and thiocyanate inhibit prostatic acid phosphatase, as well as a wide variety of hydroxycarboxylic acids (46), and certain anionic surfactants (51).

The classification number (IUB) for acid phosphatase is EC 3.1.3.2, where class 3 is hydrolase, subclass 1 is acting on ester bonds, and sub-subclass 3.2 is phophoric monoester hydrolase (acid form) (45).

Carbonic Anhydrase. Carbonic anhydrase primarily catalyzes both the hydration of carbon dioxide and the dehydration of bicarbonate, as indicated by the following general reaction:

$$CO_2 + H_2O \underset{\text{dehydration}}{\overset{\text{hydration}}{\rightleftharpoons}} H^+ + HCO_3^- \qquad [12]$$

Carbonic acid, H_2CO_3, is neglected in the expression because it is a rather strong acid, therefore, its concentration is small compared to bicarbonate (52). The reaction proceeds at an appreciable rate in the absence of carbonic anhydrase. The rate constants for the catalyzed

reaction are between 10^5 and 10^6 seconds^{-1}; consequently, carbonic anhydrase possesses one of the highest measured turnover rates among all enzymes (53,54). Carbonic anhydrase catalyzes the hydrolysis of certain esters and related compounds, and the hydration of aldehydes (55,56). However, the catalytic efficiency using these substrates is much lower than the rate of catalytic hydrolysis of carbon dioxide.

Carbonic anhydrase is widespread in nature, appearing in animals, plants, and certain bacteria. The enzyme plays an important role in respiration by facilitating the transport of carbon dioxide. The physiological requirement for such a powerful catalyst becomes apparent, due to the short time required for blood to pass through the capillaries. The enzyme is most readily isolated from erythrocytes, where it has a concentration of 1 to 2 grams per liter of mammalian red blood cells. Carbonic anhydrase is also present in kidney, brain, liver, eye lens, gills, and various glands and other organs (55).

There are several forms of mammalian carbonic anhydrases, each the product of a separate gene, differing in enzymatic properties, amino acid sequence, and inhibitor binding. In human erythrocytes, there are three isoenzymes, A, B, and C, of which A and B are of low activity and C possesses high activity. Form A represents only about 5 percent of the total, form B is present in abundance at 83 percent, and C accounts for 12 percent. Bovine erythrocytes have two isoenzymes, A and B, of which A represents 20 percent and B represents 80 percent of the total. Both forms exhibit high activity (55).

The mammalian isoenzymes of carbonic anhydrase have molecular weights near 30,000. Each enzyme is composed of one essential zinc ion and contains one single polypeptide chain consisting of approximately

260 amino acid residues. Carbonic anhydrases are rich in basic and acidic amino acids and have a high proline content. The sulfur content is small, around 0.34 percent (3 atoms per molecule) in bovine carbonic anhydrase (57). The active site is a region located at the center of the molecule containing one zinc ion. The zinc is firmly bound by three histidyl residues at positions 93, 95, and 117 (55). The zinc ion can be removed from the enzyme by dialysis treatment and the use of a chelating agent such as 1,10-phenanthroline. The result is a metal-free inactive apocarbonic anhydrase (58,59). Of the large number of metal ions tested for their ability to reactivate the apoenzyme, only Co^{2+} yields a specific activity of similar magnitude to that of the zinc-bound enzyme. Mn^{2+}, Fe^{2+}, and Ni^{2+} have occasionally been found to produce low activity (59).

Carbonic anhydrases are inhibited by many aromatic sulfonamides and by certain monovalent anions. Various studies have been carried out using sulfonamide and anionic inhibitors in an attempt to characterize the active site and the binding role of zinc (12,60,61). The results suggest that the sulfonamides and anions coordinate to the zinc ion. The metal-free enzyme binds virtually no inhibitor (60). These studies are especially interesting kinetically since one of the substrates, the anion HCO_3^-, exhibits inhibitory capabilities (62). Metal-binding monodenate ions, CN^-, HS^-, NCO^-, NCS^-, and CLO_4^-, typically referred to as "metal poisons", compete with sulfonamide inhibitors at the metal binding site.

The concentrations of sulfonamide and anionic species necessary to induce carbonic anhydrase (CA) inhibition are usually very low. Davis (63) reported that 5×10^{-8} M (4 µg/ℓ) of sodium sulfide and 4×10^{-6}M

(0.7 mg/ℓ) of sulfanilamide could substantially inhibit the hydrating activity of human erythrocyte carbonic anhydrase at 2.7×10^{-8} M (0.8 mg CA/ℓ). The chelating agents, methylamine, ethylenediamine, and 1,10-phenanthroline at concentrations as high as 2.5×10^{-3} M, had no effect on carbonic anhydrase activity (63). Similar results were obtained by Lindskog (60) who reported on the action of sulfide, cyanide, and acetazolamide or Diamox (a sulfonamide). The hydrating activity of bovine carbonic anhydrase was inhibited 50 percent by 1.7×10^{-7} M (5.4 μg/ℓ) sulfide, 3.7×10^{-6} M (96 μg/ℓ) cyanide, and 9×10^{-9} M (2 μg/ℓ) Diamox. Carbon dioxide was used as the substrate and the concentration of bovine carbonic anhydrase was 1.2×10^{-8} M (0.36 mg CA/ℓ).

Christensen and Tucker (64) studied the in vitro effect of 56 chemicals, including Diamox, monovalent anions, metal cations, and several pesticides, on the carbonic anhydrase of the fish, Ictalurus puctatus. Enzyme activity was inhibited 50 percent using 3×10^{-9} M (0.67 μg/ℓ) Diamox, 9×10^{-4} M (101 mg/ℓ) Cd^{2+}, 9×10^{-3} M (530 mg/ℓ) Ni^{2+}, 1×10^{-3} M (304 mg/ℓ) diazinon, and 4×10^{-3} M (1420 mg/ℓ) DDT. It is interesting to note that there was no inhibition using 1×10^{-4} M (11 mg/ℓ) Cd^{2+}. The lethal level or LD_{50} concentration (lethal dose to kill 50 percent of the population) is reported by Roch and Maly (65) to be in the range of 0.1 to 3.8 mg/ℓ (96 hour).

Other investigators have indicated that heavy metal salts and pesticides can inhibit carbonic anhydrase activity, but again the levels required to create inhibition are usually well above the lethal level. Maguire and Watkin (65) were able to inhibit bovine CA using $HgCL_2$ and $CdCL_2$ at a 1:1 ratio of inhibitor to enzyme concentrations when p-nitrophenyl acetate was used as a substrate. Exact concentrations were not

reported. It was also cited that the pesticides, dieldrin, chlordane, and DDT, all chlorinated hydrocarbons, were fairly weak noncompetitive inhibitors. Johnson and Walker (67) reported that the hydrating activity of carbonic anhydrase, in rat and domestic fowl testis, was inhibited 50 percent *in vitro* using 0.01 M (1.8 g/ℓ) $CdCL_2$; however, no enzyme inhibition was observed *in vivo* using 0.03 mM (6 mg/ℓ) $CdCL_2$.

It is thought that the inhibition of carbonic anhydrase is involved, to some extent, in the eggshell thinning of certain organisms. Peakall (68) reported that the injection of DDE into ringdoves shortly before egg laying, severely depressed carbonic anhydrase activity and brought about a marked decrease in eggshell thickness. Various studies tend to indicate that the effect of compounds, such as DDE in birds, may be more complex than a mere inhibitory action on carbonic anhydrase. Pocker *et al.* (69,70) suggested that DDT, DDE, and dieldrin do not block the active site and are not true inhibitors of carbonic anhydrase esterase or hydrase activity. He demonstrated that impairment of catalytic activity *in vitro*, only occurs when the insecticides are present in excess of their solubility limits. Pocker suggested that the insecticides simply have the ability to coprecipitate minute amounts of enzyme from solution, thus decreasing the apparent activity. When the solubility limits of DDT, DDE, and dieldrin are exceeded, carbonic anhydrase is adsorbed during the growth of the crystal formations. It was ascertained that 10^{-5} M solutions of DDT, DDE, or dieldrin were capable of coprecipitating 10^{-8} M bovine carbonic anhydrase. It is difficult to predict the effects of these insecticides *in vivo* because DDT and DDE concentrate in fatty tissues, thereby reducing the availability for interaction with the enzyme.

The classification number (IUB) for carbonic anhydrase is EC 4.2.1.1, where EC is Enzyme Commission, class 4 is lyases, subclass 2 is carbon-oxygen lyases, and sub-subclass 1.1 is hydro-lyases (45).

Chemicals Potentially Capable of Altering Enzyme Activity

Divalent Cations. The contamination of heavy metals in the environment has stimulated much research concerning the impact of metals on biomolecules, such as enzymes. Cadmium, nickel, and zinc are three divalent ions which appear in the environment and are known to interact with amino acids and peptides (17).

Cadmium is a relatively rare element in the earth's crust, present in minute traces as the mineral, greenockite. It is found in zinc, copper, and lead ores as an impurity (up to 3%), and is generally produced as a by-product of the metal-refining processes of these ores (71). The mine production of cadmium in 1974 was 11,000 tons (71). Cadmium is used industrially as an anti-friction agent, a corrosion inhibitor, a polymerization catalyst, and a component in alkaline storage batteries. It is also present as an impurity in superphosphate fertilizers. Cadmium gains access to the environment largely through zinc-refining operations, waste streams of electroplating plants, agricultural runoff, and atmospheric washout.

The Federal 8 hour TWA standard for cadmium fumes is 0.1 mg Cd/m^3 (72). The term, TWA, refers to a time-weighted average threshold limit value which workers can be repeatedly exposed to without adverse effects. The raw surface water criteria for cadmium in public water supplies is 0.01 mg/ℓ (73). The most common route of cadmium exposure in man is

through the respiratory tract; consequently, the largest threat of exposure occurs in industrial areas where cadmium fumes may be produced. Heavy smoking has been reported to considerably increase cadmium levels in tissues also (72).

At the cellular level, the cell nucleus appears to act as a target for cadmium toxicity. Chin and Sina (74) reported that in the cells of Physarum, cadmium morphologically disrupted the integrity of the nucleoli, producing centrally located holes. It was observed that cadmium suppressed DNA and RNA synthesis by inhibiting uridine incorporation into RNA. Cadmium has been reported to potentiate the action of certain drugs. A dosage of 2 mg Cd/kg in mice can potentiate the narcotic response of certain drugs by inhibiting microsomal hydroxylase activity and other hepatic drug-metabolizing enzymes (75).

The significance of cadmium ion interaction with enzymes, can be described through several possible mechanisms. Cadmium is chemically similar to zinc and can often replace zinc in metalloenzymes. This substitution can result in either an enhancement or a reduction in enzyme activity. Cadmium readily forms complexes with ammonia and amines, and reacts with sulfur and other nonmetals such as phosphorus and selenium. Cadmium can therefore bind with functional side groups, block the active site, and/or alter the enzyme configuration.

The element, nickel, constitutes about 0.008 percent of the earth's crust and is found in meteorites (5 to 50%), and in ores combined with sulfur, antimony, or arsenic. The nickel content in seawater ranges from 0.1 to 0.5 µg/ℓ. Processing and refining nickel is usually accomplished by reacting impure nickel powder with carbon monoxide to form gaseous nickel carbonyl which is then treated to deposite high purity metallic

nickel (72). In 1974, 540,000 tons of nickel were produced (71). Nickel is used in electroplating, casting operations, magnetic alloys and tapes, soaps and oils, and in coinage. Nickel enters the environment in much the same way as cadmium; metal-refining operations, waste streams, land runoff waters, and leachates.

The Federal TWA standard for nickel is 1 mg Ni/m^3; however, NIOSH recommends adherence to 15 µg Ni/m^3 as a TWA for a 10-hour workday (72). Nickel is not listed in the 1978 EPA interim primary drinking water standards.

Nickel is considered an essential micronutrient, although its physiological role has not been completely established. Nutritionists have determined that nickel defeciencies in rats cause a reduction in erythrocytes and hemoglobin, anemia, and retarded growth (76). Although nickel is essential, excess concentrations of the metal can be toxic and/or carcinogenic. Nickel carbonyl has been thought to affect the arylhydrocarbon metabolizing enzyme system, thereby promoting lung tumorigenesis by carcinogenic polycyclic hydrocarbons (75). Nickel salts have been shown to cause defective gastro-intestinal absorption due to inhibition of the activities of alkaline phosphatase and enterokinase (75). The interaction of nickel ions with enzymes is probably similar to that of cadmium; that is, nickel may alter the enzyme by binding with ligands, or replacing or competing with a metal cofactor.

Zinc is a biologically essential element required in trace amounts. Unlike cadmium and nickel, zinc is not considered to be toxic in normal concentrations. The zinc requirement in a normal male is estimated to be around 13 mg Zn per day (77). Zinc is used industrially as a component in chemical synthesis, battery cells, textile finishing, oil and gas,

dyes, and many other products. It can enter the environment in routes similar to cadmium and nickel. The raw surface water criteria for zinc in public water supplies is 5 mg/ℓ (73). Exorbitant amounts of zinc in the diets of rats can lead to a reduction in hemoglobin synthesis by suppressing cytochrome oxidase. Excess zinc may result in abnormally high activity of the zinc-metalloenzyme, alkaline phosphatase (77). Zinc is an essential metal ion cofactor in more than 80 enzymes. The stability constants of zinc complexes with nitrogen and oxygen ligands are greater than those for cadmium complexes. Consequently, zinc is often used to prevent manifestations of cadmium toxicity. For example, pretreatment of Escherichia coli cells with zinc can alleviate cadmium toxicity ; however, it does not ameliorate toxic symptoms due to cadmium in Physarum (74).

Monovalent Anions. Many anions have been reported to inhibit enzymes. The mechanism by which this occurs is often generally categorized as their ability to complex with ligands and their affinity for an essential metal or nonmetal cofactor. Fluoride, sulfide, and nitrate ions are often present in the environment and are capable of interacting with enzymes. Therefore, the potential of using enzymes to detect the presence of these anions is important to consider.

Fluoride is often added to public drinking waters as a public health measure. Small concentrations of fluorides in drinking water impede dental caries by being incorporated into hydroxyapatite (crystalline calcium phosphate). Excessive amounts of fluorides can cause a disfigurement of teeth, characterized by the impaired calcification of the teeth and bones, which is commonly referred to as "mottling" or dental fluorosis (75). Approximately 1 mg/ℓ of fluoride ion is desirable and

now recommended in public waters for optimal dental care (73). Common chemicals used in fluoridation include sodium fluoride (NaF), sodium silicofluoride (Na_2SiF_6), and hydrofluosilicic acid (H_2SiF_6).

Consecutive subcutaneous (s.c.) injections of NaF in rats at concentrations of 100 ppm have been shown to reduce the activities of alkaline and acid phosphatase in kidney tissue (75). Injections (s.c.) of 1 to 10 mg F^-/kg, produce significant inhibition of liver ATPase and greatly suppress the succinoxidase system within the liver (75).

Hydrogen sulfide (H_2S) and sulfide ions (HS^-) are produced from sulfate (SO_4^{2-}) by the action of anaerobic bacteria. Under anaerobic conditions, the sulfate ion serves as an electron acceptor and is reduced to sulfide ion, which establishes an equilibrium with hydrogen ion to form hydrogen sulfide in accordance with its primary ionization constant ($k_1 = 9.1 \times 10^{-8}$) (78). At pH 7, the concentrations of H_2S and HS^- are relatively equal and hydrogen sulfide usually volatilizes as a gas. The gas can cause odor problems in sewer facilities because it can be detected at very low concentrations. Hydrogen sulfide gas can dissolve in water and be oxidized into sulfuric acid through the action of bacteria. This can cause corrosion problems, especially in non-ventilated sewers and wastewater facilities.

Hydrogen sulfide is also noted for its toxicity. The lethal dose for 50 percent (LD_{50} concentration) in hamsters is 50 mg Na_2S/kg (oral administration) (79). High intake levels of some soluble sulfides can produce a functionally inactive blood pigment called sulfhemoglobin; however, its formation is not promoted by inhalation of H_2S (75). The interaction of sulfides on enzymes has been narrowly investigated. However, as previously described, sulfides (4 to 5 ppb) can inhibit carbonic

anhydrase (60,63). This suggests that carbonic anhydrase may have some potential for signaling the presence of low levels of sulfides.

Nitrates are formed by the aerobic oxidation of ammonia to nitrites by Nitrosomonas and further oxidation to nitrates by Nitrobacter. Conversion of ammonia to nitrite is the rate-limiting step; therefore, nitrite concentrations are normally very low. The nitrification process is sometimes utilized in wastewater treatment plants, thereby reducing problems of toxicity due to ammonia and organic nitrogen compounds. Nitrates are often found in relatively high levels in certain ground-waters, because it rapidly percolates through soil. In 1940 it was observed that drinking waters high in nitrate content were responsible for a condition known as methemoglobinemia in infants (78). Methemoglobinemia is a condition in which the reduced ferrous iron contained in hemoglobin is converted to the ferric form. The hemoglobin is then unable to combine with oxygen and a tissue deficit is created (75). The proposed drinking water regulations require that the nitrate concentration in terms of nitrogen be less than 10 mg/ℓ in public water supplies (78). In addition, nitrate ions compete with iodide ions and prevent accumulation of iodide in the thyroid tissue (75). The effect of nitrate on specific enzymes has not been widely studied, although Guilbault et al. (27) did not observe any effect with nitrate ions on the enzyme, peroxidase.

Herbicides and Pesticides. The mode of action of organophosphorus and carbamate insecticides on cholinesterase is well established; however, the interaction of herbicides and pesticides on other enzymes has not been rigorously investigated.

2,4-Dichlorophenoxyacetic acid (2,4-D) is a chlorophenoxy herbicide widely used for the control of broad leaf weeds. In 1975, 59 million pounds of 2,4-D was used in the U.S., according to the Council for Agricultural Science and Technology (80). It is postulated that the mechanism of 2,4-D action involves a complex series of reactions initiated by the depression of the gene regulating the synthesis of the enzyme, RNAase (81). Trace amounts of 2,4-D (5 to 25 ppm) stimulate plant growth in much the same way as the auxin, indoleactic acid (IAA). Concentrations of 2,4-D necessary to inhibit growth range from 100 to 500 ppm. The LD_{50} concentration (acute oral dose) of 2,4-D in rats is 375 mg/kg (82). In mammalian skeletal muscle, 2,4-D has been observed to decrease the activities of hexokinase and phosphorylase, creating a disturbance in glycogen metabolism (75). Information concerning the action of 2,4-D on other enzymes is at best, vague.

Atrazine, 2-chloro-4(ethylamino)-6-(isopropylamino)-s-triazine, is a triazine herbicide widely used for weed control. Atrazine is assumed to inhibit CO_2 fixation by blocking photosynthesis at the photolysis of water step (81). Small concentrations of atrazine have been reported to stimulate growth (81). The LD_{50} concentration (acute oral dose) of atrazine in rats is 3080 mg/kg (82).

Diuron, 3-(3,4-dichlorophenyl)-1,1-dimethylurea, is a substituted urea herbicide which is slow-acting but effective in killing weeds. Diuron inhibits both flavin mononucleotide (FMN) and vitamin k_3 catalyzed photophosphorylation (81). The LD_{50} concentration (acute oral dose) of diuron in rats is 3400 mg/kg (82). No information is available regarding the action of atrazine or diuron on enzymes.

Malathion, O,O-dimethyl-S-(1,2-dicarboethoxyethyl) dithiophosphate, is an organophosphate insecticide used to control insect pests on fruits, vegetables, and ornamental plants (72). It has been widely used in the control of houseflies, mosquitoes, and lice. In 1971, the annual production of malathion in the U.S. totaled about 35 million pounds (72). The LD_{50} concentration (oral dose) of malathion in rats is 1000 to 1375 mg/kg (82). In vivo intoxication (acute) of rats with malathion produces a rise in the activity of tyrosine transaminase and alkaline phosphatase in their livers (75). This is a characteristic response in animals exposed to anticholinesterase compounds at sublethal, acute doses.

Carbaryl (Sevin), 1 naphyl N-methylcarbamate, is an anticholinesterase insecticide used for combating insect pests in both domestic and agricultural areas. Approximately 53 million pounds of carbaryl were produced in 1972 in the U.S. (72). The LD_{50} concentration (oral) of carbaryl in rats is 560 mg/kg (82). The toxicity of malathion and carbaryl is mainly based on their ability to inhibit cholinesterase at terminal nerve endings. Carbaryl is known to produce hepatic lesions in both acute and chronic administrations, and it stimulates liver cytochrome oxidase and succinate dehydrogenase oxidative activities after acute administration (75).

Additional Compounds. The chelating agent, ethylenediaminetetraacetic acid (EDTA), can often reversibly bind divalent cations and thus can noncompetitively inhibit some enzymes requiring such cations for activity. Sastry and Sharma (26) reported that the in vitro addition of EDTA resulted in the restoration of certain enzymes inhibited by mercury ions.

Sulfanilamide, p-aminobenzenesulfonamide ($NH_2C_6H_4SO_2NH_2$), is an antibacterial agent widely used in medicine. Sulfanilamides and other sulfonamides, inhibit the growth of many bacteria by preventing the formation of folic acid. They competitively inhibit the incorporation of p-aminobenzoic acid at the final step in the biosynthesis of folic acid (15). Sulfanilamide is a well-known inhibitor of the zinc-containing enzymes, carbonic anhydrase (12,13,14,63,83) and alkaline phosphatase (84). The mechanism of inhibitory action by sulfanilamide involves the binding of the metal ion at the active site. Because sulfanilamide has the capacity to bind to metal cations, the sulfonamide may be useful in developing indirect methods for analyzing metals; that is, sulfanilamide may preferentially bind to other metals thus inactivating it as a carbonic anhydrase inhibitor. Therefore, other metals might be analyzed by determining the amount of carbonic anhydrase inhibition alleviated by the addition of metals to a sulfanilamide-enzyme mixture.

MATERIALS AND METHODS

Efforts to evaluate the potential for an enzyme to signal toxic effects were usually conducted in three phases. The first phase involved the development and refinement of the α-chymotrypsin, acid phosphatase, and carbonic anhydrase assays from rudimentary procedures. This consisted of testing the sensitivity and response of each system by examining several enzyme and substrate concentrations. In an effort to increase the susceptibility of each enzyme system towards the chemical agents, enzyme and substrate concentrations were employed at minimum levels. The second phase entailed the exposure of the enzymes to various chemical agents, and assaying the enzyme to obtain its activity. Any substantial alteration in enzyme activity was followed by experiments to better define the response of the enzyme to a broad range of concentrations of the chemical agents. A third phase of study with two of the enzyme systems consisted of an attempt to strain the enzyme by modifying such parameters as pH and temperature. The third phase was implemented in the acid phosphatase and carbonic anhydrase systems, but not in the α-chymotrypsin system.

The following section is comprised of subdivisions describing glassware preparation, and the preparation and types of enzymes, buffers, substrates, and chemical agents used in the study. The section also provides information regarding the introduction of the enzymes to chemical agents, and the assay methods used in the determination of each enzyme activity.

Glassware Preparation

All glassware employed in this study was washed using a laboratory detergent and then rinsed thoroughly with hot tap water, followed by several rinsings with distilled water. Glassware used for enzymes, pesticides, herbicides, and other organic materials was rinsed with redistilled acetone prior to distilled water rinses. The glassware was then allowed to air dry. The distilled water was generated by means of an activated carbon, ion-exchange, glass distillation process.

The Enzymes

The enzymes used in this study were α-chymotrypsin (bovine pancreas), acid phosphatase (potato), and various carbonic anhydrases (bovine and human erythrocytes). The enzymes were purchased in purified, lyophilized form from the Sigma Chemical Co., Saint Louis, Missouri. The lot numbers for each type of enzyme are provided in Appendix A, Table A-1. Enzymes were stored in dessicant at $-20^{o}C$. Enzyme solutions were freshly prepared for each experiment by weighing 10 to 20 mg of enzyme on a digital Mettler balance (model AC 100) and quantitatively transferring the lyophilized powder to a 15 ml graduated centrifuge tube. Appropriate solvent (distilled water or dilute HCL) was added and the tubes were mixed by means of a vortex-genie stirrer.

Preparation and Types of Buffers

Several buffer solutions were utilized in the assays of α-chymotrypsin activity. A 0.4 M Tris solution was prepared by dissolving 63.2 g of Tris (hydroxymethyl) aminomethane (THAM) in distilled water and adjusting the volume to one liter. A 0.05 M Tris-chloride buffer (pH 7.6 at 37°C) was prepared by dissolving 4.88 g of Tris (hydroxymethyl) aminomethane hydrochloride (TRIZMA HCL) and 2.3 g of THAM in a liter of distilled water. A 0.05 M Tris-chloride buffer (pH 7.6 at 37°C) containing 0.03 M $CaCL_2$ was prepared by adding 3.327 g/ℓ $CaCL_2$ to the second buffer described above. A 0.08 M Tris-chloride buffer (pH 7.8 at 25°C) containing 0.1 M $CaCL_2$ was prepared by dissolving 7.67 g of TRIZMA HCL, 3.81 g of THAM, and 11.1 g of $CaCL_2$ in a liter of distilled water.

Acid phosphatase assays required the use of a 0.09 M citrate buffer in 0.01 N HCL (pH 4.8). This was prepared by dissolving 4.32 g of citric acid in 250 ml of 0.01 N HCL. The buffer was stored at 0 to 5°C to inhibit microbial growth.

The carbonic anhydrase assays required a 0.02 M Tris-chloride buffer with a pH of 8 at 0 to 4°C. This buffer was prepared by dissolving 2.014 g of TRIZMA HCL and 0.878 g of THAM in distilled water and adjusting the volume to one liter.

The composition of the Tris-chloride buffer solutions was calculated on the basis of the Henderson-Hasselbach relationship; TRIZMA HCL acting as the conjugate acid and THAM as the conjugate base. Conjugate acid and base ratios were altered accordingly to obtain the proper buffer pH at the required temperature.

Preparation and Types of Substrates

Two substrates were employed in the assays of α-chymotrypsin activity; 2-nitro-4-carboxyphenyl-N,N-diphenylcarbamate (NCDC) and benzoyl-L-tyrosine ethyl ester (BTEE). The NCDC substrate solutions were prepared by suspending 26.5 mg in 0.2 ml of 0.4 M Tris in a test tube. After gently warming in a water bath, 1.8 ml of 0.05 M Tris-chloride buffer, pH 7.6 (described in buffer section) was added to the tube and mixed. A 1.07 mM BTEE substrate solution was prepared by adding 0.3353 g of BTEE per liter of 50 percent w/w methanol solution (63 ml methanol added to 50 ml H_2O).

Acid phosphatase assays required the use of disodium p-nitrophenyl phosphate as the substrate and was purchased in the form of 5 mg tablets from Sigma Chemical Co. The substrate solution was prepared by dissolving the tablet in distilled water and then diluting to the appropriate concentration.

The substrate for carbonic anhydrase assays was prepared by saturating water at 0 to 3°C with CO_2 gas. Prior to each experiment, CO_2 gas was bubbled through an air stone into 200 mls of 0 to 3°C distilled water for 30 to 45 minutes.

The Chemical Agents

The inorganic chemical agents of concern in this study were the divalent cations, cadmium, nickel, and zinc, and the monovalent anions, fluoride, sulfide, and nitrate. In addition, many organic chemicals were also studied. These included herbicides (2,4-D, atrazine, and diuron), pesticides (malathion and carbaryl), a chelating agent (EDTA), and an antibacterial agent (sulfanilamide).

Aqueous stock solutions of the metal cations were prepared from Fisher Scientific A.C.S. certified chemicals using chloride salts. Stock concentrations of the metals were verified on a Perkin-Elmer (model 703) atomic absorption spectrophotometer.

Aqueous solutions of the anions were prepared and used in the form of sodium salts. The Fisher grade reagents, NaF and $Na_2S \cdot 9H_2O$ were used as sources of fluoride and sulfide ions, and $NaNO_3$, purchased from Baker Chemical Co., was used as a nitrate source.

Technical grade 2,4-dichlorophenoxyacetic acid (2,4-D) was obtained from Dow Chemical Co. of Midland, Michigan. Stock concentrations of the herbicide were made at 50 to 500 mg/ℓ and checked on a Microtek 220, electron capture, gas chromatograph. Atrazine and diuron were prepared by dissolving 10 mg of each herbicide in redistilled acetone and then washing the mixture into a beaker with 800 mls of distilled water. After gently heating the mixture for one hour to remove the acetone, the volume was adjusted to one liter using distilled water.

Malathion, 25 percent pure, was obtained from the Pesticide Residue Laboratory at Virginia Tech, and was prepared by dissolving 40 mg of the pesticide in a liter of distilled water to produce a concentration of 10 mg/ℓ. Carbaryl (Sevin), 85 percent pure, was obtained from Union Carbide of N.Y. A 10 mg/ℓ aqueous solution was prepared by dissolving the pesticide in distilled water.

Disodium ethylenediaminetetraacetate (EDTA) was diluted in distilled water to make a 100 mg/ℓ solution. Sulfanilamide (p-aminobenzenesulfonamide) was purchased from Baker Chemical Co. and prepared by diluting in distilled water to make a concentration of 100 mg/ℓ.

Introduction of Chemical Agents to Enzymes

Certain chemical agents, especially inorganic ions, will undergo transformations which may possibly retard the agent's ability to interact with a given enzyme. Therefore, it is necessary to discern the species distribution of certain ions in solution. Ionic strength and pH are examples of factors which govern the presence of free ions in solution. Ionic strength was considered negligible in this study due to the low levels of chemical agents used. The pH of the enzyme solutions used in this study was in the range of 6 to 7. In this range only free ion species of Cd, Ni, and Zn are present when added as chloride salts at the concentrations studied. At higher pH levels, it is important to consider that metal hydroxide complexes may form. At pH 7, H_2S and HS^- are roughly present in equimolar concentrations ($pk_1 \simeq 7$); therefore, the species concentration of HS^- was calculated as such in this work. NaF and $NaNO_3$ were assumed to completely dissociate, liberating fluoride and nitrate ions. The effect of sodium and chloride on the enzymes was determined by using NaCL as an additive agent.

The enzymes were exposed to the chemical agents in an aqueous solution containing the chemical agent of interest. The solution was mixed and the duration of exposure was noted. A small aliquot of the enzyme/chemical agent solution was then added to the assay system to determine the activity of the enzyme. Chemical agents used in the carbonic anhydrase system required equilibration at 0 to 4^oC before addition to the enzyme solution. A listof the various chemical agents used and the period of time each enzyme was exposed to the chemical agents is presented in Table 1.

Determination of Enzyme Activity

α-Chymotrypsin. Two spectrophotometric methods were employed for the determination of α-chymotrypsin activity. The first assay, developed by Erlanger and Edel (85), utilized the substrate 2-nitro-4-carboxyphenyl-N,N-diphenylcarbamate (NCDC). NCDC reacts stoichiometrically with α-chymotrypsin to produce, in equimolar quantity, the compound, 3-nitro-4-hydroxybenzoic acid (NHB), which can be determined colorimetrically using a wavelength of 410 nm (refer to Appendix B, Figure B-1). The assay was performed by adding 1.5 ml of pH 7.6, 0.05 M Tris-chloride buffer containing 0.03 M $CaCL_2$ (described in buffer section) to a test tube and placing the tube in a waterbath (Blue M Electric Co., Blue Island, Ill.) at $37^{o}C$. α-Chymotrypsin was prepared in 0.001 N HCL at a concentration of 36 mg/ml. A 0.2 ml aliquot of the enzyme solution was added to the tube using a P 200 Rainin automatic pipet, and the mixture was allowed to equilibrate for several minutes. A blank was prepared containing 0.2 ml of 0.001 N HCL. A 0.1 ml volume of the NCDC substrate solution (described in the substrate section) was added to the tube and the reaction was allowed to proceed for 45 minutes, with intermittent swirling. After 45 minutes, 1.2 ml of 0.05 M Tris-chloride buffer at pH 7.6 was added to the tube (final volume of 3 ml). The mixture was transferred to a cuvet and the absorbance at 410 nm was determined using a Bausch and Lomb, Spectronic 100 spectrophotometer.

The second method used for the determination of α-chymotrypsin activity was adopted from Worthington Biochemical Corporation (86). In this assay, the reaction velocity was determined by measuring the

increase in absorbance at 256 nm due to the hydrolysis of the substrate, benzoyl-L-tyrosine ethyl ester (BTEE). A mixture of 1.07 mM BTEE solution (described in substrate section) and 0.08 M Tris-chloride buffer containing 0.1 M $CaCL_2$ (described in buffer section) was prepared prior to each assay in the ratio of 1.4 ml BTEE to 1.5 ml Tris-CL buffer. Of this, 2.9 mls were pipetted into a silica (3 ml) cuvet and placed in a Perkin-Elmer double beam spectrophotometer (Model 124). α-Chymotrypsin solutions were prepared by dissolving the enzyme in 0.001 N HCL to make 1 mg/ml solutions. Using a 250 µℓ Hamilton syringe (22 gauge needle), 0.1 ml of the enzyme solution was added to the cuvet through a Plexiglas injection-port assemblage. The rate of increase in absorbance at 256 nm was monitored using a strip-chart recorder (Scientific Products, McGaw Park, Ill.) at a chart speed of 1 cm/min.

Acid Phosphatase. The procedure used for the determination of acid phosphatase activity was a colorimetric method developed by Sigma Chemical Co. (87). The method utilized the substrate p-nitrophenyl phosphate, which was converted enzymatically to p-nitrophenol, a compound which formed a yellow complex upon addition of alkali (refer to Appendix B, Figure B-2). The intensity of the color formed was proportional to acid phosphatase activity. The assay was performed by adding 0.5 ml of properly diluted substrate solution (described in substrate section) and 0.5 ml of 0.09 M citrate buffer (described in buffer section) to a 12 ml test tube with a P 1000 Rainin automatic pipet. The tube was incubated at 37°C in a serological water bath (Blue M Electric Co.) for 5 to 10 minutes. After the mixture was equilibrated, 0.2 ml of a 1.25 mg/ml solution of acid phosphatase in

distilled water, was added and the reaction was allowed to proceed for 15 minutes. Five mls of 0.1 N NaOH was then added to each tube to promote a yellow color. The mixture was transferred to a cuvet and the absorbance at 400 nm was measured using a Bausch and Lomb, Spectronic 100 spectrophotometer. In an effort to increase the sensitivity of acid phosphatase toward a specific chemical agent, several trials were performed at an incubation temperature of 50°C.

Carbonic Anhydrase. Carbonic anhydrase activity was determined by means of a method which was developed by Wilbur and Anderson (88) and later simplified by Worthington (86). The method permitted the computation of carbonic anhydrase activity by measuring the rate at which the pH of a buffer solution decreased at a temperature near 0°C. In order to perform the assay, a 40.6 cm long by 15.2 cm wide by 10.2 cm deep (16 in.l x 6 in. w x 4 in. d) plexiglas water bath, insulated with 2.5 cm (1 in.) styrofoam, was constucted to hold six 15 ml beakers, five test tubes, and several small flasks. A Lauda Brinkmann circulator bath (RC 3B) was used to continually supply 0 to 4°C water to the external plexiglas water bath. A 0.02 M Tris-chloride buffer at pH 8, water saturated with CO_2, and the chemical agent solutions were kept in the water bath at 0 to 4°C. Carbonic anhydrase stock solutions were prepared by dissolving the enzyme in lyophilized form in 0 to 4°C distilled water to make a concentration of 0.1 mg/ml. This stock solution was diluted with chilled water or chilled solutions of chemical agents, and a 0.1 ml aliquot was used for each assay. Enzyme solutions were diluted sufficiently so that the pH of the reaction would decrease at a rate which could be accurately timed. The assay was performed by

adding 6.0 ml of 0.02 M Tris-chloride buffer (described in buffer section) to a 15 ml beaker and allowing the solution to equilibrate at 3.0 ± 0.1°C. The pH was noted and 0.1 ml of appropriately diluted enzyme solution was added using a P 200 Rainin microliter pipet. Immediately following addition of the enzyme, 2, 3, or 4 mls of chilled, CO_2-saturated water (decribed in substrate section) was delivered into the beaker using a 5 ml syringe (20 gauge needle). The time required for the pH to drop from 7.30 to 6.30 was recorded using a Fisher digital stopwatch (graduated in 0.01 seconds). Reagent blanks were prepared by adding CO_2-saturated water to the buffer without addition of the enzyme (refer to Appendix B, Figure B-3). The pH was monitored by the use of a Honeywell, Electronik 19 strip-chart recorder. The pH electrode employed was a five inch long Fisher microprobe, which was placed in the reaction beaker along with a thermometer that was graduated in 0.2°C increments. The electrode response at 0 to 4°C was adjusted automatically with a Fisher temperature compensator.

In an effort to increase the response of carbonic anhydrase to cadmium, the enzyme was stressed by adjusting the pH of the enzyme solution to a pH of 10 with NaOH. The enzyme was then exposed to cadmium and the pH of the solution was adjusted back to 7, then the activity of the enzyme was determined. In other experiments, the enzyme solutions were adjusted to a pH of 10 and then back to pH 7 before being exposed to cadmium.

A summary of the assay conditions for the three enzyme systems is provided in Table 1.

Table 1. Assay conditions and chemical agents investigated in three enzyme systems.

ENZYMES:	α-Chymotrypsin		Acid Phosphatase	Carbonic Anhydrase
SUBSTRATE:	NCDC[1]	BTEE[2]	NPP[3]	CO_2/H_2O[4]
Conditions:				
pH of reaction	7.6	7.7	2.3	7.80-6.30
Temperature	37°C	29°C	37°C	3.0 ± 0.1°C
Duration of assay (min)	45	20	15	1-2
Amount of enzyme used per assay	7.2 mg	12.5 µg	0.25 mg	0.1 µg
Chemical Agents:				
Compounds investigated	$CdCL_2$	$CdCL_2$	$CdCL_2$	$CdCL_2$ atrazine
	$NiCL_2$	$NiCL_2$	$NiCL_2$	$NiCL_2$ diuron
	2,4-D	2,4-D	NaF	NaF malathion
			Na_2S	Na_2S carbaryl
			$NaNO_3$	$NaNO_3$ EDTA
			2,4-D	2,4-D sulfanilamide
Period of exposure (minutes)[5]	30	35-120	30	60-120

[1] 2-nitro-4-carboxyphenyl-N,N-diphenylcarbamate

[2] benzoyl-L-tyrosine ethyl ester

[3] p-nitrophenyl phosphate

[4] water saturated with CO_2

[5] approximate time enzymes were exposed to chemical agents

RESULTS

The following section is comprised of tables and figures which provide data resulting from the experiments with α-chymotrypsin, acid phosphatase, and carbonic anhydrase enzyme systems. Information concerning the amount and form of enzyme exposed and assayed, and the amount of substrate and the concentrations of chemical agents investigated, is provided in each table. Figures were generally employed to highlight pertinent details of each experiment.

α-Chymotrypsin

α-Chymotrypsin was exposed to three chemical agents, cadmium, nickel, and 2,4-D; and, its activity was determined by two assay procedures. The first assay utilized the substrate, 2-nitro-4-carboxyphenyl-N,N-diphenylcarbamate (NCDC), and was performed at pH 7.8 and 37°C. The enzyme activity was expressed as the amount (μmole) of 3-nitro-4-hydroxybenzoic acid (NHB) formed per quantity of α-chymotrypsin. The amount of product (NHB) formed was determined by means of a standard curve (see Appendix B, Figure B-1) and used as an index for discerning the effects of chemical agents on α-chymotrypsin. Table 2 presents data resulting from the exposure of α-chymotrypsin to cadmium at 18 to 27 μM (2 to 3 mg/ℓ), nickel at 85 and 170 μM (5 and 10 mg/ℓ), 2,4-D at 45 μM (10 mg/ℓ), and a mixture of all three agents, each at 5 mg/ℓ. Each trial was conducted in triplicate and the average values and the ranges are presented in Figure 3. Each panel in Figure 3 represents a separate experiment with appropriate controls. It should

Table 2. The effects of cadmium, nickel, and 2,4-D on α-chymotrypsin formation of NHB[1] using NCDC[2] as a substrate.

		μmoles NHB		Absorbance$_{410\ nm}$	
Chemical agent	Concentration mg/ℓ	Range	Average[3]	Range	Average[3]
Control	-	0.248-0.255	0.252	0.367-0.380	0.373
Cadmium	2-3	0.255-0.266	0.260	0.377-0.394	0.385
Control	-	0.234-0.235	0.234	0.346-0.348	0.347
Nickel	5	0.217-0.232	0.225	0.321-0.343	0.332
Nickel	10	0.212-0.219	0.216	0.314-0.323	0.318
Control	-	0.225-0.229	0.227	0.333-0.339	0.336
2,4-D	10	0.222-0.226	0.224	0.328-0.335	0.332
Control	-	0.256-0.267	0.261	0.378-0.395	0.386
Mixture[4]	5	0.258-0.258	0.258	0.382-0.382	0.382

Amount of α-chymotrypsin exposed = 0.30 μmole (7.2 mg)

Amount of α-chymotrypsin used per assay = 0.30 μmole (7.2 mg)

Amount of substrate (NCDC)[2] used per assay = 3.5 μmole (1.32 mg)

[1] 3-nitro-4-hydroxybenzoic acid (product)

[2] 2-nitro-4-carboxyphenyl-N,N-diphenylcarbamate (substrate)

[3] average of samples run in triplicate

[4] a combination of cadmium, nickel, and 2,4-D each at 5 mg/ℓ

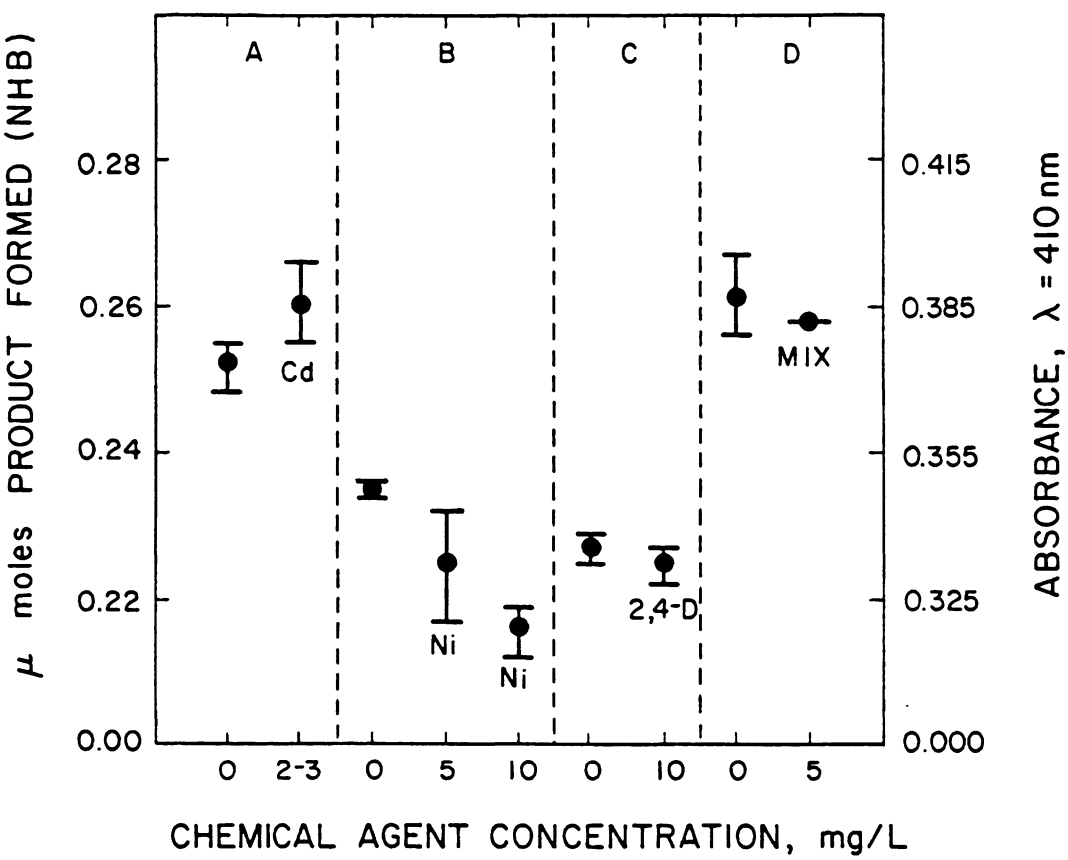


Figure 3. The response of α-chymotrypsin to (A) cadmium, (B) nickel, (C) 2,4-D, and (D) a mixture of all three agents, each at 5 mg/ℓ, when NCDC was used as the substrate.

be noted that the ranges of activity for α-chymotrypsin exposed to cadmium, 2,4-D, and the mixture of all three agents, overlap into the ranges of the controls. However, the activities of the enzyme exposed to 5 and 10 mg/ℓ of nickel appear to decrease (see Figure 3).

In another series of experiments, α-chymotrypsin was exposed to higher concentrations of cadmium, nickel, and 2,4-D, and assayed at a pH of 7.7 and at 29°C using benzoyl-L-tyrosine ethyl ester (BTEE) as the substrate. The enzyme activity was measured as the change in absorbance (λ= 256 nm) per minute using four substrate (BTEE) concentrations (see Figure 4). The activity was computed from a strip-chart recording of the hydrolysis of BTEE by α-chymotrypsin over a five minute period. α-Chymotrypsin was exposed to cadmium at 20 mM (2.2 g/ℓ), nickel at 0.75 mM (44 mg/ℓ), and 2,4-D at 0.20 mM (44 mg/ℓ). The results of these experiments are presented in Table 3. Note that the activity values obtained for the exposed enzyme are very nearly the same as those obtained for the controls, when the enzyme was assayed at lower BTEE concentrations. Figure 5 graphically presents the activity of α-chymotrypsin following exposure to cadmium, nickel, and 2,4-D using 0.50 mM of substrate, BTEE. It is interesting to note that the initial activity of the enzyme,when assayed using 0.50 mM BTEE, was approximately doubled resulting from exposure to 2,4-D (see Figure 5). However, there was no apparent change in the activity of α-chymotrypsin due to the chemical agents when the enzyme was assayed at lower substrate (BTEE) levels (see Table 3).

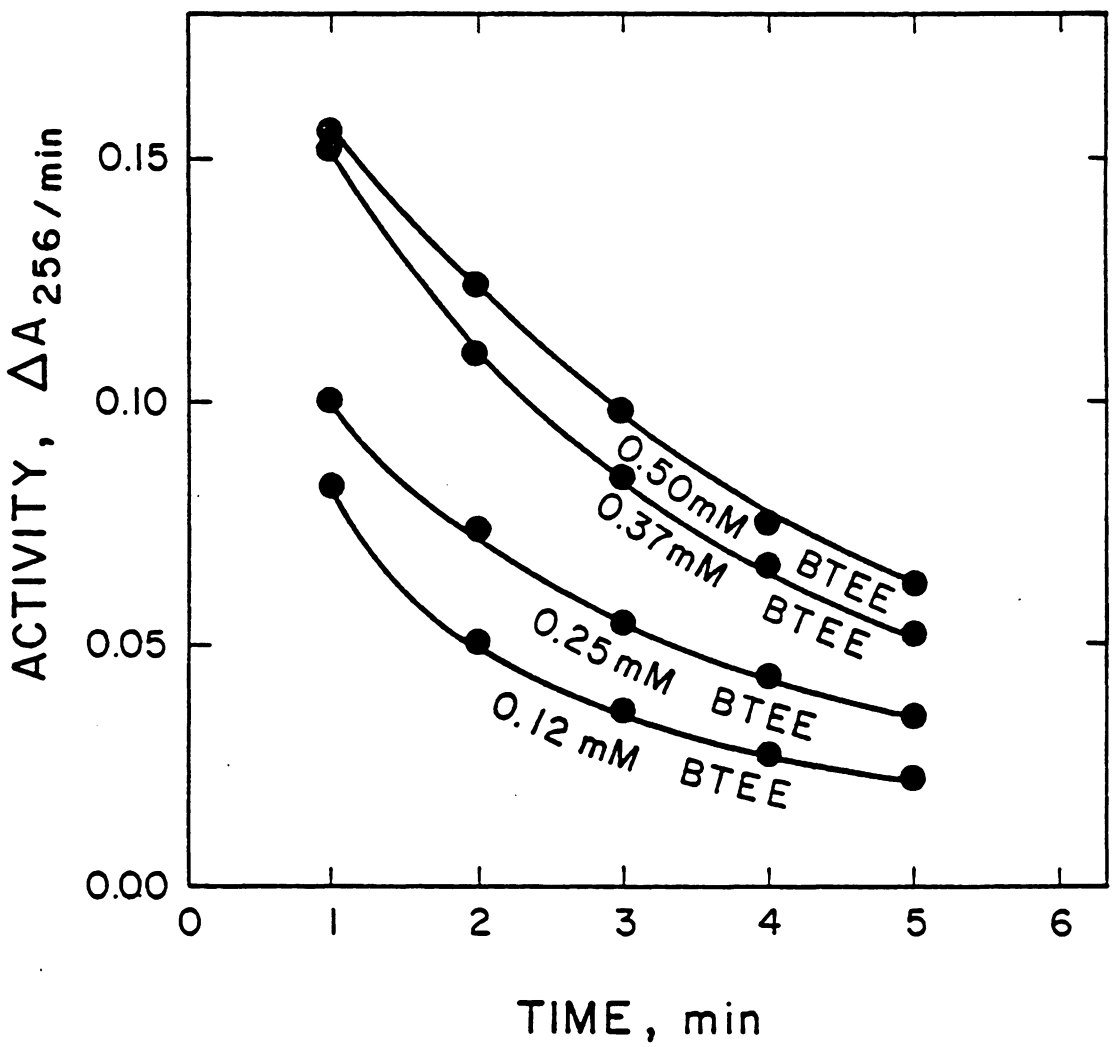


Figure 4. The effect of substrate (BTEE) concentrations on the activity of α-chymotrypsin when assayed at 29°C.

Table 3. The effect of cadmium, nickel, and 2,4-D on α-chymotrypsin activity using BTEE[1] as a substrate.

BTEE Concentration	Time (min)	CHANGE IN ABSORBANCE (256 nm)/MIN			
		Control	Cadmium[2]	Nickel[2]	2,4-D[2]
0.125 mM	1	0.083	0.060	0.050	0.060
	2	0.050	0.040	0.040	0.040
	3	0.037	0.030	0.030	0.024
	4	0.028	0.024	0.022	0.024
	5	0.022	0.019	0.018	0.020
0.250 mM	1	0.100	0.110	0.100	0.100
	2	0.075	0.080	0.075	0.075
	3	0.056	0.058	0.053	0.057
	4	0.043	0.045	0.041	0.045
	5	0.034	0.036	0.033	0.036
0.374 mM	1	0.151	0.140	0.150	0.149
	2	0.110	0.115	0.110	0.110
	3	0.082	0.087	0.082	0.085
	4	0.064	0.068	0.062	0.064
	5	0.052	0.054	0.050	0.051
0.500 mM	1	0.156	0.170	0.170	0.300
	2	0.125	0.150	0.132	0.210
	3	0.097	0.118	0.103	0.155
	4	0.075	0.092	0.081	0.118
	5	0.062	0.076	0.066	0.096

Amount of α-chymotrypsin exposed = 20 nmole (0.5 mg)

Amount of α-chymotrypsin used per assay = 0.5 nmole (12.5 μg)

[1] benzoyl-L-tyrosine ethyl ester (substrate)

[2] concentrations of chemical agents:

Cadmium	20	mM (2250 mg/L)
Nickel	0.75	mM (44 mg/L)
2,4-D	0.20	mM (44 mg/L)

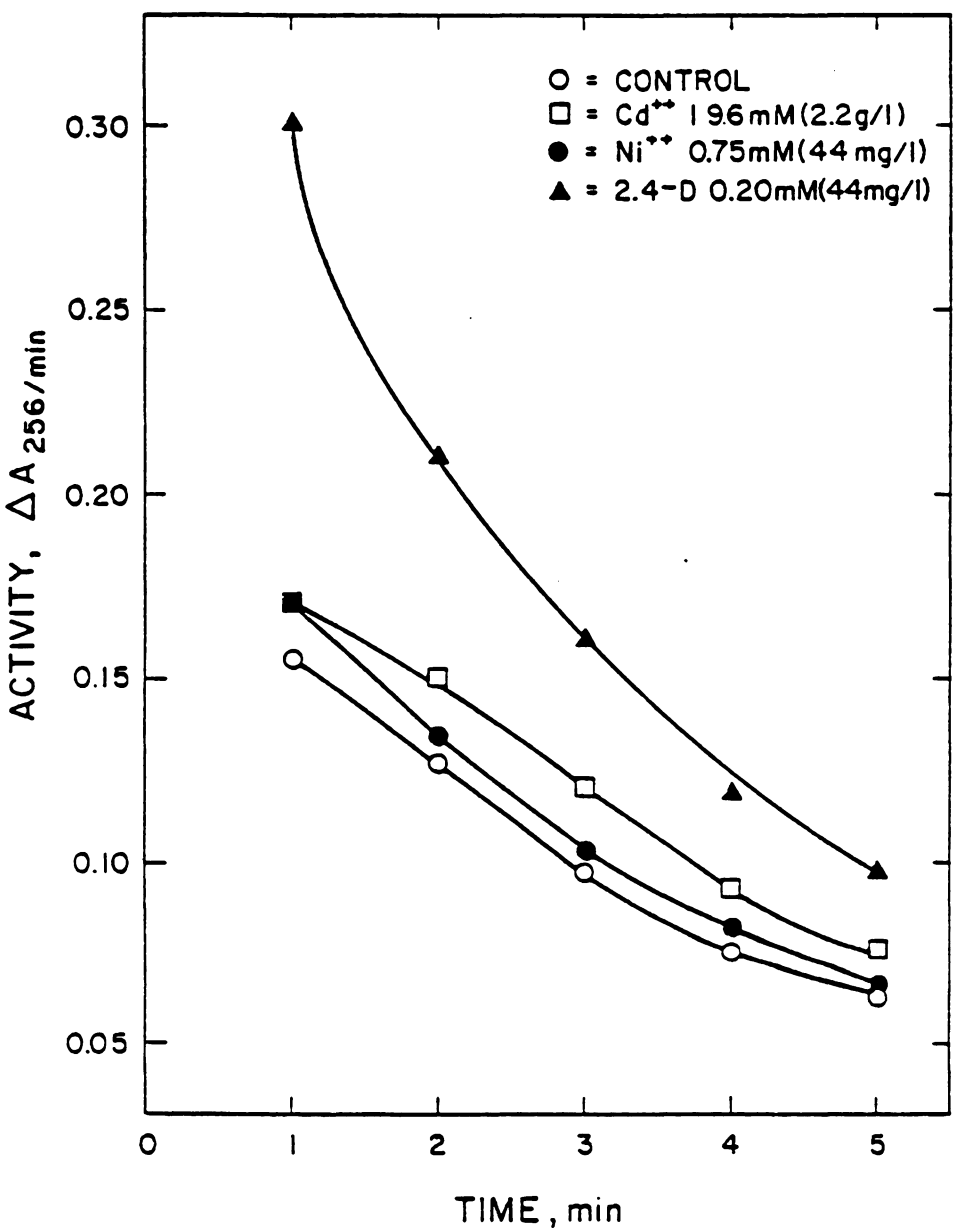


Figure 5. The effect of cadmium, nickel, and 2,4-D on the activity of α-chymotrypsin when assayed at 29°C and using a substrate (BTEE) concentration of 0.50 mM.

Acid Phosphatase

The activity of acid phosphatase was assayed utilizing p-nitrophenyl phosphate (NPP) as a substrate at pH 2.3 and 37°C. Enzyme activity was determined as the amount (nanomoles or micromoles) of product, p-nitrophenol, formed per quantity of acid phosphatase during a 15 minute incubation period. The amount of p-nitrophenol formed by the enzyme was determined from a standard curve, which is presented in Appendix B, Figure B-2. Preliminary experiments were performed to obtain suitable assay concentrations of acid phosphatase and substrate (NPP). Figure 6 illustrates how three different levels of acid phosphatase responded to various substrate levels. The results of similar studies in which both the enzyme and substrate levels were increased are presented in Figure 7. The raw data from which Figure 6 and 7 were constructed are presented in Appendix A, Table A-2. It was observed that the efficiency of product formation (activity) increased with increasing amounts of enzyme at any given substrate level (refer to Figure 6). The amount of acid phosphatase selected for use in this study was 4.5 nmole because this appeared to be the smallest amount of enzyme that exhibited a rather high level of activity (see Figure 6). The levels of substrate (NPP) employed in the assay were typically in the range of 50 to 500 nmoles. It should be noted that with each formation of product vs. substrate level curve (Figure 6 and 7), there is a characteristic initial linear increase in product formation which reaches a peak, and then declines until it reaches a point where it levels off at higher substrate levels.

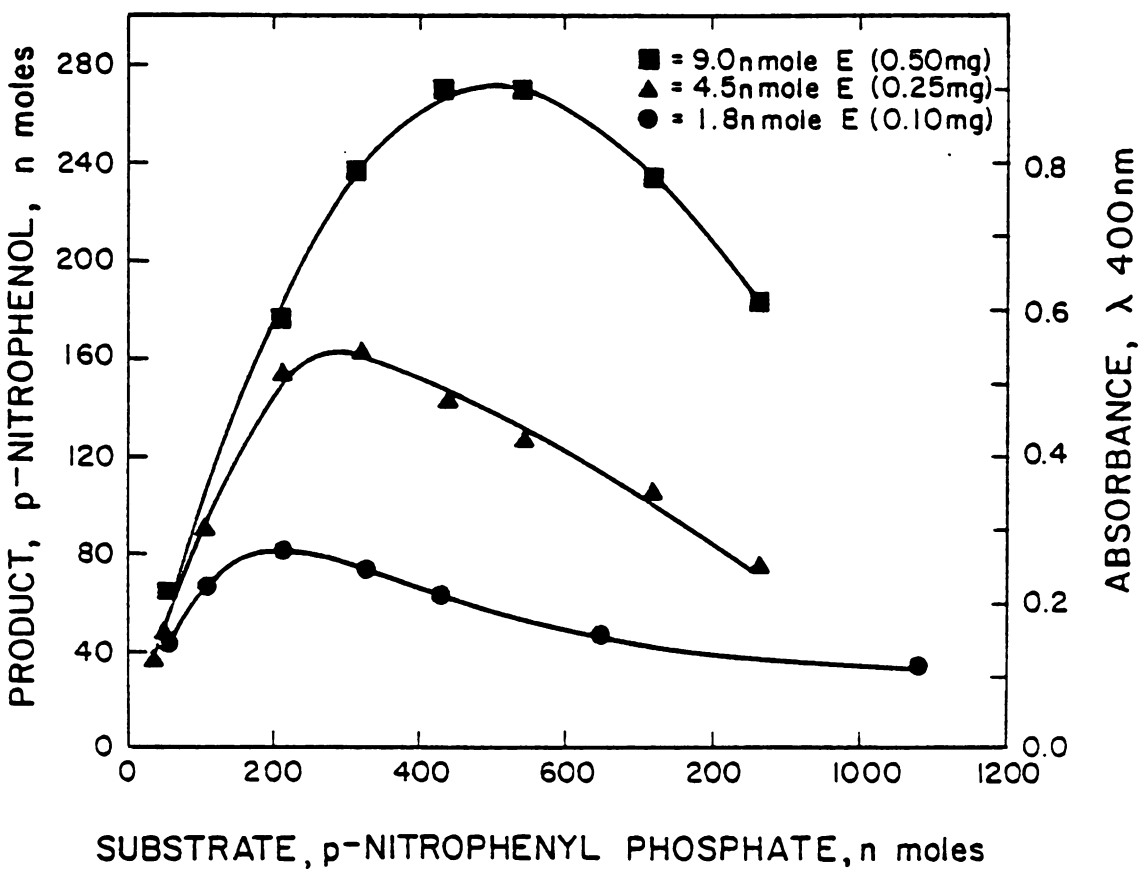


Figure 6. The formation of p-nitrophenol as a function of substrate and acid phosphatase (E) levels.

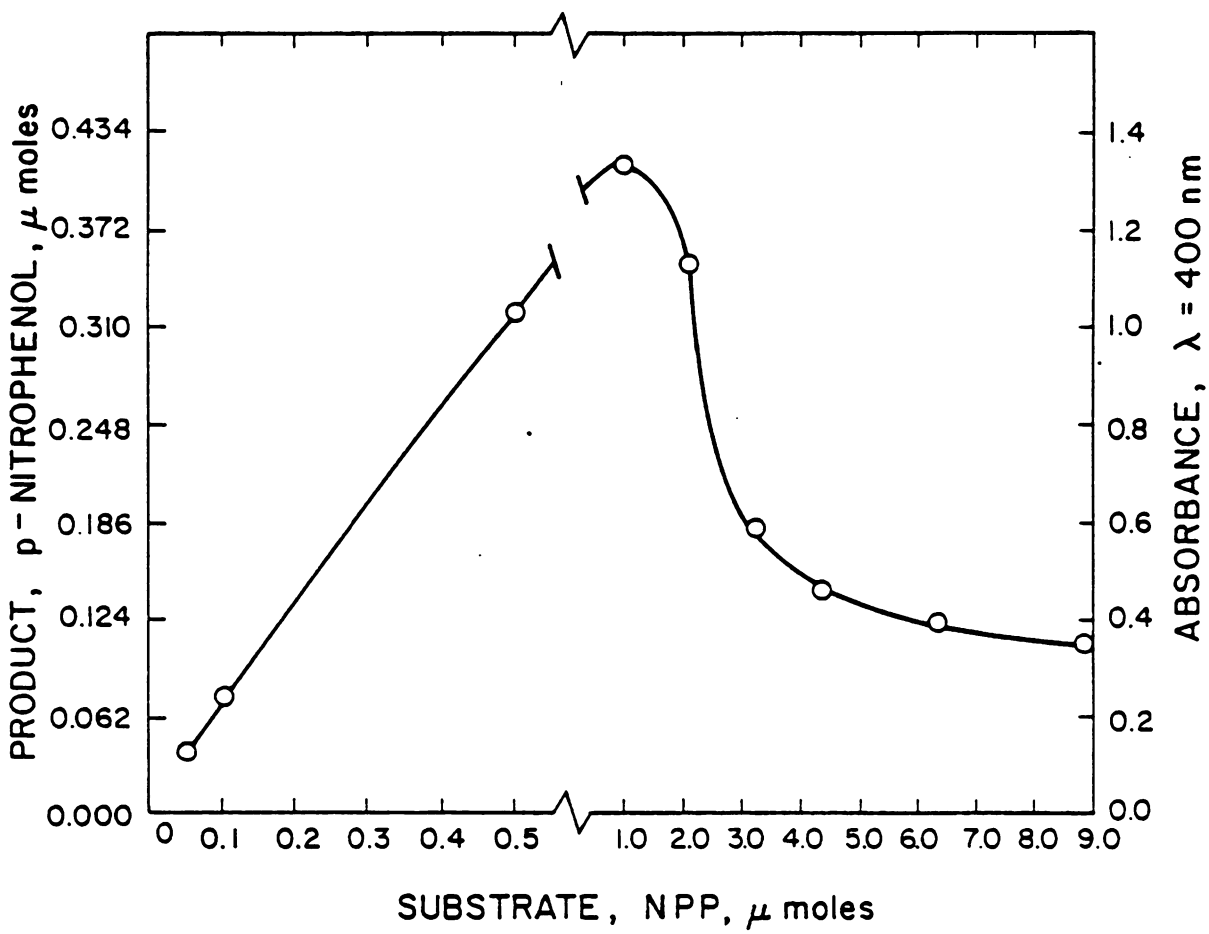


Figure 7. The conversion of p-nitrophenyl phosphate (NPP) into p-nitrophenol by 18 nmoles (1 mg) of acid phosphatase.

Acid phosphatase was exposed to six chemical agents: cadmium, nickel, fluoride, sulfide, nitrate, and 2,4-D. Table 4 provides acid phosphatase activities following exposure to cadmium at 0.09 mM and 11 mM (10 and 1250 mg/ℓ), nickel at 0.85 mM (50 mg/ℓ), and 2,4-D at 0.45 mM (100 mg/ℓ). The activity of acid phosphatase following exposure to cadmium at 1250 mg/ℓ is graphically depicted in Figure 8. Note that the decrease in activity or inhibition due to cadmium becomes more apparent as the substrate level is increased. The activity of acid phosphatase when assayed at 37°C following the exposure to fluoride at 0.12 and 0.24 mM (2.2 and 4.5 mg/ℓ), sulfide at 0.015 mM (0.5 mg/ℓ), and nitrate at 0.03 to 0.12 mM (1.8 to 7.3 mg/ℓ) is presented in Table 5. In addition, Table 5 also provides data concerning the effects of fluoride on acid phosphatase when the enzyme was assayed at 50°C. The effects of 2.2 and 4.5 mg/ℓ of fluoride on acid phosphatase activity when the enzyme was assayed at 37°C and 50°C are illustrated in Figure 9. Noting that there was an inhibitory effect using fluoride, experiments were performed in which acid phosphatase was exposed to a range of fluoride concentrations and assayed at 37°C using 323 nmole of substrate (NPP). The results of this work are illustrated in Figure 10 and the raw data is presented in Appendix A, Table A-3. The response of acid phosphatase to sulfide at 0.5 mg/ℓ and nitrate at 10 mg/ℓ are presented in Figure 11. Note that only nitrate at 10 mg/ℓ appeared to produce inhibition in activity, whereas sulfide and lower concentrations of nitrate did not have an affect on activity (see Table 5 and Figure 11). It should also be noted that the degree of enzyme inhibition induced by fluoride and nitrate, occurred at a substrate level of 323 nmole (see Figure 9 and 11).

Table 4. The effect of cadmium, nickel, and 2,4-D on acid phosphatase formation of p-nitrophenol.

Chemical agent	Conc. mg/ℓ	Substrate, NPP[1] (nmole) 54	65	86	108	130	142	172	215
		Product, p-nitrophenol (nmole)							
Control[2]	-	44	52	68	85		112	130	153
Cadmium	10	44	52	68	87		112	132	156
Control[3]	-		53	71	88	105	116	134	155
Cadmium	1250		49	66	82	98	108	123	140
Control[4]	-		42		61		74		135
Nickel	50		42		61		74		133
2,4-D	100		42		61		71		137

[1] p-nitrophenyl phosphate

[2] 4.5 nmole enzyme assayed; 180 nmole enzyme exposed to 0.09 mM (10 mg/ℓ) cadmium; samples run singly.

[3] 4.5 nmole enzyme assayed; 45 nmole enzyme exposed to 11 mM (1250 mg/ℓ) cadmium; samples run in duplicate.

[4] 4.5 nmole enzyme assayed; 45 nmole enzyme exposed to 0.85 mM (50 mg/ℓ) nickel and 0.45 mM (100 mg/ℓ) 2,4-D; samples run singly.

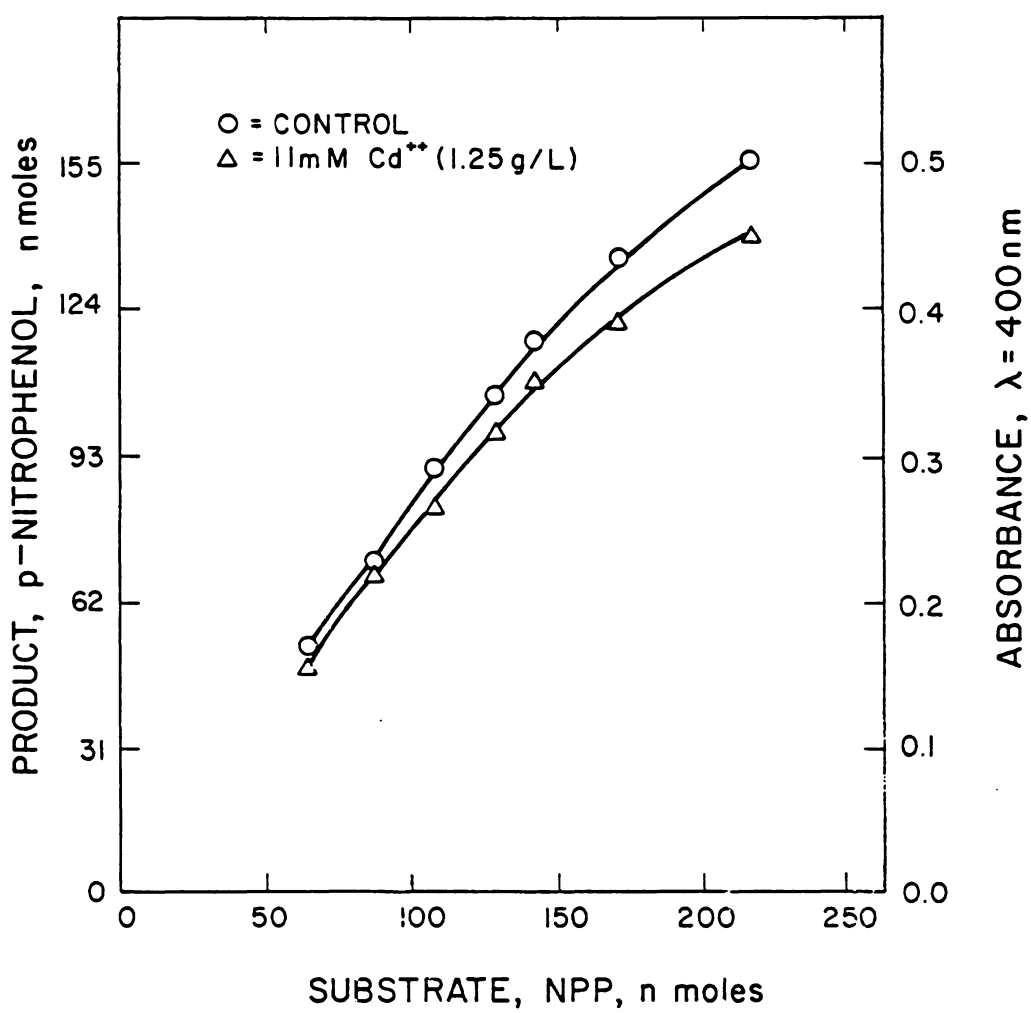


Figure 8. Response of acid phosphatase to cadmium.

Table 5. The effects of fluoride, sulfide, nitrate, and assay temperature on acid phosphatase activity.

Chemical agent	Conc. mg/ℓ	Assay temp.	Substrate, NPP[1] (nmole) 108	215	323	431	539
			Product, p-nitrophenol (nmole)[2]				
Control	-		78	135	147	145	139
Fluoride	4.5	37°C	78	122	130	121	98
Fluoride	2.2		76	116	119	109	92
Control	-			49	41	36	35
Fluoride	4.5	50°C		43	38	33	29
Fluoride	2.2			39	36	31	29
Control	-	37°C	87	141	144	141	
Sulfide	0.5		87	139	146	132	
Control	-			137	139		
Nitrate	7.3			127	125		
Nitrate	5.5	37°C		133	133		
Nitrate	3.6			135	132		
Nitrate	1.8			136	130		

Amount of acid phosphatase assayed = 4.5 nmole (0.25 mg)

Amount of acid phosphatase exposed = 45 nmole (2.5 mg)

[1] p-nitrophenyl phosphate (substrate)

[2] average values obtained from samples run in duplicate

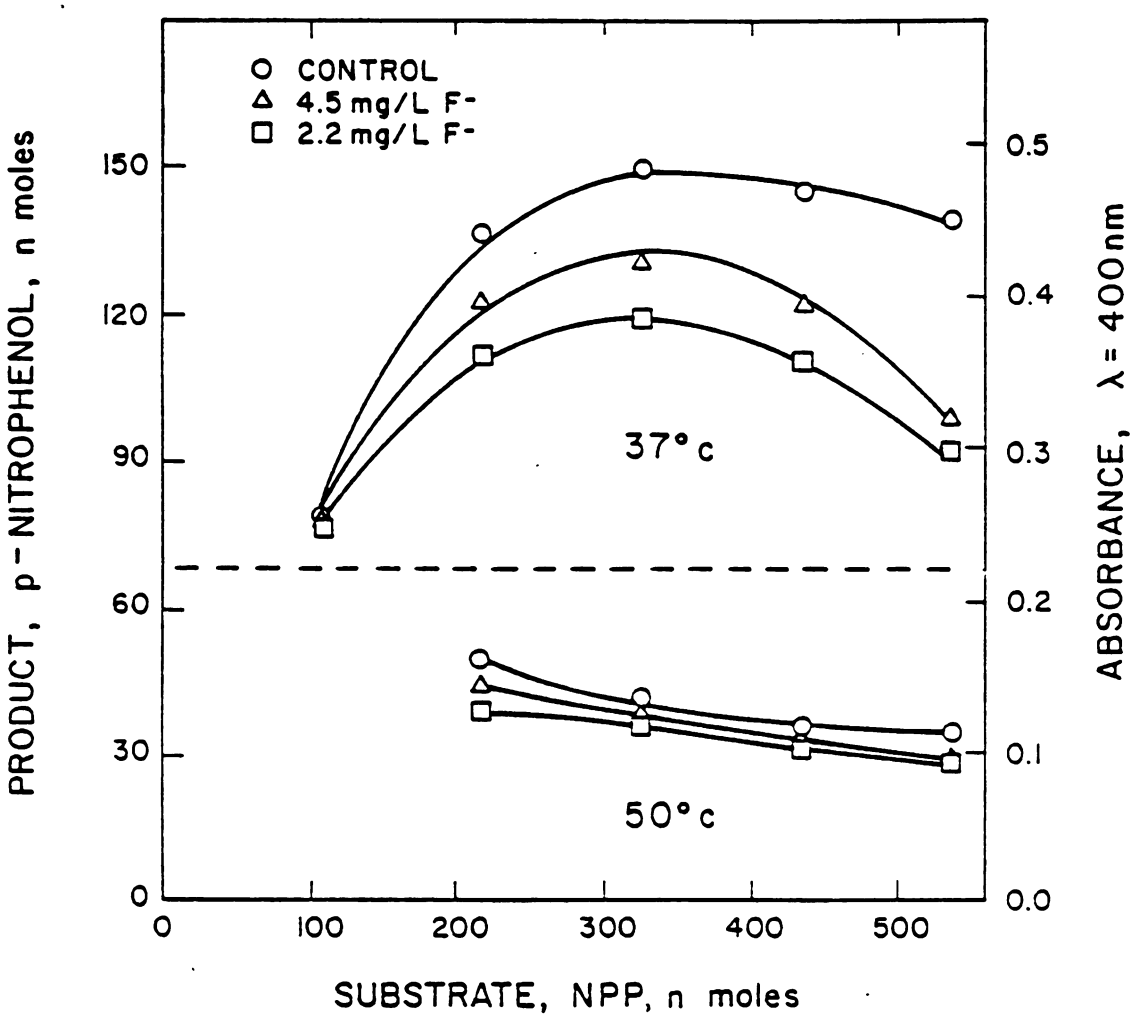


Figure 9. The effects of temperature and fluoride on acid phosphatase activity.

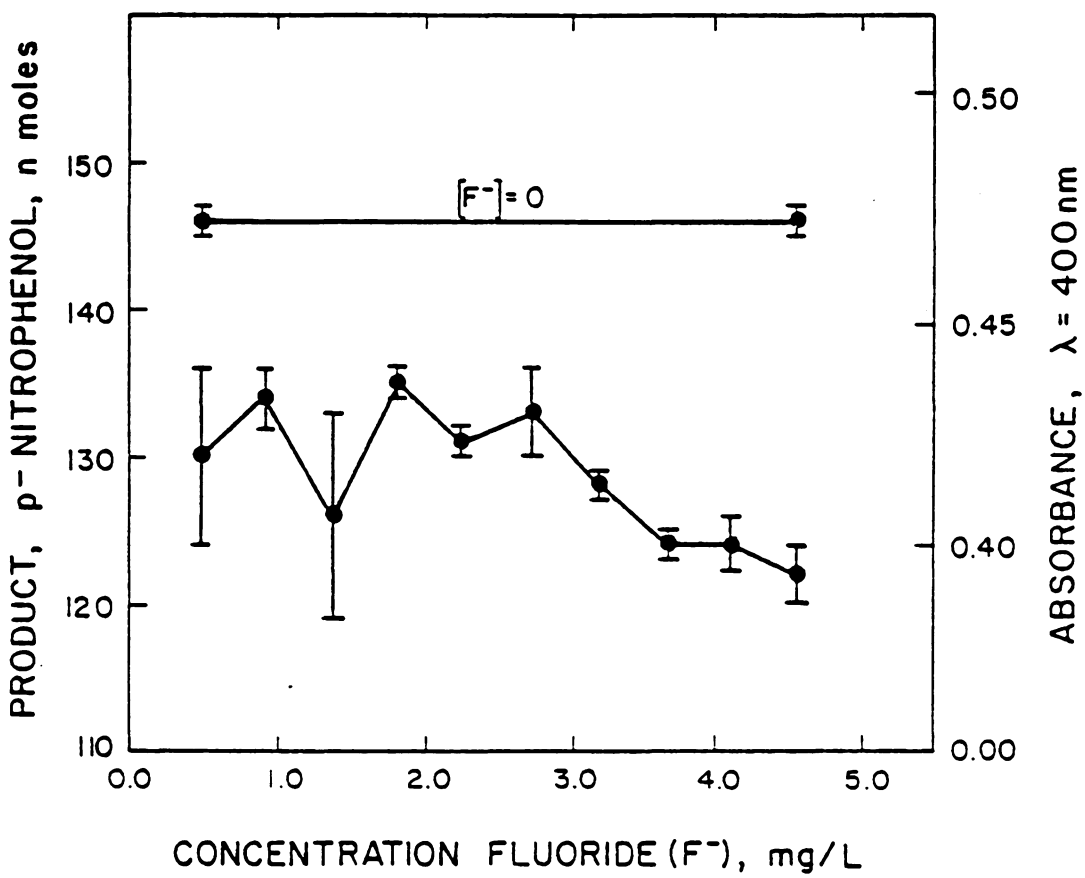


Figure 10. The response of acid phosphatase at 37°C to various levels of fluoride.

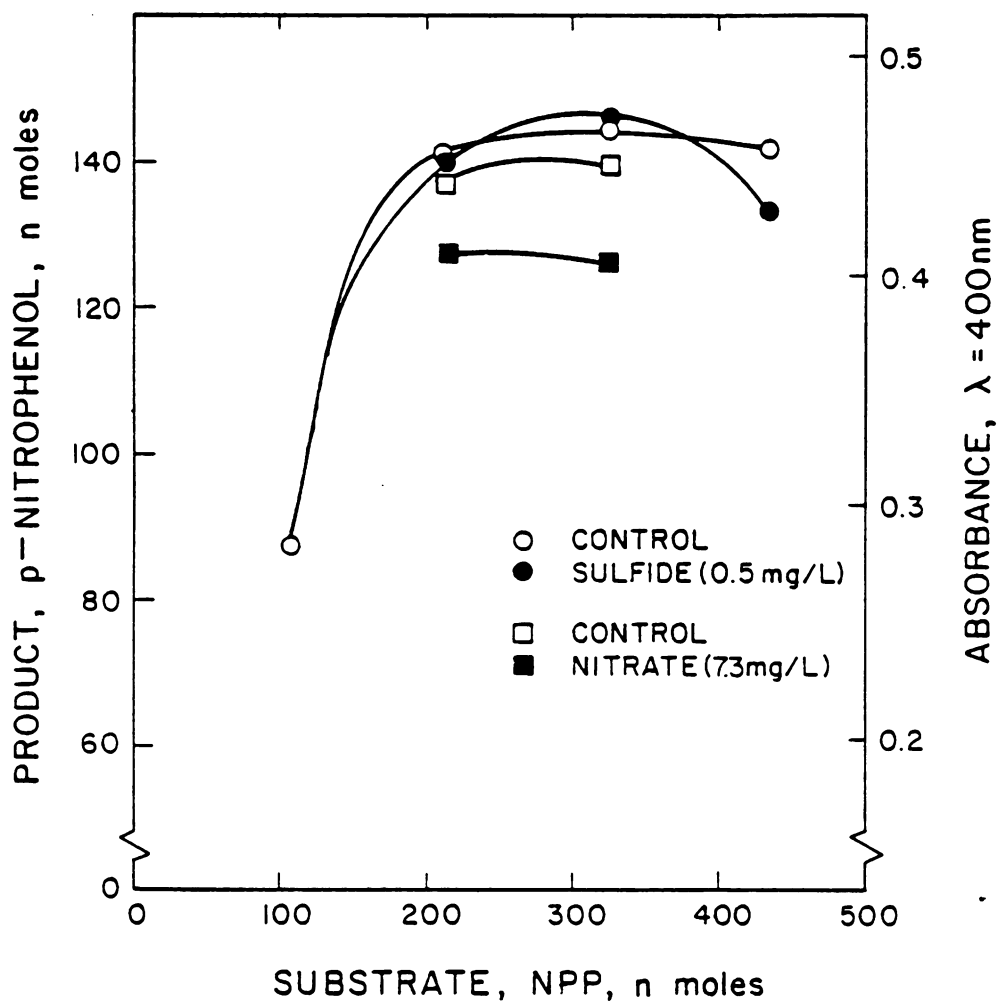


Figure 11. The response of acid phosphatase to sulfide and nitrate.

Carbonic Anhydrase

The activity of carbonic anhydrase was determined at an assay temperature of 0 to 4°C using water saturated with CO_2 as a substrate (CO_2/H_2O). The CO_2 hydrating activity of carbonic anhydrase was then determined by measuring the time in seconds for the pH of a reaction mixture (containing the enzyme) to decrease from 7.80 to 6.30. A blank was determined by recording the rate of the uncatalyzed reaction (without the enzyme). Average time values for blank samples ranged from 130 to 170 seconds using 2 mls of CO_2/H_2O and 62 to 80 seconds when 3 mls of CO_2/H_2O was employed. The relationship between the response of a typical blank sample and 0.1 μg of bovine carbonic anhydrase (mixture), over a range of CO_2/H_2O levels, is presented in Appendix B, Figure B-3. Enzyme activity was expressed as units per milligram of carbonic anhydrase assayed. The equation, as adopted from Worthington (86), is as follows:

$$\text{units/mg} = 2 \times \frac{T_b - T_E}{T_E \ (E_{mg})} \qquad [13]$$

where T_b is the time in seconds for the uncatalyzed reaction (blank), T_E is the time in seconds for the catalyzed reaction (enzyme), and E_{mg} is the amount of carbonic anhydrase assayed in milligrams.

Five forms of carbonic anhydrases from bovine and human erythrocytes were employed in the carbonic anhydrase enzyme system. The activities of each form of enzyme are presented in Table 6. The activities of bovine (mixture), bovine type A, bovine type B, human type A, and human type B were typically in the range of 8750 to 9360, 4210 to 4730, 9230 to 9230, 160 to 180, and 250 to 270 units per mg enzyme,

Table 6. Activities of various types of carbonic anhydrases at different CO_2 levels.

Form of carbonic anhydrase[2]	Volume of H_2O saturated with CO_2		
	2 mls	3 mls	4 mls
	ENZYME ACTIVITY[1]		
Bovine (mixture)			
Range	16300-11000	8750-9360	4610-6670
Average	13700	9060	5600
Bovine, type A			
Range		4210-4730	2380-3330
Average		4470	3000
Bovine, type B			
Range	8840-9400	9230-9230	
Average	9120	9230	4600
Human, type A			
Range		160-180	
Average	260	170	140
Human, type B			
Range		250-270	150-200
Average	260	260	180

[1] units/mg carbonic anhydrase

[2] from erythrocytes

respectively, when assayed using 3 mls of substrate (CO_2/H_2O). The most commonly employed form of carbonic anhydrase was a mixture from bovine erythrocytes, termed bovine (mixture). This form exhibited the highest activity (see Figure 12) and was used throughout most of the carbonic anhydrase experiments.

Twelve different chemical agents were investigated concerning their impact on carbonic anhydrases (see Table 1). Tables 7 through 10 provide activities of carbonic anhydrases exposed to the various agents. Each table contains information concerning the amount and form of carbonic anhydrase, the amount of substrate (CO_2/H_2O), and the concentrations of the chemical agents. Raw data obtained from these experiments are presented in Appendix A, Table A-4. Table 7 provides data obtained from the experiments resulting from the exposure of carbonic anhydrases to cadmium and nickel ions at a pH of 7 and a pH of 10. In the experiments performed at pH 10, the pH of the enzyme-toxicant mixture was adjusted to 7 before the activity of the enzyme was determined. In these experiments, the enzyme solution was raised to a pH of 10 using NaOH, exposed to 10 mg/ℓ of cadmium for about 10 minutes, and then adjusted back to pH 7. In another series of experiments, the enzyme solution was exposed to cadmium (10 mg/ℓ) after the pH of the enzyme solution was elevated to 10 and adjusted back to 7. The results of these experiments are graphically presented in Figure 13. Note that the activity of carbonic anhydrase was enhanced by cadmium and the elevated pH solution; however, major differences in activity appeared to be due to the elevated pH solution alone.

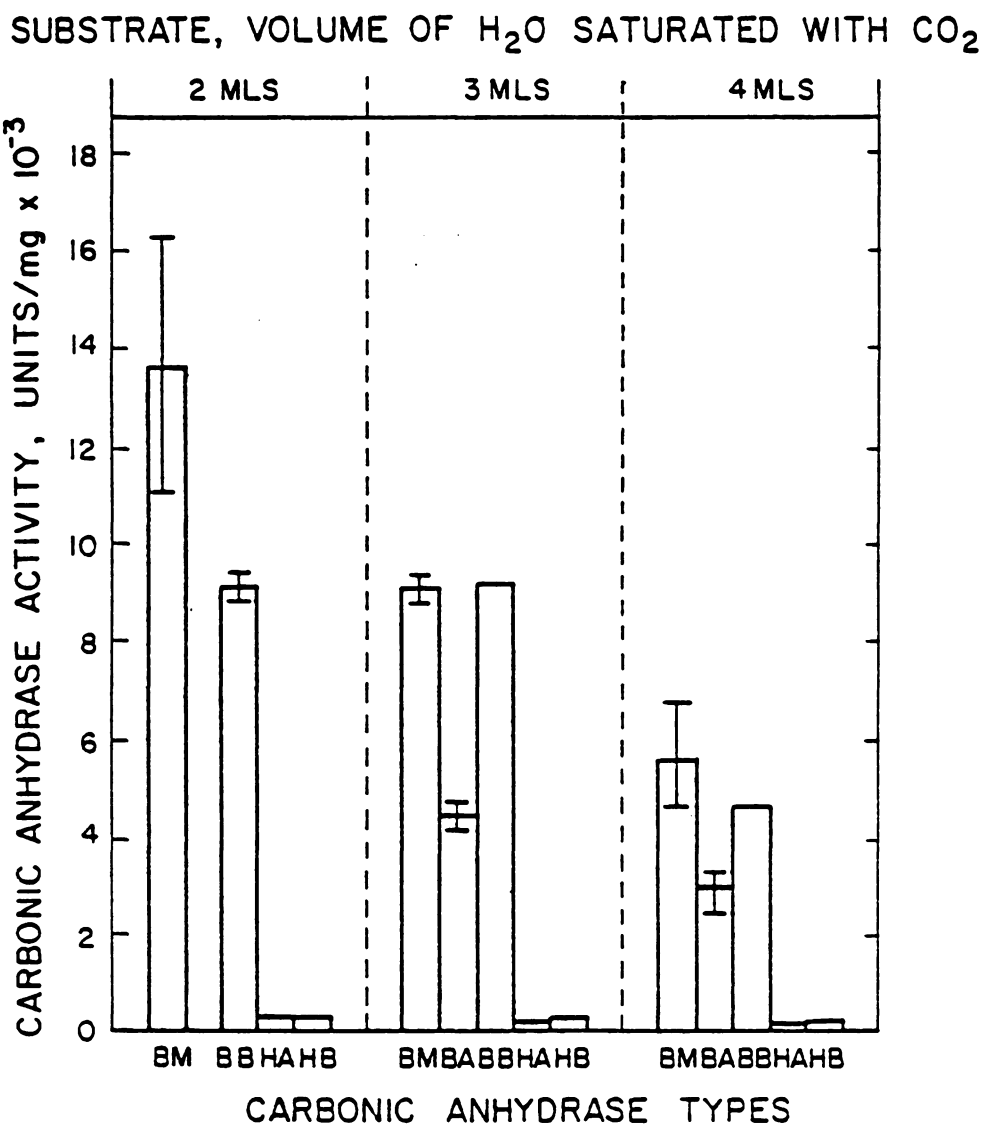


Figure 12. The activity of several carbonic anhydrases from bovine and human erythrocytes at different CO_2 levels: BM, bovine mixture; BA, bovine type A; BB, bovine type B; HA, human type A; HB, human type B.

Table 7. The effects of cadmium, nickel, and elevated pH on various types of carbonic anhydrases.

		Volume of H_2O saturated with CO_2					
		2 mls			3 mls		
Chemical agent	Conc. mg/ℓ	Enzyme Activity[1] Range	Avg.	No.	Enzyme Activity[1] Range	Avg.	No.
Control[2]	-	11000-16300	13660	2	8750-9360	9050	2
Cadmium	2250	11000-13660	12300	2	6540-9360	7950	2
Nickel	45	11360-17300	14300	2	8750-10670	9710	2
Control[3]	-	4470-8100	6300	2	6970-8250	7610	2
Cadmium	100		6000	1	7380-10170	8780	2
Nickel	1000		6000	1		4700	1
Control[4]	-				160-180	170	2
Cadmium	90				150-180	160	1
Nickel	90					180	1
Control[5]	-	8840-9400	9120	2		9230	2
Cadmium	1000		4290	1		9740	1
Nickel	1000		5850	1		5620	1
Control[2]	-		13900	2		3700	1
pH 10*		15860-20500	18050	5	3730-6900	5450	4
pH 10/cadmium$^{\phi}$		17600-21000	19000	4	5900-8570	7240	2
pH 10+cadmium$^{\Delta}$		14300-25900	20100	2		4140	1

[1] units/mg carbonic anhydrase

[2] bovine carbonic anhydrase (mixture); 0.3 nmole enzyme exposed.

[3] bovine carbonic anhydrase, type A; 3 nmole enzyme exposed.

[4] human carbonic anhydrase, type A; 17 nmole enzyme exposed.

[5] bovine carbonic anhydrase, type B; 3 nmole enzyme exposed.

* pH of the enzyme solution was raised to 10 using NaOH.

ϕ pH of the enzyme solution was raised to 10 using NaOH and then lowered to 7 before exposure to 10 mg/ℓ cadmium.

Δ enzyme solution was exposed to 10 mg/ℓ cadmium while at pH 10.

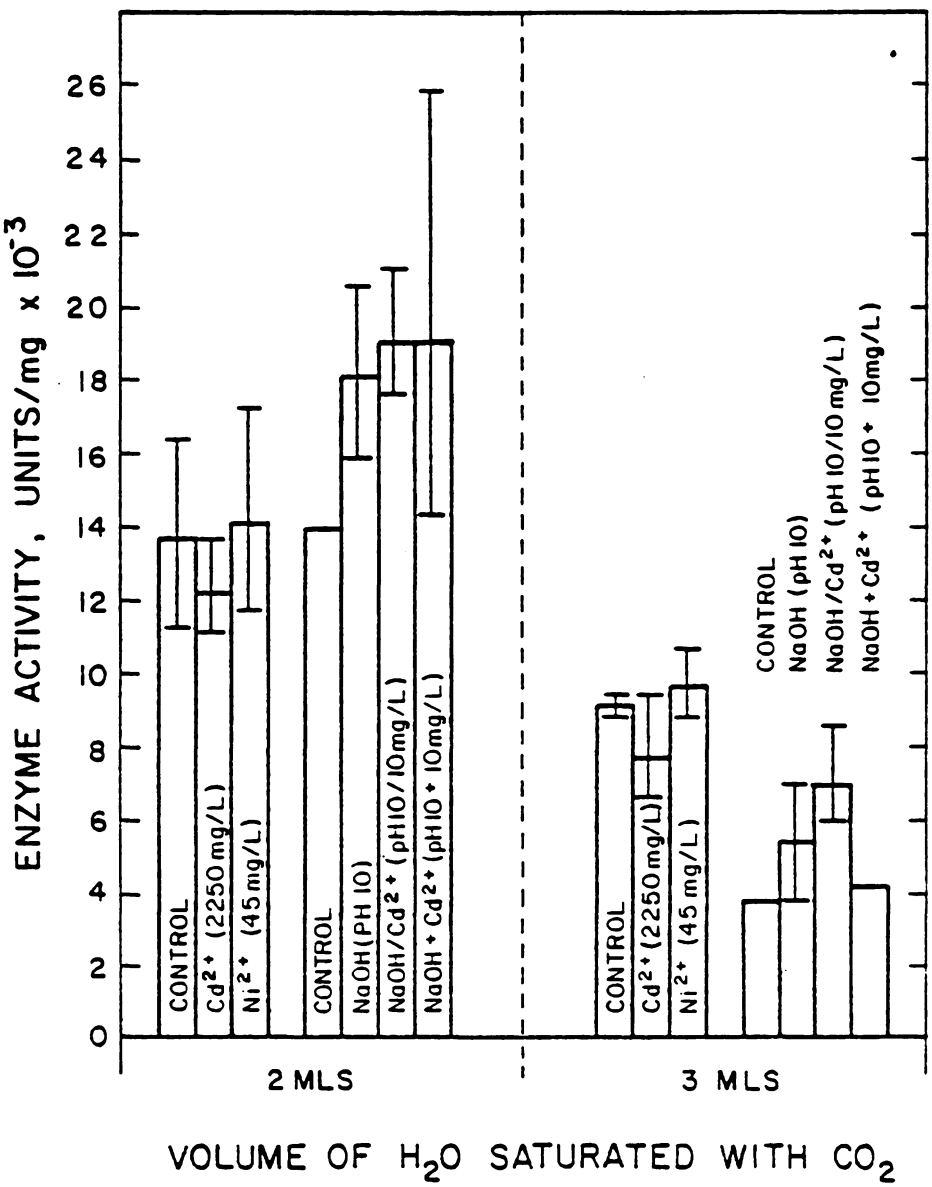


Figure 13. The effects of cadmium, nickel, and elevated pH on bovine carbonic anhydrase (mixture) activity.

Carbonic anhydrase activities resulting from the exposure to anions (fluoride, sulfide, and nitrate), herbicides (atrazine and diuron), pesticides (malathion and carbaryl), and the chelating agent, EDTA, are presented in Table 8. Included in Table 8 is a column indicating the percent inhibition (-) or enhancement (+) in enzyme activity resulting from these chemical agents. Note that carbonic anhydrase activity appeared to be inhibited by anions such as fluoride, sulfide, and nitrate, and by the pesticides, malathion and carbaryl, while the chelating agent, EDTA, appeared to enhance activity. Graphical representations of the effects of these chemical agents on carbonic anhydrase activities are provided in Figures 14 and 15. Table 9 presents data concerning the response of bovine carbonic anhydrase (mixture) to various concentrations of the herbicide, 2,4-D. It is interesting to observe that enzyme inhibition occurred at 2,4-D levels of 20 to 25 mg/ℓ and 150 to 500 mg/ℓ, but not at 50 to 100 mg/ℓ of 2,4-D. This anomaly will be discussed in the following chapter. The impact of the various levels of 2,4-D on carbonic anhydrase when the enzyme was assayed using 2 mls of CO_2/H_2O, is graphically depicted in Figure 16. Each of the three panels contained in Figure 16 represent a separate experiment with appropriate controls. The activities obtained from the exposure of carbonic anhydrase to sulfanilamide (ABSA) alone and in combination with metal cations (Cd^{2+}, Ni^{2+}, and Zn^{2+}) are provided in Table 10. The table contains a percent decrease column which denotes the inhibition of carbonic anhydrase activity caused by sulfanilamide by itself and combined with the metals. It was observed that the combination of the metal ions with ABSA produced a slightly

Table 8. The effect of various anions, herbicides, pesticides, and a chelating agent on carbonic anhydrase activity.

Chemical agent	Concentration mg/ℓ	Volume of H_2O saturated with CO_2 (2 mls) Enzyme Activity[1] Range	Average	Trials	Percent alteration[2]
Control[3]	-	8970-10540	9740	3	
Fluoride	4.5	2380-8970	6280	4	-35
Control[3]	-	10600-18500	13550	4	
Sulfide (HS^-)	0.05	15450-21600	18050	3	
Sulfide	0.25	11520-12500	11840	2	
Sulfide	0.50	6900-9700	8360	2	-38
Control[4]	-		5580	1	
Nitrate	73		3950	1	-29
Control[3]	-	15300-17670	16450	3	
Atrazine	10	9350-17050	12290	3	-25
Diuron	10	12750-15870	14240	2	
Diuron	100	14770-18960	12860	2	
Control[3]	-	14070-22460	18870	5	
Malathion	10	11360-16800	13660	2	-27
Carbaryl	10	10670-16300	12860	4	-32
Control[3]	-	11000-16300	13660	2	
EDTA	90	16300-17300	16800	2	+19
		Volume of H_2O saturated with CO_2 (3 mls)			
Control[3]	-	6380-7560	6960	2	
Fluoride	4.5	5830-6960	6380	2	
Control[3]	-	2950-4140	3730	4	
Sulfide	0.05		4560	1	
Sulfide	0.25	1540-5450	3330	2	-10
Sulfide	0.50	590-2580	1540	3	-58
Control[4]	-		4070	1	
Nitrate	73		3100	1	-24
Control[3]	-	6380-7560	6960	2	
Atrazine	10	6380-7560	6960	2	
Diuron	100		8840	1	
Control[3]	-	7450-13330	10440	4	
Malathion	10	9170-11820	10440	2	
Carbaryl	10	5000-5930	5460	2	-48
Control[3]	-	8750-9360	9050	2	
EDTA	90	10670-12860	11360	2	+20

[1] units/mg carbonic anhydrase
[2] (-) indicates inhibition, (+) indicates stimulation
[3] bovine carbonic anhydrase (mixture); 0.3 nmole exposed
[4] bovine carbonic anhydrase, type A; 0.7 µmole exposed

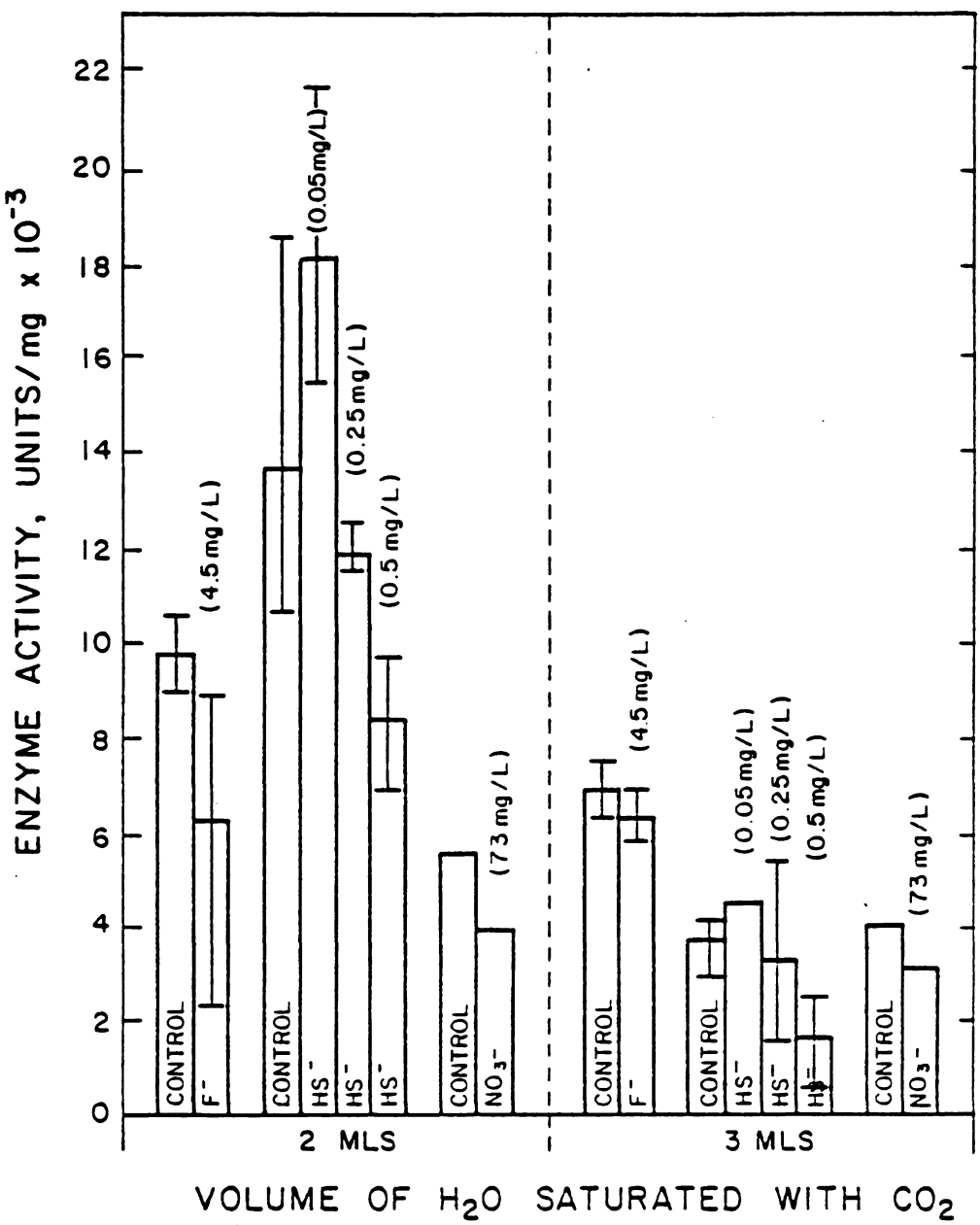


Figure 14. The activity of carbonic anhydrases following exposure to fluoride, sulfide, and nitrate.

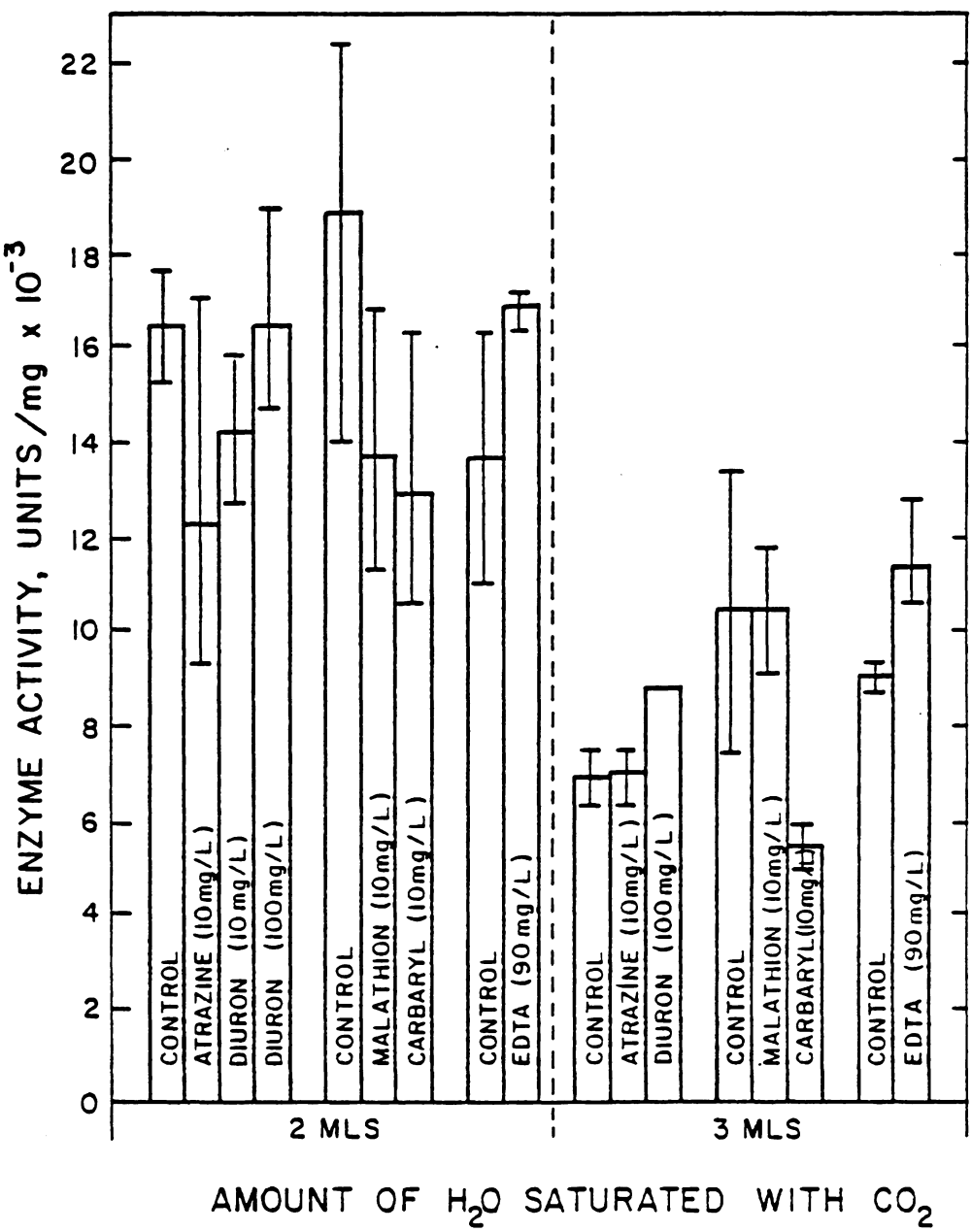


Figure 15. The activity of bovine carbonic anhydrase (mixture) following exposure to: herbicides, atrazine and diuron; pesticides, malathion and carbaryl; and the chelating agent, EDTA.

Table 9. The effects of various concentrations of 2,4-D on bovine carbonic anhydrase (mixture)[1] activity.

Concentration of 2,4-D mg/ℓ	Enzyme Activity[2] Range	Average	Trials	Percent decrease
	Volume of H_2O saturated with CO_2 (2 mls)			
Control	8600-12050	9890	4	
0.01	9230-10230	9730	2	
0.10	8600-9230	8900	2	
1.0	8600-12050	10230	2	
Control	10320-15250	13570	4	
5		15700	1	
10		13200	1	
15		12400	1	
20		7650	1	43
25		6860	1	49
Control	12820-18800	15070	4	
50		15070	1	
100		14130	1	
150		9770	1	35
200		4150	1	72
250	3270-6120	4620	3	70
400	4150-4150	4150	2	72
500	3270-3920	3600	2	75
	Volume of H_2O saturated with CO_2 (3 mls)			
Control	3790-6040	5090	3	
0.01	2260-4200	3000	2	
0.10	4640-5560	5090	2	
1.0	4210-4640	4420	2	
Control	7640-9230	8150	3	
5		9230	1	
10		8680	1	
15		3380	1	58
20		2030	1	75
25	3380-3750	3570	2	56
Control	7920-9600	8460	4	
50		9020	1	
100	11500-11500	11500	2	
150	5970-6430	6200	2	27
200	1450-2770	2090	2	75
250		1140	1	86
400		2770	1	67
500	1450-2420	1930	2	77

[1] 3 nmole enzyme exposed
[2] units/mg carbonic anhydrase

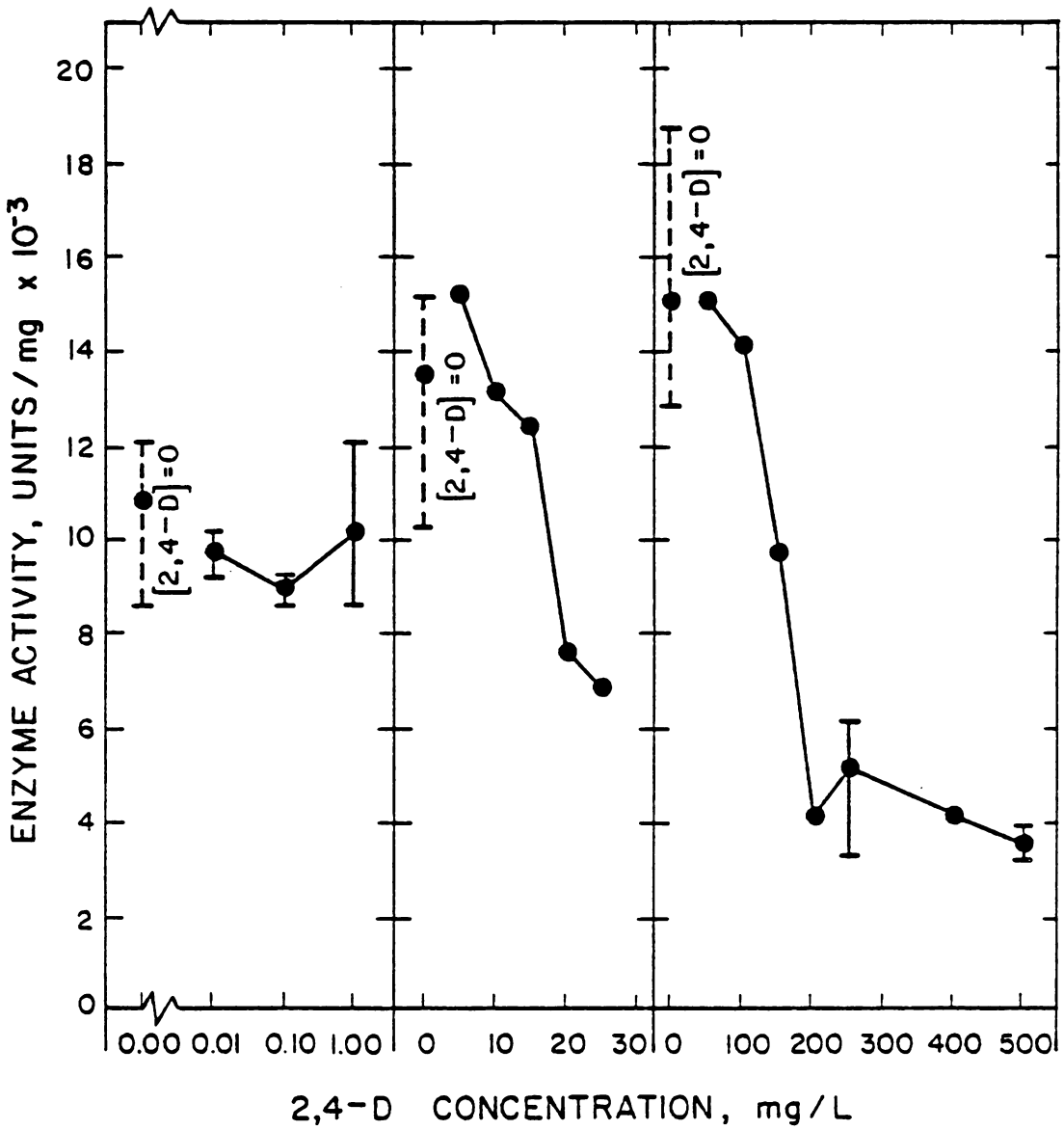


Figure 16. The effect of various concentrations of the herbicide, 2,4-D, on the activity of bovine carbonic anhydrase (mixture) using 2 mls of substrate (H_2O saturated with CO_2).

Table 10. The effects of sulfanilamide (ABSA)[1] and heavy metals on three different carbonic anhydrases.

Chemical agent	Conc. mg/ℓ	Enzyme Activity[2] Range	Average	Trials	Percent decrease
		Volume of H_2O saturated with CO_2 (2 mls)			
Control[3]	-	8840-9400	9120	2	
ABSA	10		3540	1	61
Control[4]	-	9820-18200	13660	6	
ABSA	10	5000-7870	6360	2	53
ABSA with Cd	10 50		4290	1	68
ABSA with Ni	10 50		4110	1	70
Control[4]	-		20480	1	
ABSA	10		10630	1	48
ABSA with Zn	10 5		10360	1	49
		Volume of H_2O saturated with CO_2 (3 mls)			
Control[3]	-		9230	2	
ABSA	10		3890	1	58
Control[4]	-	6670-8570	8070	3	
ABSA	10	2860-3530	3190	2	60
ABSA with Cd	10 50		2540	1	68
ABSA with Ni	10 50		1330	1	83
Control[4]	-		11370	1	
ABSA	10		5400	1	60
ABSA with Zn	10 5		4620	1	59
Control[5]	-	4210-4730	4460	2	
ABSA	100	2660-3060	2860	2	36

[1] p-aminobenzenesulfonamide
[2] units/mg carbonic anhydrase
[3] bovine carbonic anhydrase, type B; 33 nmole enzyme exposed
[4] bovine carbonic anhydrase (mixture); 3 nmole enzyme exposed
[5] bovine carbonic anhydrase, type A; 33 nmole enzyme exposed

greater inhibitory effect on carbonic anhydrase than was observed using ABSA alone. Figure 17 presents a graphical illustration of the inhibition of bovine carbonic anhydrase (mixture) due to 10 mg/ℓ of ABSA and 10 mg/ℓ of ABSA combined with 50, 50, and 5 mg/ℓ of cadmium, nickel, and zinc, respectively. It should be noted that the percent of enzyme inhibition caused by sulfanilamide (ABSA) combined with metal cations was very nearly the same when the assay was performed using either 2 or 3 mls of substrate (CO_2/H_2O) (see Table 10).

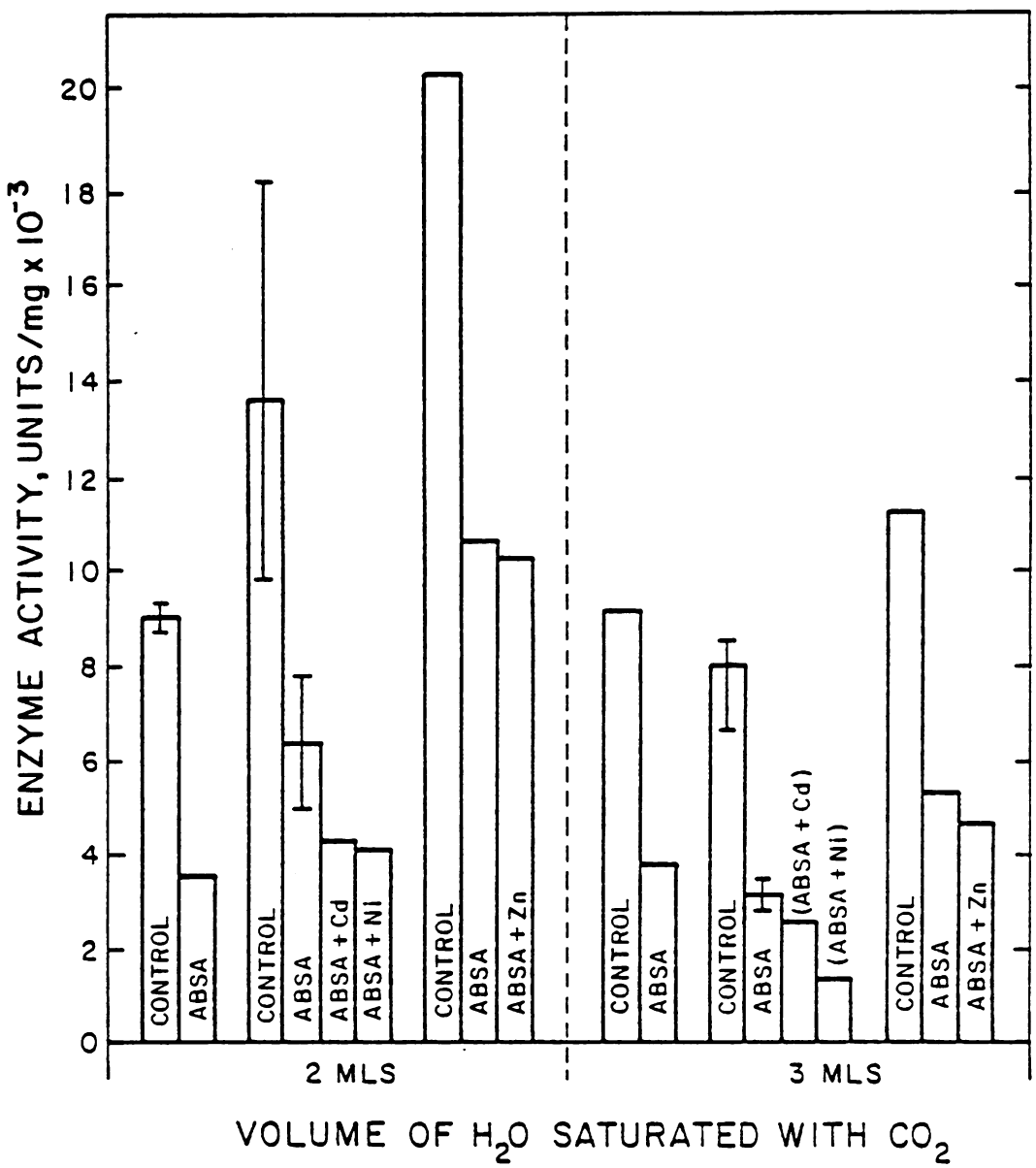


Figure 17. The effect of sulfanilamide (ABSA), 10 mg/ℓ, and ABSA (10 mg/ℓ) combined with the metal cations, Cd (50 mg/ℓ), Ni (50 mg/ℓ), and Zn (5 mg/ℓ), on the activity of bovine carbonic anhydrase (mixture).

DISCUSSION

The following section consists of three subdivisions which discuss the results obtained from experiments with α-chymotrypsin, acid phosphatase, or carbonic anhydrase. Each subdivision contains an evaluation of the response of the enzyme to the various chemical agents investigated, the problems encountered, and the advantages of each enzyme system. Also included in each subdivision is a review pertaining to the potential of each enzyme for use in the early assessment of toxicity.

α-Chymotrypsin

Two methods for assaying the activity of α-chymotrypsin were investigated in this study. The first assay employed 2-nitro-4-carboxyphenyl-N,N-diphenylcarbamate (NCDC) as a substrate, and the activity of the enzyme was determined by monitoring the formation of 3-nitro-4-hydroxybenzoic acid (NHB) by 7.2 mg of α-chymotrypsin during a 45 minute period. Cadmium, nickel, and 2,4-D were the chemical agents investigated. Cadmium at 18 to 27 μM (2 to 3 mg/ℓ) did not have a significant effect on 7.2 mg of α-chymotrypsin (see Table 2). The activity values for α-chymotrypsin exposed to cadmium were slightly higher than those obtained for the controls. A decrease in α-chymotrypsin activity was noted when nickel at 85 and 170 μM (5 and 10 mg/ℓ) was administered (see Figure 3). However, this data is questionable because it was observed that a precipitate formed following the addition of nickel, as nickel chloride, and α-chymotrypsin to the reaction mixture which contained the substrate, NCDC. It was noted

that blank samples, containing nickel and NCDC, but no enzyme, would develop a precipitate which after one hour, would exhibit absorbance values up to 70 percent of the control absorbance. It was assumed that nickel interacted with the substrate, NCDC, to form a complex, since NCDC is known to form an insoluble salt with Ca^{2+} (85). There was no observable affect on α-chymotrypsin activity following exposure of 2,4-D at 45 μM (10 mg/ℓ), and a mixture of all three agents, each at 5 mg/ℓ (see Figure 3). Data obtained from the experiments with the NCDC system indicated that α-chymotrypsin was not affected by low concentrations of cadmium, nickel, 2,4-D, or mixtures of all three agents. These results suggest several possibilities. The first possibility is that the effects of the chemical agents may have been masked because too much α-chymotrypsin was employed in the assay. Also, a high substrate concentration was employed which may have prevented interaction of the chemical agents with the enzyme molecule. Further research using the NCDC assay method was discontinued due to a few drawbacks inherent in the procedure. First, it is rather costly to use the procedure because 7.2 mg of α-chymotrypsin is required per test and the enzyme in 1981, sold for $23.00/100 mg (Sigma price). Secondly, the assay was a batch test requiring 45 minutes for color development; therefore, the catalytic activity of α-chymotrypsin could not be determined until after a 45 minute period.

The second assay procedure used for the determination of α-chymotrypsin activity employed benzoyl-L-tyrosine ethyl ester (BTEE) as the substrate. This method required only 12.5 μg of enzyme per assay and therefore, was more cost effective than the NCDC method and permitted

experimentation at lower amounts of enzyme per amount of toxicant. Furthermore, it was possible to monitor α-chymotrypsin activity over the entire period of the assay. In this assay system, α-chymotrypsin was exposed to cadmium at 20 mM (2.2 g/ℓ), nickel at 0.75 mM (44 mg/ℓ), and 2,4-D at 0.2 mM (44 mg/ℓ). The enzyme did not appear to be significantly affected by any of these agents at substrate (BTEE) levels of 0.12, 0.25, and 0.37 mM (see Table 3). However, all three agents appeared to enhance enzyme activity at 0.50 mM BTEE (see Table 2 and Figure 5). It was observed that the initial activity of α-chymotrypsin was almost doubled resulting from exposure to 2,4-D. These results are interesting because it was thought that high concentrations of heavy metal cations would interact with the two disulfide bonds present in α-chymotrypsin to cause inhibition, as described by Vallee and Ulmer (17) and Passow et al. (89). Also recall that Green et al. (44) observed inhibition in α-chymotrypsin resulting from 65 ppm Zn^{2+}, 64 ppm Cu^{2+}, and 200 ppm Hg^{2+}, using a similar substrate, acetyl-L-tyrosine ethyl ester, under the same conditions (pH 7.8 and 25°C). However, in this study, only one trial was performed per set of conditions and therefore, additional trials need to be conducted.

Hence, the BTEE system appears to be the most promising of the two systems for assaying the activity of α-chymotrypsin. It offers a quick and inexpensive method of assaying enzyme activity. The BTEE method also serves as a good model to keep in mind when other types of assay systems for other enzymes are considered. However, it is not fair to abandon the NCDC system because it may have the potential for use with other chemical agents.

Both assay systems may be sensitive to chemical agents that are structurally similar to their substrate, resulting in possible inhibition of enzyme activity. Furthermore, research could be directed toward the development of indirect techniques using these systems; such as chemically converting a specific chemical agent of interest into a form which would strongly inhibit α-chymotrypsin activity. Further research in these and other areas is actually needed in order to realize the true potential of these two α-chymotrypsin systems for use in the early assessment of toxicity.

Acid Phosphatase

The acid phosphatase assay system employed the substrate, p-nitrophenyl phosphate (NPP), at 37°C and the enzyme activity was determined by monitoring the formation of the product, p-nitrophenol. In the initial experiments with four levels of acid phosphatase (1.8, 4.5, 9.0, and 18 nmole), it was observed that the efficiency of product formation, or activity, increased as the enzyme level was increased at any given substrate level (see Figures 6 and 7). This type of curve was characterized by an initial sharp increase in activity until it reached a peak, where it then gradually declined until it reached a constant level at higher substrate concentrations. The gradual decline in activity is a phenomena often referred to as substrate inhibition, product inhibition, or enzyme poisoning. In this study the inhibition phenomena was considered to be associated with one or more of the following possibilities. First, the substrate (NPP) was purchased in the form of tablets; therefore, the inhibition in activity at higher substrate levels may have

been associated with the filler material contained in the tablets. At lower substrate concentrations, the filler material was diluted enough so that there was no effect on enzyme activity. Secondly, the product, p-nitrophenol, may have acted as an inhibitory agent as it reached a certain concentration; whereby, it prevented further formation of product by inhibiting enzyme activity. Product inhibition is a type of mechanism often apparent in complex biochemical reactions to control a catalytic reaction. However, as stated earlier, only the initial linear portion or low levels of substrate region was considered in this study; therefore, further experimentation to clarify the inhibited region at higher substrate levels was not performed.

Acid phosphatase was exposed to six chemical agents: cadmium, nickel, fluoride, sulfide, nitrate, and 2,4-D. Enzyme activity was not affected by cadmium at 0.09 mM (10 mg/ℓ), nickel at 0.85 mM (50 mg/ℓ), or 2,4-D at 0.45 mM (100 mg/ℓ) (refer to Table 4). This appears to be consistent with the findings of Guilbault et al. (25) who did not observe a change in the activity of acid phosphatase following exposure to 2,4-D. However, in this study a decrease in enzymatic activity was observed following exposure to cadmium ions (1250 mg/ℓ) (see Figure 8). It was observed that the decrease in activity, or inhibition caused by cadmium, appeared to become more pronounced as the substrate level was increased (refer to Figure 8). However, the actual values of inhibition over the entire curve ranged from 7 to 10 percent. This indicates that the enzyme inhibition due to cadmium, appears to be independent of the substrate. Additional

experiments using a broader range of substrate levels are needed to characterize the type of enzyme inhibition.

The activity of acid phosphatase was inhibited by about 20 and 10 percent at fluoride levels of 2.2 and 4.5 mg/ℓ, respectively, when the substrate level was 323 nmole NPP (see Table 5 and Figure 9). Below this substrate level, the amount of inhibition was not apparent. It is interesting to note that the inhibition produced by 2.2 mg/ℓ of fluoride was two times as much as that produced by higher amounts of fluoride (4.5 mg/ℓ). Recall that a similar phenomena at higher levels of fluoride was observed by Reiner et al. (50) who demonstrated that acid phosphatase inhibition increased, then decreased, then increased again as the fluoride level was increased from 60 to 600 mg/ℓ. Noting that there was a strange inhibitory effect with fluoride, experiments were conducted in which acid phosphatase was exposed to a range of fluoride concentrations (0.45 to 4.5 mg/ℓ) and assayed at 37°C using 323 nmole of the substrate, NPP (see Figure 10). Enzyme inhibition was observed at all fluoride levels employed. However, it was observed that most of the fluoride samples did not exhibit the same degree of reproducibility as the control samples displayed (see Figure 10).

In another experiment, acid phosphatase was assayed at 50°C, rather than the normal 37°C incubation, following exposure to 2.2 and 4.5 mg/ℓ of fluoride in an attempt to enhance inhibition induced by fluoride (refer to Table 5). Instead, it was observed that the overall enzyme inhibition remained between 10 and 20 percent (see Figure 9). Therefore, there was no difference in enzyme inhibition induced by fluoride at the two different assay temperatures.

The effects of other anions on acid phosphatase activity were also investigated. Upon exposure to sulfide at 15 μM (0.5 mg/ℓ), acid phosphatase did not appear to be affected (see Figure 11). However, a decrease in enzyme activity of 10 percent was observed following exposure to a 0.12 μM (7.3 mg/ℓ) nitrate solution (refer to Figure 11). In contrast, lower concentrations of nitrate (1.8, 3.6, and 5.5 mg/ℓ) did not produce any observable effect on enzyme activity (see Table 5).

It was observed in the acid phosphatase system that the inhibition resulting from fluoride and nitrate was at a maximum when the enzyme was assayed using 323 nmole of substrate (NPP) at 37°C. Recall that the activity of 4.5 nmoles of acid phosphatase is also at a maximum in this substrate region (refer to Figure 6). Most of the inhibition values were obtained from experiments run in duplicate; so, it probably would not be correct to regard the inhibition values as absolute numbers. However, the trends appear to be consistent and veritable. Additional research is needed to accurately quantitate and characterize the types of enzyme inhibition resulting from fluoride and nitrate anions.

Hence, the acid phosphatase assay system demonstrates potential for use in the assessment of anion toxicity as it appears to be useful in signaling the presence of relatively low concentrations of fluoride and nitrate. It does not appear to be useful in evaluating the toxicity of cadmium, nickel or 2,4-D. It was shown that the enzyme system was inhibited by fluoride at 0.45 mg/ℓ, and it is possible that even smaller levels of fluoride may affect the enzyme and therefore be

detected using this system. Furthermore, the enzyme system may be quite sensitive to other chemical agents such as compounds structurally similar to the substrate, NPP, or to agents that possess an anionic group capable of possibly inhibiting enzyme activity. Also, indirect methods such as the incorporation of co-factors, regulators, or other components which may be present in the physiological cellular enzyme, may be developed which would enhance the system's ability to detect toxicity due to certain chemicals. Additional research is warranted to fully develop the acid phosphatase enzyme system and to define its sensitivity to the chemical agents investigated in this study and to others.

Carbonic Anhydrase

Several preliminary experiments were performed to establish suitable concentrations of carbonic anhydrase and substrate (water saturated with CO_2, CO_2/H_2O). It was determined that 3×10^{-3} nmole (0.1 μg) of bovine carbonic anhydrase (mixture) was sufficient to decrease the pH of the reaction mixture at a rate which could be effectively timed and was significantly more rapid than the uncatalyzed reaction (see Appendix B, Figure B-3). The amount of substrate (CO_2/H_2O) selected for use in the assay was generally 2 or 3 mls. A volume of 4 mls was recommended by Worthington (86); however, the rate of pH decrease using 4 mls of CO_2/H_2O was too rapid for this investigation. The rate of pH change was slower when 2 to 3 mls of CO_2/H_2O was used, which served to amplify differences between the controls and the samples containing chemical agents. Also, it was envisioned

that a volume of 2 or 3 mls of CO_2/H_2O would stress the system somewhat, thereby enhancing any response induced by a chemical agent. In a typical trial the time required for the pH to decrease from 7.80 to 6.30 was recorded. A pH value of 7.80 was selected as the initial pH because the initial pH of the buffer varied between 8.25 and 7.95, thereby decreasing the reproducibility of the replicates. A pH value of 6.30 was used as the end point because the rate of pH decrease leveled off below this point.

In the experiments with five forms of bovine and human erythrocyte carbonic anhydrases (see Table 6 and Figure 12), bovine (mixture) exhibited the highest activity and was the form most commonly employed. All bovine types exhibited high activity, while the two human types possessed low activity. The activity of each type of enzyme decreased as the substrate (CO_2/H_2O) was increased (see Figure 12). This was due to the fact that at higher CO_2/H_2O levels, the difference between the catalyzed reaction and the uncatalyzed reaction becomes smaller (refer to Appendix B, Figure B-3). As a result, a lower activity is calculated at the higher substrate level (refer to equation 13).

As previously described, the various carbonic anhydrases were exposed to twelve different chemical agents (see Table 1). The effect of cadmium and nickel cations on carbonic anhydrase was studied for the reason that these ions, especially in high concentrations, might be capable of interacting with the zinc binding site contained in the enzyme. Recall that carbonic anhydrase contains one zinc ion per molecule which is bound at the active site. There was no observable effect on the activities of bovine (mixture), bovine type A, and human

type A carbonic anhydrases following exposure to 0.8 to 20 mM (90 to 2250 mg/ℓ) of cadmium and 0.76 to 17 mM (45 to 1000 mg/ℓ) of nickel (see Table 7). However, the activity of bovine carbonic anhydrase, type B substantially decreased following exposure to cadmium at 8.9 mM (1000 mg/ℓ) and nickel at 17 mM (1000 mg/ℓ); but, only one trial with each cation was conducted. These findings appear to be somewhat consistent with that of Maren (14) who reviewed the action of inhibitory agents on carbonic anhydrase. Maren stated that metals including Na, K, and Co are without effect, and that Cu, Ag, Au, Zn, Hg, and Va are active in the 0.1 mM range, but have not been systematically explored. However, the levels of metal ions necessary to induce an inhibitory effect on carbonic anhydrase are generally much greater than their reported aquatic lethal level. Recall that the LD_{50} concentration of cadmium in fish was reported to be in the range of 0.1 to 3.8 mg/ℓ (96 hour) (65).

An effort was made to stress carbonic anhydrase and increase its susceptibilty to cadmium through pH adjustment. In some experiments, the pH of the enzyme solution was elevated to a pH of 10 with NaOH and subsequently exposed to 10 mg/ℓ of cadmium. The solution was then adjusted to a pH of 7 and the enzyme was assayed. In other experiments, cadmium (10 mg/ℓ) was added to the enzyme after the pH of the enzyme solution had been elevated to a pH of 10 and adjusted to pH 7 (refer to Table 7). The results of these experiments showed that cadmium had little affect on carbonic anhydrase; however, the activity of the enzyme appeared to be stimulated somewhat as a result of the elevated pH level (pH 10) (see Figure 13). A cloudy white color was observed

in the elevated pH solution containing cadmium, due to the formation of cadmium hydroxide precipitates.

The response of carbonic anhydrase to several monovalent anions such as fluoride, sulfide, and nitrate was also studied. The activity of bovine carbonic anhydrase (mixture) was decreased or inhibited approximately 35 and 38 percent by fluoride at 0.2 mM (4.5 mg/ℓ) and sulfide at 15 μM (0.5 mg/ℓ), respectively (see Table 8). Recall that Lindskog (61) observed 50 percent inhibition in the hydrating activity of bovine carbonic anhydrase at a sulfide level of 0.17 μM (5.4 mg/ℓ). Maren (14) stated that sulfide ions are usually active in the range of 1 μM. However, it is difficult to compare inhibition values and sulfide levels between various studies because of differences in experimental methods and conditions; but, it is important to realize that the trends are consistent. In this study there was no observable affect on enzyme activity following exposure to sulfide at 1.5 μM (50 μg/ℓ) or 7.5 μM (250 μg/ℓ), which represent levels 10 to 50 times greater than the values reported by Lindskog (61). The response of carbonic anhydrase to nitrate ions was also studied. These studies indicated that enzyme activity of bovine type A was inhibited by approximately 26 percent by a 1.2 mM (73 mg/ℓ) solution of nitrate (see Table 8). However, this level of nitrate is well above the water quality standard of 10 mg/ℓ and suggests that this system may not be useful for signaling the presence of nitrate in most natural aquatic systems.

The activity of carbonic anhydrase following exposure to the herbicides, atrazine and diuron, was also investigated. Bovine carbonic anhydrase (mixture) activity was inhibited 25 percent by atrazine at 46 μM (10 mg/ℓ) when assayed using 2 mls of CO_2/H_2O. However, no

inhibition was observed when the enzyme was assayed using 3 mls of CO_2/H_2O (see Table 8). These results suggest that the carbonic anhydrase system may be useful for signaling the presence of atrazine when the system is employed at substrate limiting conditions. The herbicide, diuron, at 43 and 430 μM (10 and 100 mg/ℓ), did not appear to affect the activity of bovine (mixture) at either substrate level studied (see Table 8 and Figure 15).

The response of bovine carbonic anhydrase (mixture) to the pesticides, malathion and carbaryl (Sevin), was also studied. Malathion at 30 μM (10 mg/ℓ) inhibited enzyme activity by about 27 percent when assayed using 2 mls of CO_2/H_2O; however, no inhibition was observed when the enzyme was assayed using 3 mls CO_2/H_2O (see Table 8). Therefore, as with atrazine, malathion appears to be a carbonic anhydrase inhibitor when the enzyme is assayed at substrate limiting conditions. Carbonic anhydrase activity was inhibited approximately 32 and 48 percent by carbaryl at 50 μM (10 mg/ℓ) when assayed at 2 and 3 mls CO_2/H_2O, respectively (see Table 8 and Figure 15). The 96-hour TLM value (acute toxicity) for malathion and carbaryl in bluegills has been reported as 0.9 and 5.3 mg/ℓ, respectively (90). Therefore, carbonic anhydrase may be useful in signaling potential toxicity problems associated with malathion or carbaryl, and warrants additional research to establish the sensitivity and reliability of this enzyme for detecting pesticides.

The results of the experiments using the chelating agent, EDTA, indicate that this agent appears to enhance the activity of carbonic anhydrase. The activity of bovine (mixture) following exposure to

0.24 mM (90 mg/ℓ) of an EDTA solution was stimulated approximately 20 percent when the enzyme was assayed using both 2 and 3 mls of CO_2/H_2O (see Table 8 and Figure 15). This is interesting because it was thought that EDTA would bind the zinc ion and inhibit activity.

The response of bovine carbonic anhydrase (mixture) to various concentrations of the herbicide, 2,4-D, was also studied. The activity of the enzyme did not appear to be significantly affected by the following concentrations: 0.01, 0.10, 1.0, 5.0, and 10 mg/ℓ of 2,4-D (see Table 9 and Figure 16). However, enzyme inhibition (43 to 75 percent) was produced at 2,4-D concentrations of 15, 20, and 25 mg/ℓ and inhibition (27 to 77 percent) was noted at 150 to 500 mg/ℓ, but no inhibition was observed at intermidiate levels such as 50 to 100 mg/ℓ 2,4-D. Upon comparing the inhibition values obtained at substrate levels of 2 and 3 mls CO_2/H_2O, it was noted that 58 percent inhibition occurred at 15 mg/ℓ 2,4-D when 3 mls CO_2/H_2O was used, but no inhibition occurred at a CO_2/H_2O level of 2 mls. These data suggest that there was a fluctuation in enzyme inhibition over the range of 20 to 150 mg/ℓ 2,4-D, and warrants additional research to accurately assess the impact of these levels of 2,4-D on carbonic anhydrase.

The effect of sulfanilamide (ABSA), a known inhibitor of carbonic anhydrase, was investigated individually and in combination with metal cations. The activity of carbonic anhydrase was inhibited by as much as 61 percent by ABSA at 58 μM (10 mg/ℓ) when 2 mls of CO_2/H_2O was used. Recall that it was shown by Lindskog and Nyman (60) and Lindskog (61) that the zinc ion at the active site in the enzyme, will bind to ABSA. It was envisioned that other cations such as Cd^{2+} and Ni^{2+}

might preferentially bind with ABSA, thus alleviating the inhibitory affect of ABSA on carbonic anhydrase. Therefore, it was thought that this may be useful as an indirect test for signaling the presence of these metals. To examine this possibility, ABSA at 58 μM, was combined with Cd^{2+} at 444 μM, Ni^{2+} at 852 μM, and Zn^{2+} at 76 μM, and then added to carbonic anhydrase to determine the inhibitory affect (see Table 10 and Figure 17). The results of these experiments indicated that the inhibition of carbonic anhydrase was not alleviated by the addition of metals to ABSA, but instead the inhibition was 2 to 23 percent greater (see Table 10). Therefore, it appeared that there was an additive affect on enzyme inhibition due to both the cations and ABSA. This suggests that carbonic anhydrase may be more responsive to metal cations in the presence of ABSA. Further research is needed to examine various other levels of these and other cations in combination with sulfanilamide.

The degree of reproducibility present in the carbonic anhydrase assay system might be enhanced by employing additional equipment. For example, the concentration of CO_2 dissolved in water (substrate) probably varied somewhat throughout a given series of experiments. Through the course of this study, CO_2 levels were not monitored. The use of CO_2 electrodes or another suitable tool to accurately measure the amount of dissolved CO_2 in water before a given trial would be advisable. Also, the temperature of the assay was critical to the rate of the reaction, as even slight differences (± 0.1°C) caused a fluctuation in activity between replicates. The use of a well insulated water bath along with a constant temperature atmosphere would improve

the degree of consistency between replicates.

The carbonic anhydrase enzyme system offers several advantages over the other systems investigated for use in the early assessment of toxicity. First, the assay procedure is very rapid and simple to perform and requires the use of relatively inexpensive equipment. Secondly, a very small amount of enzyme (bovine CA) is required per assay due to the extremely high catalytic activity of carbonic anhydrase. Also, it would be very easy to develop an automated system for monitoring carbonic anhydrase activity using this type of methodology.

From the data obtained in this investigation, the carbonic anhydrase enzyme system appears to be the most useful for the early assessment of anion, pesticide, and sulfonamide toxicity, as it demonstrates potential for signaling the presence of these chemical agents at environmentally significant concentrations. It does not appear to be useful in evaluating the toxicity of cations, such as cadmium, nickel, or zinc. The enzyme assay system may be useful in detecting high concentrations of herbicides, such as 2,4-D and atrazine; however, these compounds generally do not appear in high concentrations in environmental samples. Further research using the carbonic anhydrase enzyme system is warranted in the following areas. First, indirect techniques should be examined, such as chemically derivatizing chemical agents of interest to produce an active enzyme inhibitor. Secondly, research should be directed toward the incorporation of co-factors, regulators, or other cellular components to simulate *in vivo* conditions, thereby possibly enhancing the response of the enzyme system towards

specific chemical agents. Also, general refinement of the assay system might enhance reproducibility and enzyme response. Additional research in these and other areas would better define the limits of the carbonic anhydrase enzyme system and establish its reliability as a technique for the early assessment of toxicity.

SUMMARY AND CONCLUSIONS

This study was conducted to determine the potential of α-chymotrypsin, acid phosphatase, and carbonic anhydrases for use in the early assessment of toxicity. The study was primarily associated with the development of suitable assay methods for the enzymes and the determination of enzyme response to several chemical agents. This was accomplished by measuring the activities of purified enzymes following exposure to several inorganic and organic agents. The activities of α-chymotrypsin and acid phosphatase were measured spectrophotometrically, while carbonic anhydrase activities were determined by monitoring a shift in pH. The chemical agents investigated included several toxic compounds such as heavy metals, herbicides, and pesticides, and various environmentally significant anions.

Several techniques were explored to amplify the response of the enzyme systems to certain chemical agents through modifying temperature or pH. It was observed that the percent of acid phosphatase inhibition induced by fluoride (2 and 4 mg/ℓ), was not altered by increasing the assay temperature from 37°C to 50°C; however, the overall enzyme activity was reduced approximately 70 percent. Studies were also conducted in which the pH of a carbonic anhydrase solution was elevated to 10 in an attempt to increase the response of the enzyme to cadmium (10 mg/ℓ). Preliminary experiments indicate that there may be some potential in altering the pH of an enzyme solution in order to create or potentiate a given response. Further research

is required to fully appreciate how temperature and pH might be used to enhance the response of enzymes to toxic agents.

In addition, some indirect methods of assessing toxic compounds were investigated through characterizing the response of carbonic anhydrase to the chelating agent, EDTA, and to sulfanilamide. It was thought that the effect of EDTA or sulfanilamide on carbonic anhydrase activity might be altered by the addition of metal cations. Therefore, either system may have the potential for indicating the presence of metal cations and be useful for indirectly assessing toxic activity due to heavy metals. The results of these studies indicated that carbonic anhydrase activity was stimulated somewhat by EDTA and inhibited by sulfanilamide. Addition of the metals, cadmium, nickel, or zinc, to the sulfanilamide-enzyme mixture appeared to cause greater inhibition than when sulfanilamide was administered alone. The use of purified enzymes to indirectly signal toxic compounds appears to show some potential; however, additional research is needed to establish the sensitivity and reliability of these types of indirect tests in the early assessment of toxicity.

Data obtained from the exposure of various chemical agents to α-chymotrypsin, acid phosphatase, and carbonic anhydrase suggests the following conclusions:

1. α-Chymotrypsin activity was not significantly affected by cadmium (2 to 3 and 2250 mg/ℓ), nickel (5,10, and 44 mg/ℓ), or 2,4-D (10 and 44 mg/ℓ) under the conditions of this study. Hence, the α-chymotrypsin enzyme system does not appear to be useful for signaling potential toxicity problems associated with these agents.

2. Acid phosphatase activity was inhibited by fluoride (0.45 to 4.5 mg/ℓ) and nitrate (7.3 mg/ℓ). However, the enzyme did not respond to cadmium (10 mg/ℓ), nickel (50 mg/ℓ), or 2,4-D (100 mg/ℓ). Therefore, acid phosphatase does not appear to be useful for indicating toxic activity due to these agents.

3. Carbonic anhydrase activity was inhibited by 10 mg/ℓ solutions of atrazine, malathion, and carbaryl, and by 150 to 500 mg/ℓ of 2,4-D. The monovalent anions, F^-, HS^-, and NO_3^- also produced enzyme inhibition at 4.5, 0.5, and 73 mg/ℓ, respectively. However, carbonic anhydrase did not respond to cadmium (90 to 2250 mg/ℓ) or nickel (45 to 1000 mg/ℓ). Therefore, carbonic anhydrase appears to demonstrate some potential for indicating toxic activity associated with herbicides, pesticides, and certain anions.

4. The activity and inhibition of acid phosphatase and carbonic anhydrase were observed to be a function of substrate concentration.

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APPENDIX A

Table A-1 Characteristics and lot numbers of several purified enzymes.

ENZYME	ORIGIN AND TYPE	LOT NUMBER[1]
α-Chymotrypsin	bovine pancreas, type VII	70F-8000
Acid Phosphatase	potato, type IV	80F-0073
Carbonic Anhydrase	bovine erythrocyte (mixture)	69C-9335
Carbonic Anhydrase	bovine erythrocyte type A	125C-8080
Carbonic Anhydrase	bovine erythrocyte type B	80F-9350
Carbonic Anhydrase	human erythrocyte type A	25C-8200
Carbonic Anhydrase	human erythrocyte type B	110F-9331

[1] all enzymes purchased from Sigma Chemical Co., Saint Louis, Mo.

Table A-2. Activities of four different amounts of acid phosphatase using a range of substrate (NPP)[1] levels.

NPP[1]		AMOUNT OF ACID PHOSPHATASE ASSAYED[2]							
		0.1 mg		0.25 mg		0.50 mg		1.0 mg	
mg	nmole	A_{400}[3]	NP[4]	A_{400}	NP	A_{400}	NP	A_{400}	NP
0.010	43			0.108	33.6	0.122	37.9		
0.012	54	0.136	42.3	0.150	46.6	0.201	62.3	0.123	38.2
0.025	108	0.213	66.0	0.285	88.3	0.288	89.2	0.240	74.4
0.050	215	0.254	78.7	0.498	154.1	0.571	176.6		
0.075	323	0.231	71.6	0.522	161.5	0.772	238.7		
0.100	431	0.200	62.0	0.457	141.4	0.869	268.6		
0.125	539			0.414	128.1	0.874	270.2	1.036	320.2
0.150	647	0.151	46.9						
0.167	720			0.336	104.0	0.752	232.5		
0.200	862			0.240	74.4	0.583	180.3		
0.250	1078	0.107	33.3					1.332	411.6
0.500	2150							0.925	285.9
0.750	3234							0.592	183.1
1.000	4312							0.454	140.5
1.500	6468							0.390	120.7
2.083	8969							0.341	105.6

[1] p-nitrophenyl phosphate (substrate)

[2] 1.8, 4.5, 9.0, 18 nanomoles of acid phosphatase

[3] absorption values at a wavelength of 400 nm

[4] p-nitrophenol (product), nanomoles

Table A-3. Values of acid phosphatase activity following exposure to a range of fluoride (F^-) levels.

Concentration				
NaF (mg/ℓ)	F^- (mg/ℓ)	F^- (mM)	ABSORBANCE[1]	p-nitrophenol nanomole
Control[2]	-	-	0.473 ± .002	146 ± 1
1	0.45	0.02	0.419 ± .019	130 ± 6
2	0.90	0.05	0.434 ± .006	134 ± 2
3	1.36	0.07	0.407 ± .023	126 ± 7
4	1.81	0.09	0.436 ± .003	135 ± 1
5	2.26	0.12	0.423 ± .003	131 ± 1
6	2.71	0.14	0.429 ± .009	133 ± 3
7	3.17	0.17	0.413 ± .001	128 ± 1
8	3.62	0.19	0.399 ± .002	124 ± 1
9	4.07	0.21	0.401 ± .005	124 ± 2
10	4.52	0.24	0.395 ± .004	122 ± 2

Amount of acid phosphatase assayed = 5 nmole
Amount of acid phosphatase exposed = 50 nmole
Amount of substrate (NPP)[3] used per assay = 323 nmole

[1] wavelength = 400 nm; values corrected for fluoride and substrate absorbance; samples run in duplicate.

[2] enzyme not exposed to fluoride.

[3] p-nitrophenyl phosphate.

Table A-4. Raw data obtained from the exposure of carbonic anhydrase to various chemical agents.

Chemical agent	Conc. mg/ℓ	MLS OF H_2O SATURATED WITH CO_2 2.0 Time (seconds)		Trials	3.0 Time (seconds)		Trials
Control[Δ]	-	76-89*	82**	2	47-48*	48**	2
Cadmium	2250	82-89	86	2	47-52	50	2
Nickel	45	74-88	81	2	45-48	46	2
Control[Δ]	-	74-78	76	3	45-47	46	2
NaF	10	78-101	86	4	46-48	47	2
Control[Δ]	-	81-102	93	4	58-61	59	4
HS^-	0.05	75-88	82	3		57	1
HS^-	0.25	96-99	98	2	55-65	60	2
HS^-	0.5	105-116	110	2	62-68	65	3
Control[⊖]	-		77	1		54	1
$NaNO_3$	100		86	1		58	1
Control[Δ]		83-93	89	4	53-58	55	3
2,4-D	0.01	88-91	90	2	57-62	60	2
2,4-D	0.10	91-93	92	2	54-56	55	2
2,4-D	1.0	83-93	88	2	56-57	56	2
Control[Δ]	-	80-93	89	4	52-55	54	3
2,4-D	5		79	1		52	1
2,4-D	10		85	1		53	1
2,4-D	15		87	1		65	1
2,4-D	20		102	1		69	1
2,4-D	25		105	1		64	1
Control[Δ]	-	66-78	73	4	50-53	52	4
2,4-D	50		73	1		51	1
2,4-D	100		75	1		47	2
2,4-D	150		86	1	56-57	56	2
2,4-D	200		106	1	65-69	67	2
2,4-D	250	98-110	104	3		70	1
2,4-D	400		106	2		65	1
2,4-D	500	107-110	108	2	66-69	68	2
Control[Δ]	-	60-64	62	3	45-47	46	3
Atrazine	10	61-77	70	3	45-47	46	2
Diuron	10	63-69	66	2			
Diuron	100	58-65	62	2		43	1
Control[Δ]	-	65-81	71	5	42-51	46	4
Malathion	10	75-88	82	2	44-48	46	2
Carbaryl	10	76-90	84	4	54-56	55	2
Control[Δ]	-	76-89	82	2	47-48	48	2
EDTA	90	74-76	75	2	42-45	44	2

Table A-4 continued

Control[Δ]	-	89-114*	101**	6	56-60*	57**	3
ABSA	10	122-136	129	2	68-70	69	2
ABSA/Cd	10/50		140	1		71	1
ABSA/Ni	10/50		141	1		75	1
Control[Δ]	-		84	1		51	1
ABSA	10		111	1		63	1
ABSA/Zn	10/5		112	1		65	1
Control[Θ]	-	67-69	68	2		39	2
ABSA	10		96	1		54	1
Cadmium	1000		91	1		38	1
Nickel	1000		82	1		48	1
2,4-D	50		111	1		65	1
Control[Δ]			92	2		59	1
pH 10		77-87	82	5	52-59	55	4
pH 10/Cd		76-83	80	4	49-54	52	2
pH 10 + Cd		68-91	80	2		58	1

* range of values

** average of all values

Δ bovine carbonic anhydrase (mixture)

Θ bovine carbonic anhydrase, type A

APPENDIX B

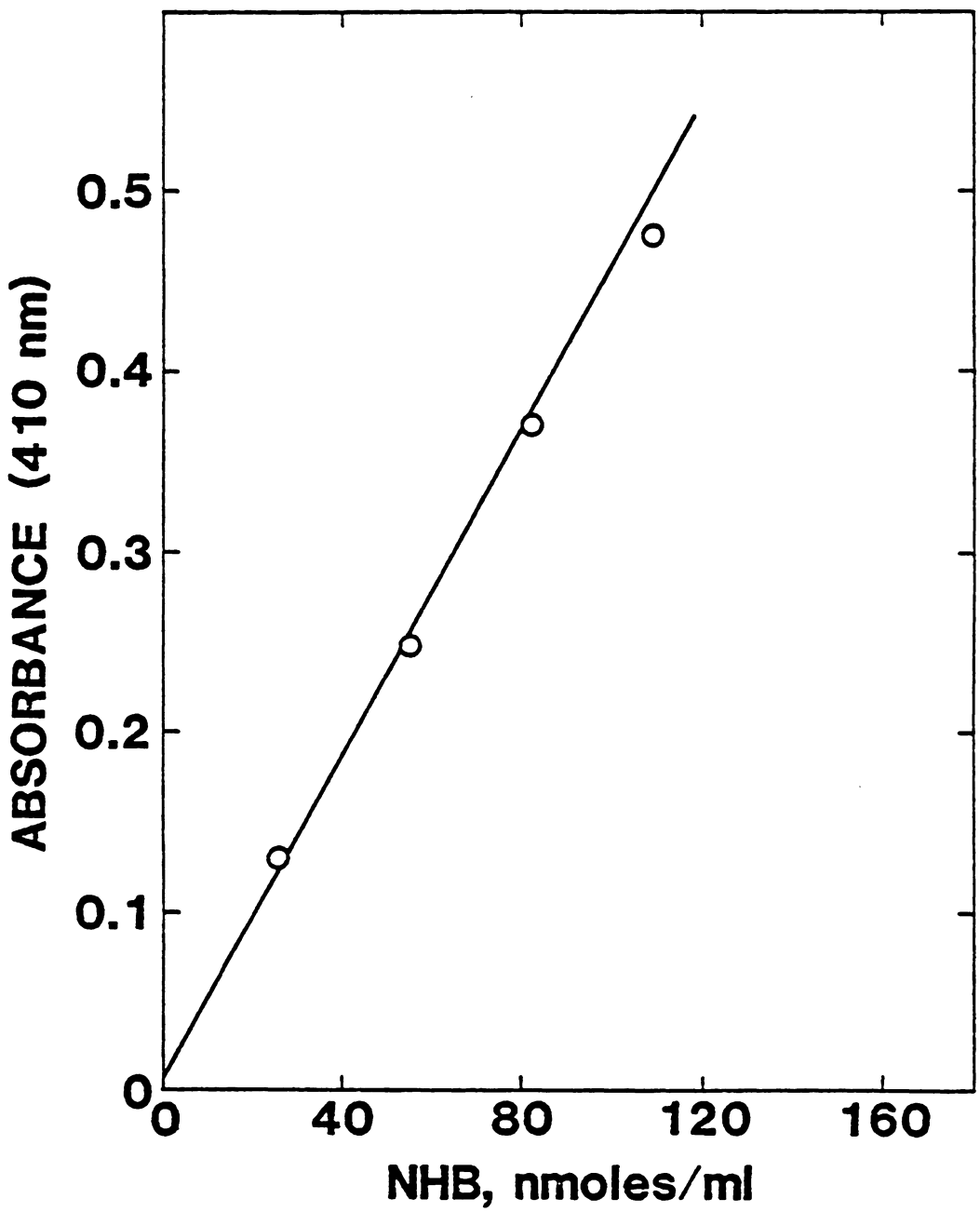

Figure B-1 Standard curve for the determination of 3-nitro-4-hydroxybenzoic acid (NHB).

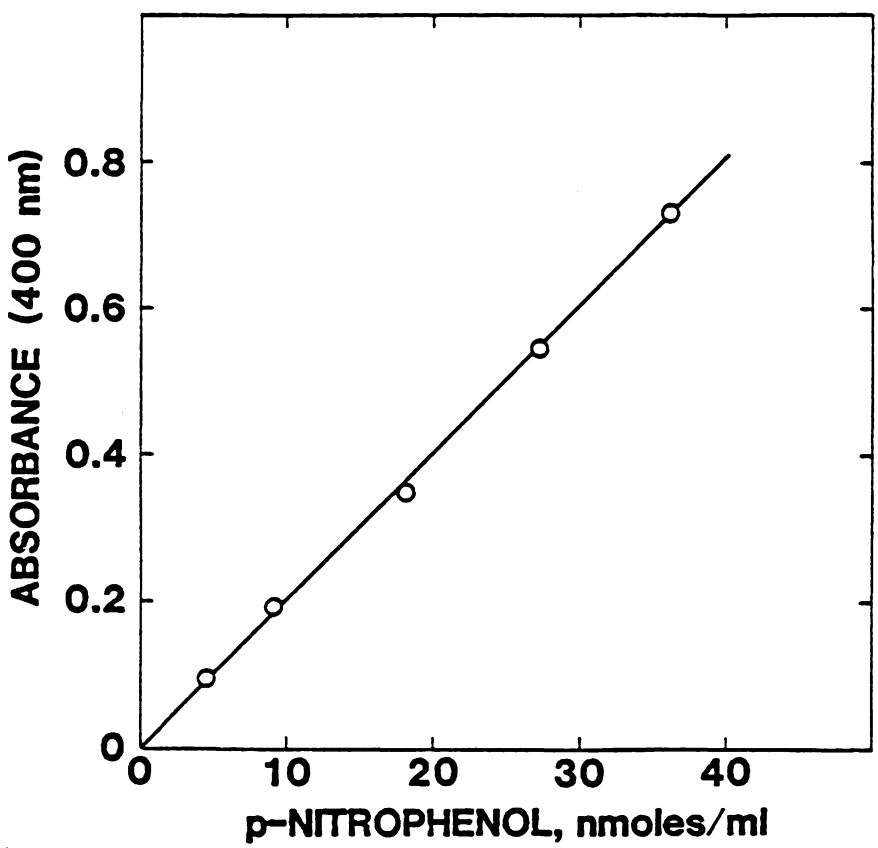

Figure B-2 Standard curve for the determination of p-nitrophenol.

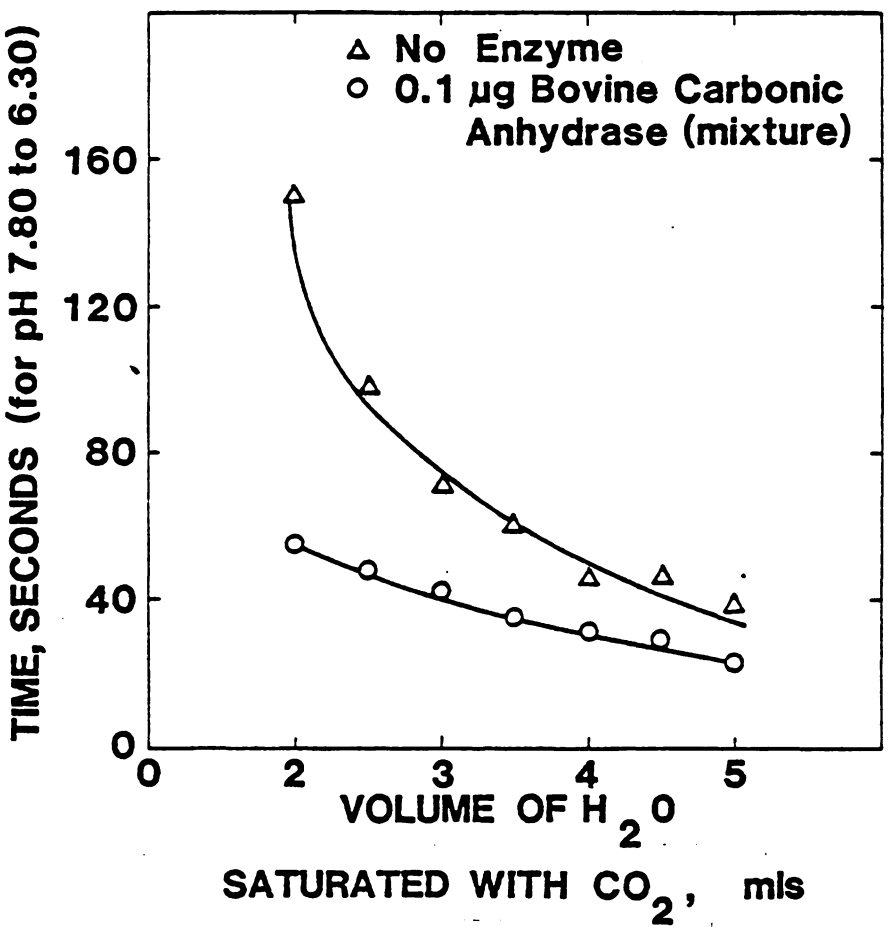

Figure B-3 A graphical representation of the enzyme-catalyzed hydration of CO_2 in relation to the uncatalyzed reaction.

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THE USE OF PURIFIED ENZYMES FOR THE EARLY ASSESSMENT OF TOXICITY

by

John F. Pfaff

(ABSTRACT)

The increasing level and dispersion of toxic chemicals in the environment has stimulated a need for accurate methods capable of detecting and quantifying the activity of toxicants. The primary objective of this study was to determine, through in vitro tests, the potential of three purified enzymes: α-chymotrypsin, acid phosphatase, and carbonic anhydrase for use in the early assessment of toxicants at environmentally significant levels. Activities of α-chymotrypsin and acid phosphatase were measured spectrophotometrically, while carbonic anhydrase activities were determined through monitoring a pH change. The chemical agents investigated included several heavy metals, common herbicides and pesticides, and various environmentally significant anions. In addition, several techniques were explored to amplify enzyme response to chemical agents.

The results of the study demonstrated that α-chymotrypsin did not significantly respond to cadmium, nickel, or 2,4-D, and consequently, does not appear to be useful in indicating potential toxicity problems associated with these agents.

The acid phosphatase enzyme system appears to be useful in signaling the presence of low levels of certain anions (fluoride at

0.45 mg/ℓ and nitrate at 7.3 mg/ℓ), but does not appear to have potential for detecting toxic activity due to cadmium, nickel, or 2,4-D. Enzyme inhibition induced by fluoride at 37°C was not altered by changing the assay temperature to 50°C.

The results of the experiments with carbonic anhydrase show that the enzyme does not appear to be affected by cadmium or nickel ions. However, enzyme activity was inhibited by fluoride (4.5 mg/ℓ), sulfide (0.5 mg/ℓ), and nitrate (73 mg/ℓ). Enzyme inhibition was also induced by 10 mg/ℓ of atrazine, malathion, or carbaryl, and 150 to 500 mg/ℓ of 2,4-D. Inhibitory effects induced by sulfanilamide appeared to be slightly enhanced by the addition of Cd^{2+}, Ni^{2+}, or Zn^{2+} cations. These findings, although preliminary, suggest that carbonic anhydrase demonstrates potential for signaling the presence of anions, and appears to be useful in indicating potential toxicity problems due to pesticides and herbicides.