ABSTRACT

SUPEROXIDE DISMUTASES AND SUPEROXIDE RADICAL:
OCCURRENCE IN HIGHER PLANTS AND
POSSIBLE ROLE IN THE ACTION OF HERBICIDES

By

Constantine Nicholas Giannopolitis

The photochemical assay for superoxide dismutase (SOD) consisting of methionine, riboflavin, and *p*-nitro blue tetrazolium chloride (NBT) was adjusted for quantitation of the enzyme in crude extracts. The enzyme units can be accurately determined from the ratio of NBT reduction in absence *versus* in presence of SOD and not from the percent inhibition of NBT reduction. An equation derived from the kinetics of the reaction and confirmed with various crude extracts can be used for calculating enzyme units from the above ratio. Interferences with enzyme assays of crude extracts were examined. Only peroxidase at high concentrations was shown to interfere. Peroxidase was easily inactivated by heat and SOD was heat-stable, allowing correction for peroxidase interference by heating the crude extracts.

Shoots, roots, and seeds of corn (*Zea mays* L., cv. Michigan 500), oats (*Avena sativa* L., cv. Au Sable), and peas (*Pisum sativum* L., cv. Wando) were analyzed for their SOD content. The enzyme is present in the shoots, roots and seeds of all three species. Quantitative differences exist between species and between organs within a species. On a dry weight basis, shoots contain more enzyme than roots. In seeds,

the enzyme is present in both the embryo and the storage tissue. It was estimated that SOD accounts for 0.9 to 3.1% of the water-soluble protein in 10-day-old seedlings of corn, oats, and peas. The specific activity of SOD increased 3-fold during germination of oats, and 40% during greening and hook opening of the pea plumule.

Electrophoresis indicated multiple forms of the enzyme. Ten distinct enzyme bands were obtained from the three species. Corn contained seven of the bands and oats three different bands. Peas contained one of the corn, and two of the oat enzymes. Some of the SOD forms were found primarily in mitochondria or chloroplasts. Differences and similarities in the enzyme pattern of the various organs may be explained by the organelle specificity of the SOD forms.

Superoxide dismutase was purified to a maximum specific activity from pea seeds, and partially purified from corn seedlings. The purified pea enzyme eluting as a single peak from gel exclusion chromatography columns contained the three electrophoretically distinct SOD bands characterizing the crude pea extract. The purified corn enzyme eluted as the same peak as the pea enzyme, and contained five of the seven active bands found in the crude extract. The similar molecular weights and the cyanide sensitivities of these bands indicated that they are isozymes of a cuprozinc SOD. One of the remaining corn bands was shown to be a peroxidase. The other was a protein resistant to cyanide and sensitive to chloroform-ethanol treatments and may be a manganese-containing SOD.

The ability of herbicides to produce superoxide radical as well as their ability to react with this radical was examined through their effect on the superoxide-induced reduction of NBT. Paraquat enhanced and diuron inhibited the reduction of NBT. Paraquat was reduced photochemically

(riboflavin/methionine) or enzymatically (xanthine/xanthine oxidase) and produced superoxide radical upon reoxidation. Diuron and monuron interacted with photochemically produced superoxide radical, but not with enzymatically produced superoxide radical. The product of the monuron/superoxide interaction was a demethylated, dechlorinated water-soluble compound containing phenolic hydroxyl group(s), which was not toxic to oats. The enzyme SOD prevented the formation of this product. Other herbicides (atrazine, metribuzin, terbacil, 2,4-D, CDEC, diphenamid) had little effect on the NBT reduction.

SUPEROXIDE DISMUTASE AND SUPEROXIDE RADICAL: OCCURRENCE IN HIGHER PLANTS AND POSSIBLE ROLE IN THE ACTION OF HERBICIDES

By

Constantine Nicholas Giannopolitis

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INTRODUCTION

It is generally accepted that herbicides kill plants by inhibiting fundamental processes such as photosynthesis, respiration, and protein and nucleic acid synthesis (6)[1]. A simple inhibition of such processes, however, does not explain some important phases of the herbicidal action (5,44,53). Inhibition of these processes may not be the real factor causing death, although it may be the primary site of action of herbicides. The mechanism of action of herbicides, therefore, is ambiguous, and yet the practical interest it presents has abruptly increased in recent years along with efforts to obtain safer and more effective pesticides.

It has been suggested that free radicals may be involved in deteriorative mechanisms in living organisms (23). Furthermore, some herbicides have been postulated to act through generation of free radicals (5,15). Recent research supports the view that free radicals may be toxic and encourages the hypothesis that they are involved in herbicidal action.

The superoxide and hydroxyl free radical are of particular interest because they can be easily formed from oxygen during various reactions of biological significance (7,35,36,40,41), and they cause toxicity to organisms (25,26,32). Furthermore, the discovery of the enzyme

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[1] References at the end of "Literature review".

superoxide dismutase (37) has facilitated the study of the involvement of these radicals in biological processes.

Most of the information about superoxide radical and superoxide dismutase has been obtained from studies on animals and lower plants. This study was primarily designed to examine the occurrence of superoxide dismutase in higher plants, and the quantitative differences between species. Possible involvement of superoxide radical, hydroxyl radical, and superoxide dismutase in herbicidal action was also examined by *in vitro* experiments.

LITERATURE REVIEW

LITERATURE REVIEW

THE SUPEROXIDE RADICAL ($O_2^{\cdot -}$)

The superoxide free radical ($O_2^{\cdot -}$) has been known to chemists for several decades (27), but it has only recently become of interest to biologists. In 1954 biologists became interested in free radicals after Gerschman's group proposed that oxygen toxicity and radiation injury were due to the high reactivity of free radical intermediates (23). However, the involvement of $O_2^{\cdot -}$ in oxidations of biological significance was not anticipated. McCord and Fridovich first proved that $O_2^{\cdot -}$ is involved in the oxidation of sulfite and the reduction of cytochrome *c* and subsequently discovered the enzyme superoxide dismutase (36,37) indicating that $O_2^{\cdot -}$ is a possible intermediate in biological systems. Currently, a considerable amount of research is being conducted in this area as indicated in a number of recent review papers (10,19,21,28). Only topics pertinent to this study are reviewed here.

Forms of Oxygen. Taube describes the electronic structure and excited states of molecular oxygen (54). Two of his terms are to be used often in this study, namely triplet (ground) oxygen (O_2) and singlet oxygen (O_2^*). The first is the normal oxygen, the lowest energy and reactivity state of oxygen. The second is one of the excited states of oxygen, particularly the state of the highest energy and reactivity, and is produced from ground oxygen after an energy input. This latter

form can be toxic to organisms (10).

Triplet oxygen may also become $O_2^{\cdot -}$ of hydroxyl radical ($OH^{\cdot}$) after univalent and trivalent reduction, respectively (21). Superoxide radical formed from triplet oxygen further initiate production of both singlet oxygen and hydroxyl radical. Hydroxyl radical is the most potent oxidant known (43) and, therefore, its toxicity can be easily inferred.

Generation of $O_2^{\cdot -}$. Superoxide radical is the product of univalent reduction of triplet oxygen. Divalent, trivalent and tetravalent reduction will yield H_2O_2, $OH^{\cdot}$, and H_2O, respectively (21). In biological systems, many enzymes catalyze the overall tetravalent reduction of oxygen to water without the release of reactive intermediates. However, the electronic structure of oxygen favors univalent pathways of reduction (54) and so generation of $O_2^{\cdot -}$ during biological reduction of oxygen is possible. Research of recent years has provided support for this view.

There is good evidence that $O_2^{\cdot -}$ is produced during the aerobic action of several enzymes, among which are xanthine oxidase (36), numerous flavin dehydrogenases (35), and NADPH-cytochrome *c* reductase (7). Superoxide radical is also formed during autoxidation of a variety of compounds found in biological systems including ferredoxin (40), flavins and quinones (41), and haemoglobin (42).

Generation of $O_2^{\cdot -}$ by isolated chloroplasts upon illumination has been reported (1,2,12,14). The production of $O_2^{\cdot -}$ by leucocytes in mammals has also been established (8).

Properties of $O_2^{\cdot -}$. Properties of $O_2^{\cdot -}$ that are interesting from the viewpoint of this study are:

(a). Superoxide radical can act either as a reducing or as an oxidizing agent. In the first case it gives up its extra electron and

becomes O_2, *eg*:

$$\text{Cytochrome } c\ (Fe^{3+}) + O_2^{\cdot -} \rightarrow \text{Cytochrome } c\ (Fe^{2+}) + O_2$$

In the second case it becomes H_2O_2, *eg*:

$$\text{Ascorbate} + 2O_2^{\cdot -} + 2H^+ \rightarrow \text{Dehydroascorbate} + 2H_2O_2$$

This latter reaction may be responsible for the effects of ascorbate on O_2 uptake by isolated chloroplasts (14).

(b). In the absence of anything else for $O_2^{\cdot -}$ to react with, it reacts with itself giving singlet oxygen (31):

$$O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2^*$$

(c). In the presence of H_2O_2, $O_2^{\cdot -}$ produces the far more reactive hydroxyl radical (27):

$$O_2^{\cdot -} + H_2O_2 \rightarrow OH^- + OH^{\cdot} + O_2$$

(d). Compared with other oxygen radicals, $O_2^{\cdot -}$ is rather unreactive with a lifetime in the msec range.

Biological Significance of $O_2^{\cdot -}$. *In vivo* formation of $O_2^{\cdot -}$ may lead to cell damage. This can be brought about by its direct reaction with the cell components or by the generation of $OH^{\cdot}$ and O_2^*. Two additional facts support this statement: 1) the long lifetime of $O_2^{\cdot -}$ allows it to difuse away from the site of formation (15) and, 2) hydrogen peroxide, which accumulates in a system producing and dismutating $O_2^{\cdot -}$ (37), subsequently leads to the production of $OH^{\cdot}$ (27) capable of attacking any of the organic substances found in cells (43).

There is considerable evidence that peroxidation of membrane lipids causing loss of integrity of the membrane and inactivation of membrane bound enzymes involves $O_2^{\cdot -}$ (10,20). Exposure of membranes to sources of $O_2^{\cdot -}$ causes peroxidation (16,17,45,46,59).

Bacteria and viruses exposed to $O_2^{\cdot -}$ were rapidly destroyed

(25,26,32). Superoxide radical-induced inactivation of ribonuclease and of lysine *t*RNA ligase was reported (32).

Production of $O_2^{\cdot -}$ may be responsible for the nerve degeneration caused by injecting animals with 6-hydroxydopamine (29), and $O_2^{\cdot -}$ produced by phagocytizing leucocytes may be responsible for the degradation of synovial fluid and inflammation in humans (39).

Reports on beneficial effects of $O_2^{\cdot -}$ such as turnover of cell constituents and drug metabolism (22), as well as involvement in the bactericidal activity of leucocytes (8), however, suggest that $O_2^{\cdot -}$ may also be a useful cell metabolite.

SUPEROXIDE DISMUTASES (SOD)

Fridovich defines superoxide dismutases as metallo-proteins that catalyze with extraordinary catalytic efficiency the reaction:

$$O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2$$

ie, the dismutation of superoxide radical to H_2O_2 and triplet oxygen (20).

Although these proteins were known for many years as cupreins and manganins, their function was not known until 1969 when the involvement of $O_2^{\cdot -}$ in biological oxidations was substantiated (36,37). These proteins have now facilitated insight into areas unsuspected before.

Aspects relevant to this study, such as occurrence, distribution, forms, and biological role of SOD are discussed below.

Occurrence. SOD has been found in a wide range of aerobic organisms. It has been purified and characterized from bovine erythrocytes, equine liver, bovine brain, human brain, human erythrocytes, human and chicken liver, bovine heart, *Neurospora crassa*, *Fusarium oxysporum*,

yeast, pea seeds, spinach leaves, and wheat germ (20). It has also been purified and characterized from prokaryotes, mainly *Escherichia coli* (30) and *Streptococcus mutans* (55).

Various aerobes that were examined were found to contain fairly fixed amounts of SOD. Aerotolerant anaerobes also contain SOD, although slightly less. However, no SOD could be detected in strict anaerobes. This led to the conclusion that SOD is of general occurrence to all aerobic organisms and constitutes the main mechanism of defense against oxygen toxicity (38).

Types and Isozymes. The existence of 3 distinct types of SOD is well documented. They resemble each other in enzymatic activity, but they are different in metal content, structure, and in a number of other properties (20).

(a). Cupro-zinc enzymes contain two Cu^{2+} and two Zn^{2+} atoms per molecule, have a molecular weight around 32,000, and are composed of two subunits of equal size joined by non-covalent bonds.

(b). Manganoenzymes contain two Mg^{2+} atoms per molecule, have a molecular weight of 80,000, and are composed of four equal subunits non-covalently joined.

(c). Ferrienzymes contain two Fe^{3+} atoms per molecule and are characterized by a molecular weight of 39,000 and two equal subunits.

Similar cupro-zinc superoxide dismutases have been isolated from a wide range of eukaryotes, but have not been found in any prokaryotes (20). Cupro-zinc superoxide dismutases are all sensitive to cyanide and this has been used as a quick test to distinguish them from the rest of the superoxide dismutases (9,56).

Isozymes of cupro-zinc SOD have been reported. The enzyme from

cytosol of chicken liver was resolved by disc electrophoresis into a family (four or more) of isozymes (56); also two distinct isozymes of cupro-zinc SOD were isolated and characterized from wheat germ (9). Asada and co-workers have purified to a crystalline state a single form of Cu-Zn SOD from spinach leaves (3); they further found that spinach chloroplasts contain the same enzyme, and that 30-50% of the chloroplastic SOD was bound to the lamellar structure. They could determine little or no activity in mitochondria and no activity at all in peroxisomes. The existence of the same enzyme in both the stroma and lamellae of spinach chloroplasts was recently confirmed by Elstner and Heupel using a different assay (13). Lumsden and Hall, however, reported on the presence of two distinct SOD enzymes in spinach leaves (33,34). One of them, which was a Cu-Zn SOD, occurred in the stroma of isolated chloroplasts. The second one, which was described as a "cyanide-resistant SOD-like activity associated with manganese", occurred in lamellae (33,34). A single Cu-Zn SOD has also been purified from dry seeds of green peas (51).

The manganese type enzyme has been found primarily in prokaryotes. In eukaryotes, it is restricted to mitochondria (20). Thus, mitochondria from chicken liver contain a Mn-SOD similar to that from bacteria; cytosol lacks this enzyme and contains only Cu-Zn enzyme (56). It was soon revealed that mitochondria contain Mn-enzyme in the matrix and Cu-Zn enzyme in the intermembrane space (57). Wheat germ contains a cyanide-resistant SOD which may be mitochondrial manganese enzyme (9). The presence of manganese enzyme in spinach chloroplasts has not been established (13,14).

Iron containing enzyme has been found in the periplasmic space of

Escherichia coli (25). Also, the blue-green alga *Spirulina platensis* has been reported to contain a similar ferrienzyme (33). Ferrienzyme has been reported to be the major form of soluble SOD in the blue-green alga *Plectonema boryanum* (4). There has been no report of ferrienzyme in higher plants.

Isozymes of manganese and iron enzymes, although likely to exist, have not been observed.

Induction. Oxygen has been found to induce SOD activity in organisms like *Escherichia coli* and *Streptococcus faecalis* (25). Thus, a 16-fold increase of SOD in *Streptococcus faecalis* has been achieved by raising the oxygen pressure from 0 to 20 atmospheres. In *Escherichia coli*, a 25-fold increase of SOD was observed when the oxygen pressure increased from 0 to 5 atmospheres. This induced SOD activity was a response to oxygen rather than to pressure, since 20 atmospheres of nitrogen had no effect. The induction was rapid with half of the maximal level reached within 90 minutes after the transfer of the cultures from anaerobic conditions to 20 atmospheres of oxygen.

Induction of SOD by 85% oxygen occurs in rats (11). This has been referred to as a mechanism of acclimatization of rats by 85% O_2 to resist at 100% O_2.

Further investigation of the SOD induction in *Escherichia coli* revealed that the manganese enzyme and not the ferrienzyme was induced by oxygen. The level of the latter could be changed by altering the iron supply in the medium (25). From the same studies, it was shown that the manganese enzyme serves to counter the toxicity of endogenous $O_2^{\cdot -}$. The ferrienzyme functions as a defense against exogenous $O_2^{\cdot -}$.

There is no information yet concerning the induction of the Cu-Zn

enzyme. In tissues rich in Cu-Zn enzymes (*eg.* plant tissue), induction of manganese enzyme can be masked by the Cu-Zn enzyme, if the latter is not inducible.

Biological Role. The deleterious effects that can be brought about by $O_2^{\cdot -}$ have been demonstrated in a variety of systems. Superoxide dismutase provides protection in all these cases and according to Fridovich the function of the enzyme in organisms is to prevent the accumulation of this radical (19,21). Distribution studies of the enzyme are in agreement with this conclusion (38).

Catalases and peroxidases decompose H_2O_2 and prevent production of $OH^{\cdot}$ from $O_2^{\cdot -}$ and H_2O_2, and thus, they are also essential in the overall defense mechanism against O_2 toxicity (21,28). Antioxidants, such as the tocopherols and ascorbate, may function as a second line defense in scavenging these radicals (20).

The possibility that $O_2^{\cdot -}$ may also be utilized by the organism in its defense against undesirable exogenous factors has not been considered, although such cases have already been reported (8,22). Therefore, another possible role for SOD may be to regulate the concentration of a useful metabolite.

Weser and co-workers recently proved that SOD also inhibits reactions induced by O_2^* (58). Singlet oxygen arising from $O_2^{\cdot -}$ may be responsible for the effects attributed to $O_2^{\cdot -}$ (10). This prompted the above researchers to propose that the biological role of SOD may be protection from O_2^* rather than $O_2^{\cdot -}$.

SUPEROXIDE RADICAL AND HERBICIDES

Ashton and Crafts define the mechanism of action of herbicides as "biochemical and biophysical responses of the plant that appear to be associated with the herbicidal action" (6). According to the same authors, "the primary biochemical site of action (lesion) is the single enzyme or metabolic reaction that is affected at a concentration lower than any other enzyme or metabolic reaction, or the first reaction affected at a given low concentration". They further point out that the primary site of action may not be the answer to why a chemical is a herbicide, particularly because it may not be of such an importance to the plant that its inhibition will cause death.

Frear and Shimabukuro concluded that control or death of a weed may result from one or a few key processes or, in contrast, it may require the sum of injury at several sites to attain a threshold level where total injury is irreversible and complete loss of competitive ability or death results (18).

Others have concluded specifically for inhibitors of photosynthesis that, although photosynthesis is the primary site of action, accumulation of toxic intermediates following the blockage of electron transport is the real factor causing death (5,53).

The above statements indicate that effects of herbicides other than the "primary lesion" may be important in causing death. There is the possibility that formation of the toxic oxygen radicals or abolishment of the delicate defense machinery of the plant against these radicals is associated with some of these unexplained effects. There are justifications for believing that this can be possible.

Superoxide radical is easily formed. Electron transport reactions as well as enzymatic reactions can lead to formation of $O_2^{\cdot -}$ radical.

Bipyridylium herbicides become reduced to the bipyridilium radicals by the electron flow of photosynthesis and are then autoxidized to produce $O_2^{\cdot -}$ (15).

Other photosynthesis inhibitors blocking the electron transport may lead to accumulation of the reduced form of intermediate electron carriers which when autoxidized may form $O_2^{\cdot -}$. It is known that reduced ferredoxin (40), flavins and quinones (41) all form $O_2^{\cdot -}$ upon autoxidation. Good proposed that deleterious oxidative processes may be involved in the killing by photosynthesis inhibitors (24). Oorschot recently provided experimental evidence to support a herbicide-induced photooxidation of the chloroplast (44). He observed that exposure of beans to CO_2-free air and treatment with simetone caused similar symptoms. Exposure to CO_2-free nitrogen, however, delayed the onset of injury. The nature of the accumulated oxidant is not known.

Isolated chloroplasts produce $O_2^{\cdot -}$ upon illumination (2), probably by the electron flow driven reduction and subsequent autoxidation of a quinone or ferredoxin. The $O_2^{\cdot -}$ production in illuminated chloroplasts was suppressed when diuron was included in an assay mixture containing epinephrine as $O_2^{\cdot -}$ detector (2). Although this indicates that the electron transport is involved in the production of $O_2^{\cdot -}$, it does not exclude the possibility tnat diuron induces $O_2^{\cdot -}$ production at another site, perhaps the pigment system of PS II. In this case, endogenous scavengers close to that site, *eg.* carotenoids, may efficiently scavenge $O_2^{\cdot -}$ making its detection with epinephrine impossible. In support of

this hypothesis it has been shown that addition of diuron to isolated chloroplasts causes a fast degradation of carotenoids and only two hours later degradation of chlorophyll begins (52).

It is possible that enzymatic reactions, when disrupted by herbicides, proceed through alternate pathways generating $O_2^{\cdot -}$

Superoxide radical may cause pigment degradation and destruction of membranes. Rensen showed that diquat in the light causes lipid peroxidation and decrease of chlorophyll content of *Scenedesmus* (50). Diuron, the antioxidant cysteine, or nitrogen flushing all strongly suppressed the diquat-induced lipid peroxidation providing evidence that oxygen radicals were involved. The decrease of chlorophyll was also lessened by diuron and nitrogen flushing but to a smaller extent.

The defense mechanism against $O_2^{\cdot -}$ is very efficient but no defense is perfect (21). The existence of many forms of superoxide dismutase with differences in subcellular localization and probably function, as well as the inducibility of some forms of the enzyme, may permit the prediction that important differences between species exist. These differences then may be a factor for the selectivity of some herbicides. Frear and Shimabukuro showed that the enzyme glutathione-*S*-transferase catalyzes the detoxification of *s*-triazines in certain plant species. They expressed the view that a major factor responsible for herbicide selectivity may be a difference in the activities, specificities, and distribution of key enzyme systems (18).

Involvement of $O_2^{\cdot -}$ or its decay products (O_2^{*}, $OH^{\cdot}$) in biological transformations of herbicides is also possible. Superoxide radical is both a reducing and an oxidizing agent (28), and the $OH^{\cdot}$ is one of the

most potent oxidants (43). Evidence for involvement of $O_2^{\cdot -}$ in sulfoxidation of thioethers was obtained with ethionamide (49). Hydroxylation of aromatic compounds catalyzed by enzymes from *Aspergillus niger* was shown to involve $O_2^{\cdot -}$ (48). *s*-Triazine herbicides were dealkylated by an OH·-generating system to products identical to those isolated from various biological systems (47).

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SECTION ONE

OCCURRENCE OF SUPEROXIDE DISMUTASES IN HIGHER PLANTS

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ABSTRACT

The photochemical assay for superoxide dismutase consisting of methionine/riboflavin/*p*-nitro blue tetrazolium chloride was adjusted for quantitation of the enzyme in crude extracts. Shoots, roots, and seeds of corn (*Zea mays* L., cv. Michigan 500), oats (*Avena sativa* L., cv. Au Sable), and peas (*Pisum sativum* L., cv. Wando) were analyzed for their superoxide dismutase content. The enzyme is not organ specific. It is present in the shoots, roots and seeds of all three species. Quantitative and qualitative differences exist between species, while quantitative differences exist between organs within a species. On a per dry weight basis, shoots contain more enzyme than roots. Both shoots and roots contain considerably more enzyme than seeds. In seeds, the enzyme is present in both the embryo and the storage tissue. Electrophoresis indicated multiple forms of the enzyme. From the three species, a total of 10 distinct enzyme bands was obtained on gels. Corn contained seven of the bands, oats only three. Peas contained one of the corn, and two of the oat enzymes. Nine of the enzyme bands were eliminated with cyanide treatment suggesting that they may be cupro-zinc enzymes, whereas one was cyanide resistant and may be a manganese enzyme. Some of the superoxide dismutases were primarily found in mitochondria or chloroplasts.

Differences and similarities in SOD pattern of the various organs may be explained on basis of the above organelle localization of the SOD forms.

INTRODUCTION

Superoxide dismutases (EC 1.14.1.1) are metalloproteins catalyzing the reaction:

$$2O_2^{\cdot -} + 2H^+ \rightarrow O_2 + H_2O_2$$

i.e. the dismutation of the superoxide free radical ($O_2^{\cdot -}$) to molecular oxygen and hydrogen peroxide. This enzymatic activity was first described by McCord and Fridovich with a cupro-zinc protein (erythrocuprein) from bovine erythrocytes (5). Similar cupro-zinc proteins with SOD[1] activity were subsequently isolated from various eukariotic sources (8). Manganese-containing proteins with SOD activity were later found in prokaryotes and in mitochondria of eukaryotes (8). Iron proteins from *Escherichia coli* (10) and algae (13) were recently shown to possess SOD activity.

Cupro-zinc SOD has already been isolated from tissues of higher plants: pea seeds (17), spinach leaves (1,13), and wheat germ (4). Isozymes have also been reported for wheat and spinach cupro-zinc enzyme (4,13). A cyanide resistant enzyme from wheat germ has been described as manganese-SOD (4). Occurrence of manganese enzyme in spinach chloroplasts has been reported (13), but is not certain (7). There is no report of iron-enzyme in higher plants.

There is considerable evidence that $O_2^{\cdot -}$ may be an indigenous intermediate of metabolic processes which may initiate deteriorative effects

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[1] Abbreviations: SOD: superoxide dismutase; NBT: *p*-nitro blue tetrazolium chloride.

in biological systems; SOD is believed to constitute an important part of the defense mechanism against such deleterious action of $O_2^{\cdot -}$ (9,11). A comparative study of the SOD distribution in plants has not been conducted thus far. In view of the above evidence, however, distribution of the enzyme within the plant and intraspecific differences may be of interest, and were the objective of the present study.

MATERIALS AND METHODS

Plant Material. Seeds and seedlings of corn (*Zea mays* L., cv. Michigan 500), oats (*Avena sativa* L., cv. Au Sable), and peas (*Pisum sativum* L., cv. Wando) were utilized.

Seeds were treated with 0.3% (w/v) captan 80W for 5 min and germinated in plastic flats containing 3 cm turface (Turf Supplies Co., Taylor, Michigan) at the bottom and 3 cm vermiculite at the top. The seedlings were grown for 10 days in a growth chamber under 26 C, 25000 lux of light, and a 12-hr photoperiod. Half-strength Hoagland's solution was provided once after six days, and distilled water throughout growth as needed.

Embryos and scutella were excised from seeds which had been allowed to imbibe at 25 C for 10 hr and were rinsed three times with distilled water. Endosperm was obtained from dry corn and oat seeds from which the embryo-bearing end had been removed. Endosperm was drilled from the cut surface by using a size 60 wire bit fitted on a battery operated drill. The hulls of oat seeds were removed, unless the seeds were to be germinated.

Preparation of Extracts. The tissues were thoroughly ground with a cold mortar and pestle in an ice bath, until no fibrous residue could be

seen. The grinding medium consisted of 0.1 M potassium phosphate and 0.1 mM EDTA, pH 7.8, plus homogenizing glass beads. The homogenate was centrifuged twice at 13000*g* for 10 min in a Sorvall RC2-B refrigerated centrifuge at 0 to 5 C. The supernatant, hereafter referred to as crude SOD extract, was used for electrophoresis and for determination of the SOD content in the tissue.

Different volumes of buffer were used for enzyme extraction, depending on the tissue and whether the extract was to be used for electrophoresis or determination of the SOD content (Table 1). For determination

Table 1. *Volumes of Buffer Homogenized with Plant Material for SOD Extraction.*

Plant Part	Species	Extracts for Electrophoresis	Extracts for SOD Determination
Leaves, Roots, Shoots	All species	4 ml/g fresh wt	10 ml/g fresh wt
Dry seeds	Corn	6 ml/g seed wt	15 ml/10 seeds
Dry seeds	Oats	6 ml/g seed wt	10 ml/10 seeds
Dry seeds	Peas	6 ml/g seed wt	30 ml/10 seeds
Soaked seeds	All species	4 ml/g wet wt	same as dry seeds

of the SOD content in the tissue, the volumes necessary for quantitative extraction of the enzyme were predetermined.

The water-soluble protein content of all crude SOD extracts was determined by the method of Lowry *et al.* (12), after precipitation with 10% trichloroacetic acid. Bovine serum albumin was used as a standard. Extracts were diluted 10 times for SOD assays. To the extracts for electrophoresis 1 M sucrose was added.

Isolation of Organelles. Mitochondria were isolated by the method of Bonner (5) and chloroplasts by the method of Smillie (19) from shoots of 7-day-old seedlings grown in the dark and light, respectively. The organelle pellets were washed twice by resuspending in wash media and recentrifuging. The washed pellets were finally resuspended in small volumes of 0.05 M potassium phosphate, pH 7.8, and sonicated for 3 min. After dialysis against changes of the same buffer and centrifugation, the supernatants were used for electrophoresis.

Electrophoresis. Polyacrylamide gel electrophoresis of the crude SOD extracts was performed according to Davies (6). Each extract was applied to the gels at various concentrations ranging from 50 to 300 µg of protein, or 5 to 20 units of enzyme. A current of 1 mA/gel was applied during migration of the bromphenol blue marker on the spacer gel. Electrophoresis was continued under 2 mA/gel until the marker had migrated approximately 10 cm on the resolving gel.

The superoxide dismutases were localized by the photochemical procedure of Beauchamp and Fridovich (3) as modified by Weisiger and Fridovich (20). The gels were first soaked in a solution of NBT, then immersed in a solution of riboflavin and tetramethylenediamine, and illuminated in tubes containing potassium phosphate and EDTA at pH 7.8. The stained gels were photographed on Kodak Panatomic X 120 film using a 25 A red filter, or scanned with a Gilford model 220 gel densitometer.

Enzyme Assay. All extracts were assayed for SOD activity photochemically, using the assay system consisting of methionine, riboflavin, and NBT (3). The photochemical procedure was chosen as being independent of other enzymes and proteins, and therefore more reliable in the case of crude extracts than enzymatic assay systems (15).

The original assay described by Beauchamp and Fridovich (3) was modified. The reaction mixture was composed of 1.3 μM riboflavin, 13 mM methionine, 63 μM NBT, 0.05 M sodium carbonate pH 10.2, and the appropriate volume of extract. Distilled water was added to bring to the final volume of 3 ml. The mixtures were illuminated in glass test tubes selected for uniform thickness and color. Identical solutions that were not illuminated served as blanks.

The apparatus devised for exposing the tubes to light was composed of a rotating test tube holder (Rayonet MGR-100, Southern New England UV Co.) immersed in water in a cylindrical glass container thermostated at 25 C by a Forma 2095 refrigerated and heated water circulator. A circular fluorescent lamp (Sylvania, FC 12 T 10-CW-RS) was attached on the outside wall of the water-bath and the entire assembly was fitted in a box lined with aluminum foil. The reaction was initiated and terminated by turning on and off the light. There was no detectable amount of the reaction occurring under room light during preparation of the solutions and spectrophotometric measurements.

The initial rate of the reaction was determined as increase of absorbance at 560 nm. Under the described conditions, the initial rate of the reaction in absence of SOD was 0.100 absorbance units/5 min and was linear up to 15 min. In the presence of SOD the reaction was inhibited and the amount of inhibition was used to quantitate the enzyme. Each extract was assayed twice and the results varied less than ±0.005 absorbance units/5 min.

Dialysis and gel filtration (Sephadex G-50) of crude SOD extracts indicated no significant interference of small molecules with the assay.

RESULTS AND DISCUSSION

Enzyme Quantitation. The assay system used in this study utilizes the photochemical production of $O_2^{\cdot -}$ from methionine, riboflavin, and oxygen, and the subsequent reduction of NBT to blue formazan. Superoxide dismutase, by scavenging the $O_2^{\cdot -}$, inhibits the photoreduction of NBT. Beauchamp and Fridovich defined one unit of SOD as the amount that inhibits the NBT photoreduction by 50% and quantitated the enzyme on basis of the % inhibition it causes (3). Percent inhibition and SOD concentration were not linear, however. Asada *et al.* (2), using crystalline spinach SOD and the xanthine/xanthine oxidase assay system (15), established a linear relationship between the amount of the enzyme and the V/v ratio (V, v represent the rate of the assay reaction in absence and in presence of SOD, respectively). This linear relationship was also observed with crude corn SOD and the photochemical assay system used in the present study (Fig. 1). Asada *et al.* also derived from the kinetics of the assay reaction the equation:

$$V/v = 1 + K'[SOD] \quad (I),$$

which explains the linearity obtained. Asada's plot was further tested in this study with a variety of crude extracts, and the above equation was properly adapted for convenient and accurate quantitation of SOD.

The linearity between V/v ratio and SOD concentration was maintained throughout a wide range of enzyme concentrations (Table 2). The upper limit of the linear portion varied with the various extracts in the range of 67 to 84% inhibition. The linear correlation was high, as indicated by correlation coefficients close to unity. The maximum inhibition of the NBT photoreduction that could be achieved by the crude SOD used was 93 to 97%. The reason linearity is not maintained up to this maximum

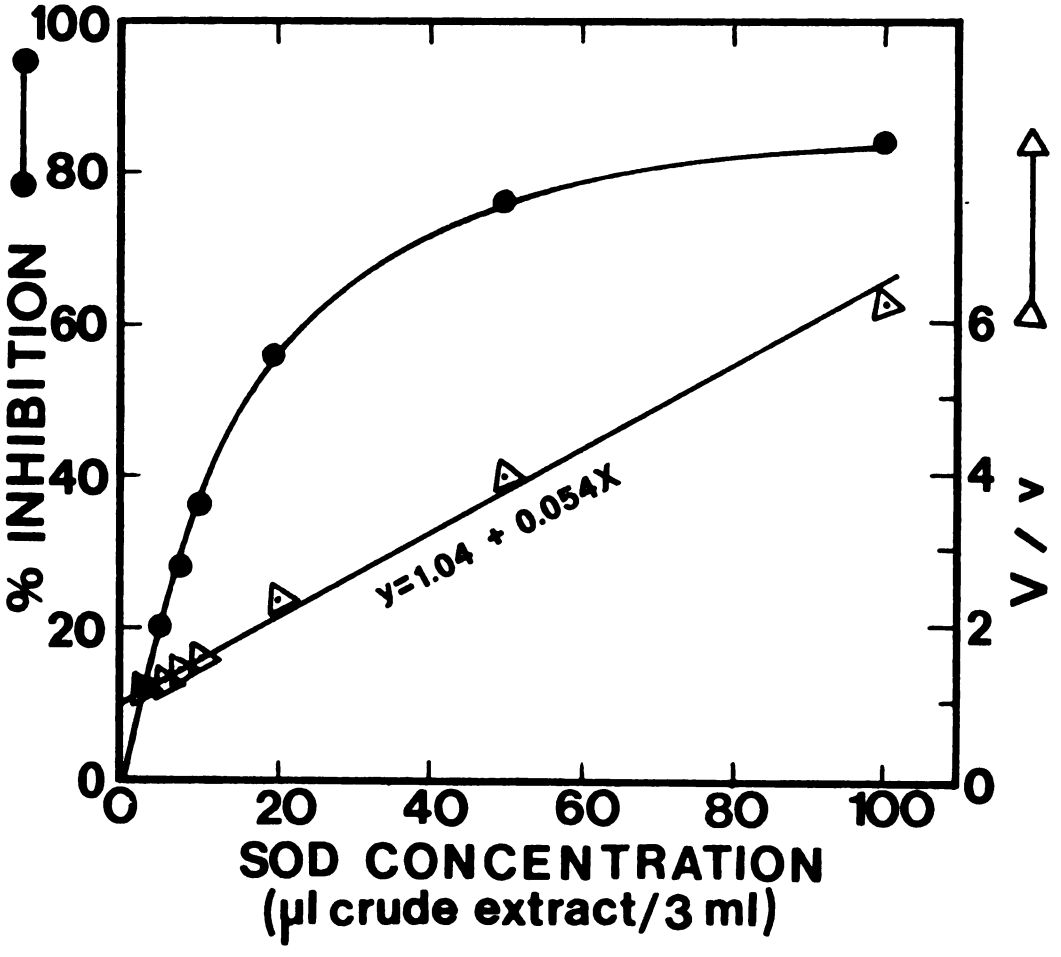

Fig. 1. Inhibition of the NBT photoreduction *versus* concentration of crude SOD from corn seeds. Crude extract prepared from 10 corn seeds homogenized with 15 ml of 0.1 M potassium phosphate, pH 7.8, was diluted various times with the same buffer and each dilution was used at 100 µl/3 ml assay solution to give the indicated SOD concentrations. Percent inhibition is not linear with SOD concentration (●—●). Linearity ($r = 0.995$) is obtained by plotting V/v ratio against SOD concentration (▷—▷), V and v representing the rate of the reaction in absence and presence of enzyme, respectively.

is not known.

Table 2. *Relationship of V/v with SOD Concentration in Crude Extracts.*

Crude extracts were diluted and assayed to obtain the V/v *versus* [SOD] curve as in Figure 1. The apparent linear portion was determined between the lower limit (always 0%) and the upper limit (the maximum % inhibition up to which the linear relationship was attained). Regression analysis of the X,y pairs within the linear portion (at least 6 pairs) was conducted to obtain the line equation and the correlation coefficient.

Extract Species	Part	Upper Limit (% inhibition)	Line Equation	Correlation Coefficient (r)
Corn	Seeds	84	y=1.04 + 0.054X	0.995
Oats	Seeds	68	y=1.02 + 0.140X	0.994
Peas	Seeds	67	y=1.08 + 0.047X	0.998
Oats	Roots	69	y=1.09 + 0.045X	0.997
Peas	Roots	67	y=1.06 + 0.041X	0.986
Oats	Shoots	73	y=0.96 + 0.317X	0.997
Peas	Shoots	70	y=0.96 + 0.058X	0.994

The intercept of all lines obtained from the various extracts (Table 2) approximates unity, this being in agreement with equation (I) of Asada *et al.* The slope (b) of the lines, corresponding to the constant K' in equation (I), varies with the various extracts as they are different in SOD content, and fits the equation:

$$y - 1 = bX \quad \text{or} \quad (V/v) - 1 = b\,[SOD] \qquad (II)$$

One unit of SOD has been defined as the amount of enzyme that causes 50% inhibition of the assay reaction (3,15). The relationship between V/v,b[SOD], and % inhibition of the NBT photoreduction is illustrated in Table 3. Apparently, V/v and b[SOD] are linearly related. One SOD unit

Table 3. *Relationship between V/v, b[SOD], and % Inhibition.*

V/v[1]	b[SOD][2]	% Inhibition[3]
1	0	0.0
2	1	50.0
3	2	66.6
4	3	75.0

[1] V = rate of the NBT photoreduction in absence of SOD (uninhibited reaction); v = rate of the NBT photoreduction in presence of SOD (inhibited reaction).

[2] b[SOD] = (V/v) - 1 (see text).

[3] % Inhibition = [(V-v)/V] 100.

can be defined as the amount that either causes 50% inhibition or gives a product b[SOD] equal to unity. If the latter definition is adopted, advantage can be taken of the more linear curve of Asada *et al.*, and further, the enzyme can be directly quantitated from the V/v ratio according to the equation:

SOD units/ml = [(V/v) - 1] (dilution factor) (III).

Superoxide Dismutase Content of Plant Organs and Tissues. Botn enzyme assays and electrophoresis established that extractable SOD was present in seeds and various seed parts, roots, leaves and shoots of all three species. Electrophoresis also indicated that SOD activity is composed of a number of distinct bands.

Within a species, the same active bands with apparent quantitative differences were obtained from seeds, roots and shoots (Fig. 2). From the different species, a different number of bands were obtained. In corn, six bands were visually observed, in contrast to only three in oats and

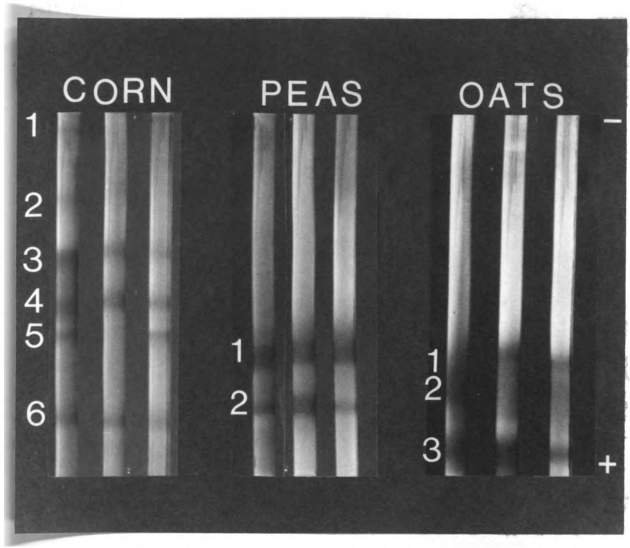

Fig. 2. Superoxide dismutases of corn, peas, and oats. Extracts from seeds (left gel in each group), leaves (middle gels), and roots (right gels) were applied for each species. The enzymes appeared as achromatic bands on the blue-stained gels. The negative image used here, in which the bands appear black, shows their positions more clearly. The corn SOD was resolved into at least six visually observable bands, whereas that of peas was resolved into two, and that of oats into three major bands (numbers on the left).

two in peas. Only bands of high relative mobility were obtained from oats and peas, whereas corn also contained bands of intermediate and low relative mobilities (Fig. 2). High concentrations of oat and pea extracts did not produce additional bands, even though the observed bands overlapped. The corn bands of intermediate and low relative mobility were also observed at considerably lower concentrations of extract. It may be concluded that the above differences in band numbers are real and not due to concentration effect. The same banding patterns were obtained under various extraction conditions, such as pH 6 to 8, potassium phosphate concentrations 0.005 to 0.4 M, and presence or absence of 0.1 mM EDTA. Dialysis and ammonium sulfate or acetone fractionation of the crude extracts did not alter the banding patterns. However, freeze-thawing and aging in the cold resulted in additional minor bands.

In seeds, SOD was present in both the embryo and the storage tissue (Table 4). In this study the units of enzyme per seed were determined photochemically from extracts of whole seeds, embryos and storage tissue. It was also established that during imbibition of seeds from 0 to 25 hr, the total activity of enzyme per seed as well as the activity per embryo and storage tissue were not altered. Evidence is provided that a fixed amount of SOD is always present in the embryo and the storage tissue and no *de novo* synthesis or activation needs to occur upon imbibition.

Most of the enzyme in oat and pea seeds was found in the storage tissue; the oat endosperm containing 62% and the pea cotyledons 82% of the total activity (Table 4). By contrast, in the corn seed, more enzyme was found in the embryo than in the storage tissue. This may be attributed to the fact that the corn embryo (including the scutellum) constitutes a considerably larger portion of the whole seed than the oat or pea embryo.

Table 4. *Superoxide Dismutase Content of Seeds and Seed Parts.*

Each value is the average of 8 non-significantly different values obtained from seeds soaked in water for 0, 5, 10, and 25 hr (2 samples each).

Species	Whole Seed[1]	Embryo[2]	Storage Tissue[3]
		SOD units/seed	
Corn	40.7	26.7	17.7
Oats	20.5	8.0	12.7
Peas	792.2	142.0	652.0
		SOD units/g dry wt	
Corn	194	1057	96
Oats	884	4819	565
Peas	4681	39776	3739

[1] Purification of the enzyme from pea seeds did not indicate any interference of impurities with the crude extract assays (Table 7).

[2] The scutellum of corn and oats is included in the embryo.

[3] Storage tissue: the remaining part of the seed after removal of the embryo.

On a per dry weight basis, the embryo of all three species contains approximately 10 times more enzyme than the storage tissue. Therefore, the embryo is richer in SOD regardless of containing more or less enzyme than the storage tissue on a per seed basis.

The occurrence of the enzyme in both the embryo and the storage tissue was further confirmed by electrophoresis (Fig. 3). The active bands were obtained from the embryo of all three species. Scutella excised from the corn seeds contained the same SOD bands as the corn embryo. No attempt was made to demonstrate the presence of enzyme in scutella of

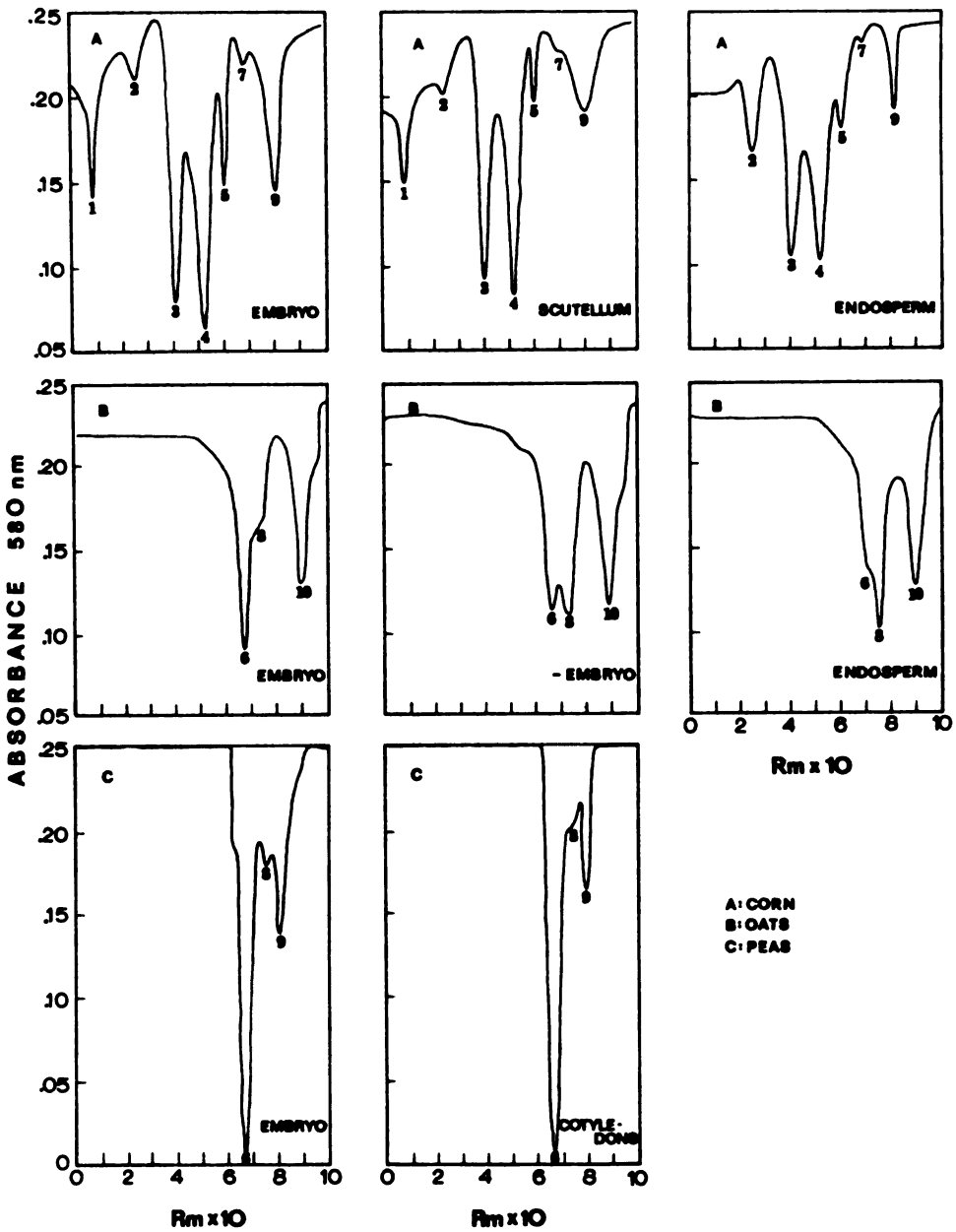

Fig. 3. Densitometer tracings of gels showing the seed superoxide dismutases. Extracts from the indicated seed parts were used at 10 µl per gel, except for the extracts from oat and corn endosperm, which were used at 50 and 100 µl respectively. The enzymes, which appeared as achromatic bands on blue-stained gels, are shown here as negative peaks, and are numbered in order of increasing relative mobility. Rm: relative mobility (migration distance of band/migration distance of marker).

oats. Endosperm drilled out of dry corn and oat seeds, and cotyledons excised from dry pea seeds contained the same SOD bands as their embryos. Quantitative differences between the SOD bands will be discussed in the next section. The occurrence of the enzyme in all seed parts, and more importantly in the endosperm is of particular interest. If the proposed biological role for this enzyme (9,11) is accepted, then its distribution may demonstrate that $O_2^{\cdot -}$ can be formed in seeds, and may be involved in aging and reduced viability of seeds, as suggested by Pammenter *et al.* (16).

The enzyme was found in both shoots and roots of the seedlings (Table 5). In this study the seedlings were grown up to the stage that the shoots

Table 5. *Superoxide Dismutase Content of 10-day-old Seedlings*[1].

Each value is the average of two samples extracted and assayed independently.

Species	Organ	Dry wt (mg/plant)	Protein (mg/g dry wt)	Units/ plant	Units/mg dry wt	Units/mg protein
Corn	Shoot	143	57.5	579.1	4.0	69.5
Corn	Root	146	20.8	124.1	0.9	41.3
Oats	Shoot	13	132.8	89.7	6.9	51.9
Oats	Root	13	32.2	31.8	2.5	76.2
Peas	Shoot	67	208.4	304.8	4.6	22.0
Peas	Root	62	51.2	127.1	2.0	20.0
LSD at 0.05		...	18.6	206.5	1.7	19.6

[1] The enzyme has been purified from pea seeds and corn seedlings and interferences with the assay are negligible for crude extracts from seedlings of the three species (see section two).

and roots of each species accumulated the same amount of dry matter at harvest. On a per dry weight basis, the shoots of all three species contain more protein and SOD than the roots. There is no statistically significant difference between SOD content of shoots and roots of peas on a per protein basis. The most important difference among the three species is that the least SOD-active protein is present in roots for corn, in shoots for oats, and in both shoots and roots for peas.

The enzyme of roots is compared with that of leaves in Figure 4. Differences in quantities of enzyme on a per protein basis can be judged from the area below the bands. The two grass species, particularly corn, contain more enzyme in the roots. Peas contain more enzyme in the leaves. Simon *et al.* (18) also found more enzyme in leaves than in roots of beans.

Multiple Forms of SOD. Ten distinct SOD bands were obtained from extracts of the three plant species in a single electrophoretic run. They are numbered in order of increasing relative mobility in Figures 3 and 4, and summarized in Table 6.

It has also been concluded from their relative mobilities that SOD 6 and SOD 8 are common to peas and oats, while SOD 9 is common to peas and corn. In two different plant species, therefore, some SOD forms may be identical, and some may be different.

The above 10 bands may correspond to different proteins with SOD activity, or different isozymes of a single protein, or a combination of both. Proteins containing copper-zinc, manganese, or iron have already been described to function as superoxide dismutases (8). Isozymes of cupro-zinc SOD have also been reported (4,13).

Cupro-zinc enzymes are sensitive to cyanide, whereas manganoenzymes are resistant to cyanide and sensitive to treatment with a chloroform-

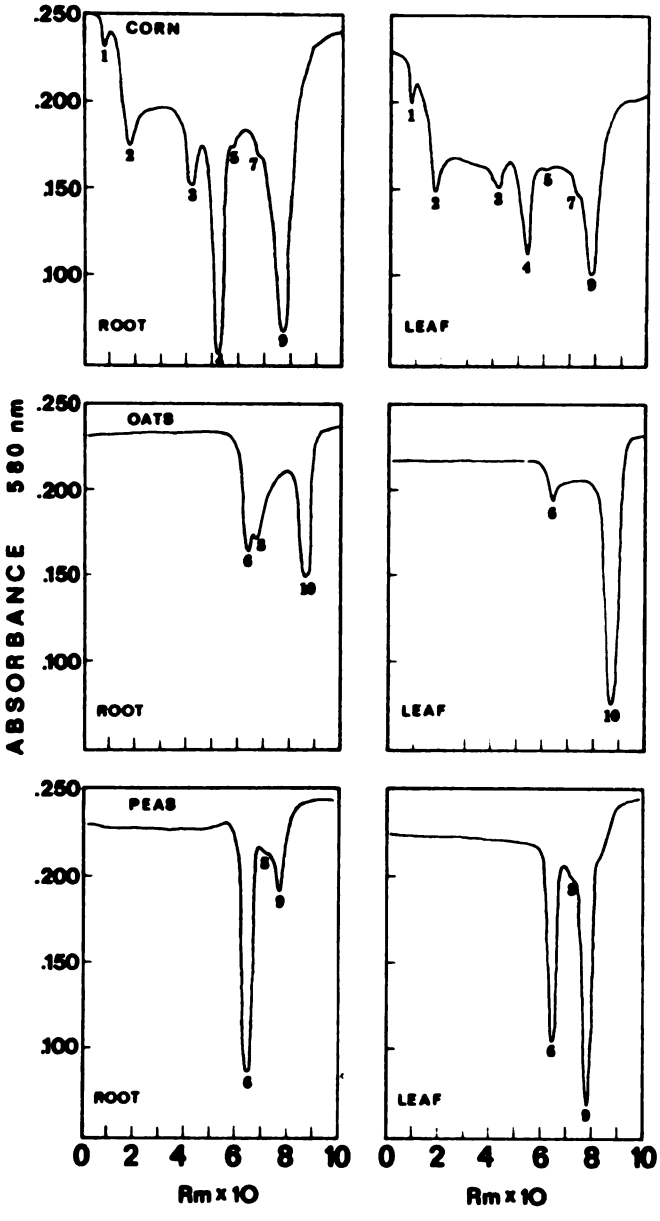

Fig. 4. Densitometer tracings of gels showing the seedling superoxide dismutases. Extracts from roots and leaves were used at 50 µg of protein per gel, except for the extract from corn leaves, which was used at 100 µg of protein. The enzymes, which appeared as achromatic bands on blue-stained gels, are shown here as negative peaks, and are numbered in order of increasing relative mobility. Rm: relative mobility (see Fig. 3).

ethanol mixture, thus allowing their discrimination on gels (4,20). The only enzyme that was found resistant to cyanide was SOD 5 of corn (Fig. 5). It seems likely, therefore, that SOD 5 is a manganoenzyme, all the remaining being isozymes of cupro-zinc SOD.

Table 6. *Relative Mobility and Occurrence of Various Superoxide Dismutases in Corn, Oats, and Peas.*

SOD Band[1]	Relative Mobility[2]	Occurrence
1	0.08 - 0.09	corn
2	0.18 - 0.25	corn
3	0.41 - 0.42	corn
4	0.52 - 0.54	corn
5	0.61 - 0.62	corn
6	0.65 - 0.67	corn,peas
7	0.69 - 0.70	corn
8	0.74 - 0.75	oats,peas
9	0.78 - 0.80	corn,peas
10	0.87 - 0.91	oats

[1] Numbers correspond to the SOD bands as in Figures 3 and 4.

[2] Defined as in Figure 3.

The same SOD enzymes were obtained from the various organs or tissues within a species (Figs. 3 and 4), and this supports the reproducibility of the bands. However, quantitative differences of the bands among organs or tissues are evident from the "band area".

The following differences between seed parts can be seen in Figure 3. Corn endosperm does not contain any detectable amount of SOD 1, and compared to the other seed parts is lower in SOD 3 and 4, but higher in SOD 2.

A similar situation occurs in oat seeds; the endosperm is low in SOD 6 and high in SOD 8, exactly the opposite of the embryo. The remaining part of the oat seed after the removal of the embryo, which includes aleurone and endosperm, is high in both SOD 6 and 8 suggesting that the aleurone must be similar to the embryo. There is no significant difference between pea embryo and cotyledons.

The localization of some of the SOD enzymes in organelles was exploted as a possible explanation of the above differences. Mitochondria were isolated from etiolated seedlings and the extracts from them were subjected to electrophoresis at various concentrations. Results are presented in Figure 5. The SOD enzymes in which endosperm is rich (SOD 2 and 8) were not detected in mitochondrial extracts. The SOD enzymes in which embryo, aleurone, or cotyledons are rich (SOD 1,3,4, and 6) were major bands in mitochondria. Therefore, a possible explanation for the above differences may be the fact that SOD 1,3,4, and 6 are primarily, but not exclusively, localized in mitochondria. Embryo, scutellum, aleurone, and cotyledon tissue contains a large number of mitochondria, in contrast to the endosperm.

Major similarities between the SOD pattern of the roots (Fig. 4) and that of the embryos (Fig. 3) are in agreement with the localization of certain SOD forms in mitochondria. The leaves, compared to both seeds and roots, present the major difference that SOD 9, or 10, is the major band in them. Chloroplasts isolated from leaves of young seedlings contained more of these two enzymes than mitochondria (Fig. 5). This difference in SOD pattern between mitochondria and chloroplasts may be the reason for the difference in SOD pattern between leaves and roots.

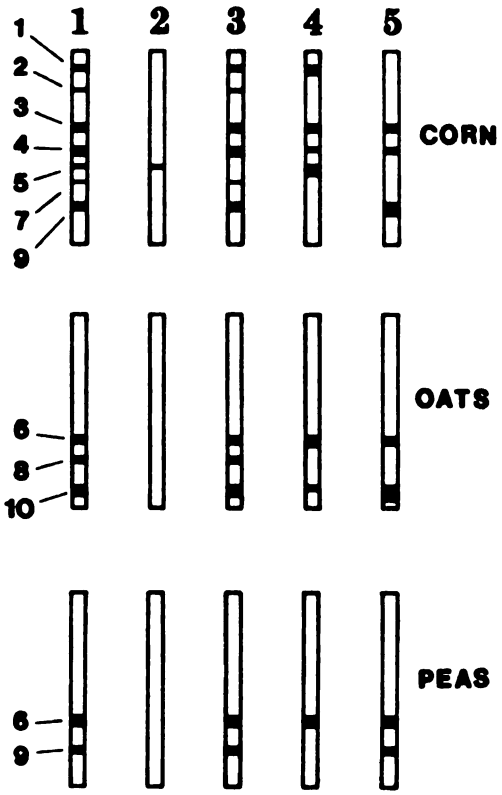

Fig. 5. Sensitivity to cyanide and chloroform-ethanol treatments, and organelle specificity of the superoxide dismutases. Treatments (numbers at the top) are as follows: (1) leaf extract, (2) leaf extract and cyanide treatment, (3) leaf extract and chloroform-ethanol treatment, (4) mitochondrial extract, (5) chloroplastic extract. Potassium cyanide at 1 mM was included in the staining solutions for treatment (2). For treatment (3), the extracts were mixed with chloroform (0.15 volume) and ethanol (0.25 volume), and centrifuged before applied on the gels. The enzyme bands are numbered (on the left) as in Figures 3 and 4.

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SECTION TWO

PURIFICATION AND PROPERTIES OF SUPEROXIDE DISMUTASE FROM HIGHER PLANTS

PURIFICATION AND PROPERTIES OF SUPEROXIDE DISMUTASE FROM HIGHER PLANTS

ABSTRACT

Superoxide dismutase was purified to a maximum specific activity from pea (*Pisum sativum* L., cv. Wando) seeds, and partially purified from corn (*Zea mays* L., cv. Michigan 500) seedlings. The purified pea enzyme eluting as a single peak from gel exclusion chromatography columns contained the three electrophoretically distinct bands of superoxide dismutase characterizing the crude extract. The purified corn enzyme eluted as the same peak as the pea enzyme, and contained five of the seven active bands found in the crude extract. The similar molecular weights and the cyanide sensitivities of these bands indicated that they are probably isozymes of a cupro-zinc superoxide dismutase. One of the remaining corn bands was shown to be a peroxidase.

It was estimated that in 10-day-old seedlings of corn, peas and oats (*Avena sativa* L., cv. Au Sable) 0.9 to 3.1% of the water soluble protein is accounted for by superoxide dismutase. Interferences with the enzyme assays of crude extracts were examined. Peroxidase at high concentrations interferes with the assay. Peroxidase was easily inactivated by heat, whereas superoxide dismutase was relatively heat-stable. In crude extracts from seedlings of all three species the interference by peroxidase was negligible. A 3-fold increase of specific activity

(units/mg water-soluble protein) was observed during germination of oats, and a 40% increase during greening and hook opening of the pea plumule.

INTRODUCTION

In a previous study, considerable amounts of SOD[1] were found to be present in roots, shoots, seeds and seed parts of oats, corn and peas (see section one). Electrophoresis indicated multiple forms of the enzyme. Significant differences in quantity and forms of the enzyme were observed between species and between organs within a species. The objective of this study was to further substantiate the occurrence of the enzyme in higher plants and to examine the observed differences between species. For this purpose the enzyme was purified. Impurities interfering with the assays of crude extracts, and changes of SOD specific activity during seedling growth were also studied.

MATERIALS AND METHODS

Enzyme Purification. Unless otherwise stated all operations were performed at 0 to 4 C. Dry pea seeds (*Pisum sativum* L., cv. Wando) were soaked in distilled water for about 15 hr. The resulting 1650 g wet weight was crushed with an electric mortar and pestle, and homogenized with 1 liter of 0.1 M K_2HPO_4 in a Waring blender. After stirring, the slurry was filtered squeezed through six layers of cheese-cloth. The

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[1] Abbreviations: SOD: superoxide dismutase; NBT: *p*-nitro blue tetrazolium chloride.

filtrate was centrifuged twice at 13000*g* for 30 min in a Sorvall RC2-B refrigerated centrifuge.

The supernatant was subjected to the Tsuchihashi (chloroform-ethanol) treatment essentially as described by Weisiger and Fridovich (13). It was established that none of the pea SOD enzymes is inactivated by this treatment. The supernatant was mixed with 0.25 volume of ethanol and 0.15 volume of chloroform and stirred for 15 min. It was then clarified by centrifugation at 13000*g* for 15 min. Chloroform that was separated out during centrifugation was removed by suction. The supernatant was decanted, solid K_2HPO_4 (20 g/liter) was added, and the two phases were separated after 30 min. The less dense, ethanol rich phase was collected, chilled and centrifuged at -15 C. Additional chloroform separating out during centrifugation was removed by suction, and the ethanolic phase was decanted.

Chilled acetone (0.5 volume) was added to the ethanolic phase while stirring. The precipitate was removed by centrifugation. Additional acetone (1.5 volume) was added to the supernatant, and the second precipitate was collected and redissolved in a minimal volume of 0.05 M potassium phosphate buffer pH 7.8. Solid $(NH_4)_2SO_4$ was added to the supernatant to bring it to 70% saturation. After 1 hr, the second precipitate was collected, and resuspended in and dialyzed against 0.1 M KCl, 0.005 M potassium phosphate, and 0.01 mM EDTA, pH 7.8.

The enzyme was further purified by gel exclusion chromatography. It was first applied on a Sephadex G-100 column (2 x 90 cm) equilibrated with the dialysis buffer. The void volume of the column was 74.5 ml and the flow rate 0.2 ml/min. This column was calibrated with proteins of known mol wt (11). Fractions with a specific activity greater than

300 units/mg protein were pooled and salted out from 70% saturated $(NH_4)_2SO_4$ solution. The precipitate was collected by centrifugation, dissolved in a small volume of distilled water and dialyzed against the eluting buffer. It was then applied on a column (1 x 60 cm) of Biogel P-30 equilibrated with the same buffer. Some impurities of higher mol wt were removed by this column. Fractions whose specific activity exceeded 700 units/mg were pooled and concentrated as above. The enzyme was rechromatographed on the same Biogel P-30 column. Impurities of slightly lower mol wt were partially separated from the enzyme. Only the two fractions of maximum specific activity (around 2000 units/mg) were pooled this time. Additional impurities were removed by fractionation with chilled acetone. The most active fraction was obtained between 1.5 to 2.0 volumes of acetone. The precipitate from the last fraction was redissolved in 0.05 M potassium phosphate, pH 7.8.

Enzyme from corn (*Zea mays* L., cv. Michigan 500) seedlings was partially purified. The seeds were treated with 0.3% (w/v) captan 80W for 5 min, and germinated for 7 days on moist paper towels in the dark at room temperature. The seed remnants were removed, and the seedlings (182 g) were rinsed with distilled water and cut into 1-cm sections with a stainless steel razor blade. The tissue was homogenized with 400 ml of 0.1 M potassium phosphate and 0.1 mM EDTA, pH 7.8, in a Waring blender. After 1 hr in the cold and occasional stirring, the homogenate was filtered squeezed through four layers of cheese-cloth. The filtrate was centrifuged twice at 13000*g* for 20 min. This supernatant was not subjected to the Tsuchihashi treatment, since it has been shown that this treatment inactivates one of the corn enzymes (Fig. 5). The enzyme was successively purified by acetone fractionation (0.75 to 2.0 volumes),

$(NH_4)_2SO_4$ fractionation (45 to 95% saturation) and chromatography on Sephadex G-100 in a manner similar to the pea enzyme.

Protein concentration was determined throughout according to Lowry *et al.* (9), using bovine serum albumin as a standard. Enzyme assays and electrophoresis were performed as previous described.

Interference Experiments. Crude extracts used in these experiments were prepared from shoots and roots of 10-day-old seedlings as previously described. Catalase (beef liver, Nutritional Biochemicals Corporation) proved to be contaminated with SOD, and required purification by gel exclusion chromatography. Horse radish peroxidase (A grade, Calbiochem) was not contaminated with SOD. Peroxidase activity was determined with the guaiacol test (12). Gels were stained for peroxidase localization according to Hart *et al.* (7). Crude extracts were heated in portions of 1.0 ml in Korex 17-ml centrifuge tubes kept in a boiling water bath (95 to 97 C) for 0.5 to 20 min. Precipitated protein was removed by centrifugation.

Changes of SOD Specific Activity. The changes in specific activity during greening were studied with excised oat (*Avena sativa* L., cv. Au Sable) and pea plumules. Seeds, treated with 0.3% (w/v) captan 80W for 5 min, were soaked in distilled water for 10 hr and planted 2-cm deep in 10 x 14.5 cm styrofoam pots containing vermiculite. Seedlings were grown in a growth chamber in complete darkness at 25 C for 7 days. Plumules were excised by cross-secting with a stainless steel razor blade above the first node from the apex. Uniform plumules were transferred into 9-cm petri dishes (10 plumules/dish) containing 10 ml of 1% sucrose solution. Three replicate dishes for each treatment were prepared. Half of the dishes were placed in a growth chamber with all lights off.

The other half were placed in another growth chamber with only the fluorescent lights on (10 and 7 $\mu W/cm^2$ blue and red light, respectively). The temperature in both chambers was maintained at 25 C. At various time intervals, petri dishes were removed from the chambers, the plumules were rinsed with distilled water, blotted and weighed. Extracts were prepared and the assays were performed immediately. All operations with etiolated plumules were conducted under dim green safelights.

The changes of specific activity during germination were studied with oat and pea seedlings. Seeds were treated as in the previous test, and placed on two layers of filter paper in 9-cm petri dishes (15 seeds/dish). Five ml of distilled water was added to each dish, and the dishes were transferred into a dark growth chamber at 25 C. After two days, 10 uniform seedlings/dish were harvested daily at a fixed time. The roots and shoots were separated, rinsed and weighed prior to extraction and assaying. The experiment was repeated three times.

RESULTS AND DISCUSSION

Tsuchihashi treatment of the pea extract resulted in a 5-fold purification and insignificant loss of activity, which is in agreement with earlier observation that pea SOD is resistant to this treatment (Table 7). Subsequent acetone and $(NH_4)_2SO_4$ fractionation gave a 20-fold purification of the pea enzyme with good recovery. Purification of the corn enzyme with the Tsuchihashi treatment was not attempted due to the elimination of one of the SOD bands observed on gels. Only part of the enzyme could be precipitated from crude corn extract, and this explains the poor recovery after acetone fractionation (Table 8). Fractionation of the

Table 7. *Purification of SOD from 500 g of Pea Seeds*

Purification Step	Volume (ml)	Total Protein (mg)	Total Units	Units/mg protein	Yield (%)	Purification (-fold)
Crude extract	1580	55774	780678	14.0	....	...
Ethanolic phase[1]	1543	10492	761316	72.6	97.5	5.2
Acetone fraction	401	4210	701750	166.7	89.9	11.9
$(NH_4)_2SO_4$ fraction	15	1842	528948	287.2	67.8	20.5
Sephadex G-100	637	567	319297	563.1	40.9	40.2
Biogel P-30 (1st)	88	231	235765	1020.6	30.2	72.9
Biogel P-30 (2nd)	12	38	76506	2002.8	9.8	143.1
Acetone Fraction	5	29	59331	2039.0	7.6	145.6

[1] Separated out after the crude extract was treated with a chloroform - ethanol mixture (Tsuchihashi treatment).

Table 8. *Purification of SOD from 182 g of Corn Seedlings.*

Purification Step	Volume (ml)	Total Protein (mg)	Total Units	Units/mg protein	Yield (%)	Purification (-fold)
Crude extract	404	2513	30102	12	....	....
Acetone fraction	121	231	14109	61	46.9	5.1
$(NH_4)_2SO_4$ fraction	26	60	13013	216	43.2	18.0
Sephadex G-100	208	18	9482	539	31.5	44.9

corn crude extract with $(NH_4)_2SO_4$ also resulted in a low recovery.

Chromatography on a Sephadex G-100 column resolved the pea enzyme into a major and a minor peak (Fig. 6). Addition of mercaptoethanol to the eluting buffer, however, converted the minor peak to the major one. The pooled fractions of the major peak, after they were concentrated and dialyzed, were rechromatographed twice on a Biogel P-30 column. The enzyme eluted as a single peak from this column (Fig. 7). Enzyme from both the major Sephadex peak and the single Biogel peak were compared with freshly prepared crude enzyme by electrophoresis. All three enzyme preparations gave banding pattern on gels identical to that previously described for pea SOD (Figs. 2,3,4).

The corn enzyme eluted from the Sephadex column also as a major and a minor peak (Fig. 8). Enzyme from each peak was compared with freshly prepared crude enzyme by electrophoresis. The banding pattern previously described for corn SOD (Figs. 2,3,4) was again obtained with the crude enzyme. The enzyme from the major Sephadex peak gave this banding also, except that SOD 2 and 5 were not present. The enzyme from the minor Sephadex peak gave three new faint bands which were not similar to any from the crude enzyme.

The following conclusions may be made from the chromatographic and electrophoretic behavior of the enzyme. The minor peaks eluted from the Sephadex column represent a small portion of the enzyme which was altered during purification. This is supported by the observation that electrophoresis indicates that these proteins are not present in the crude extracts. Weisiger and Fridovich reported that chicken liver enzyme elutes also as a minor and a major peak from Sephadex, and that mercaptoethanol converts the former to the latter (13). They also have shown that the

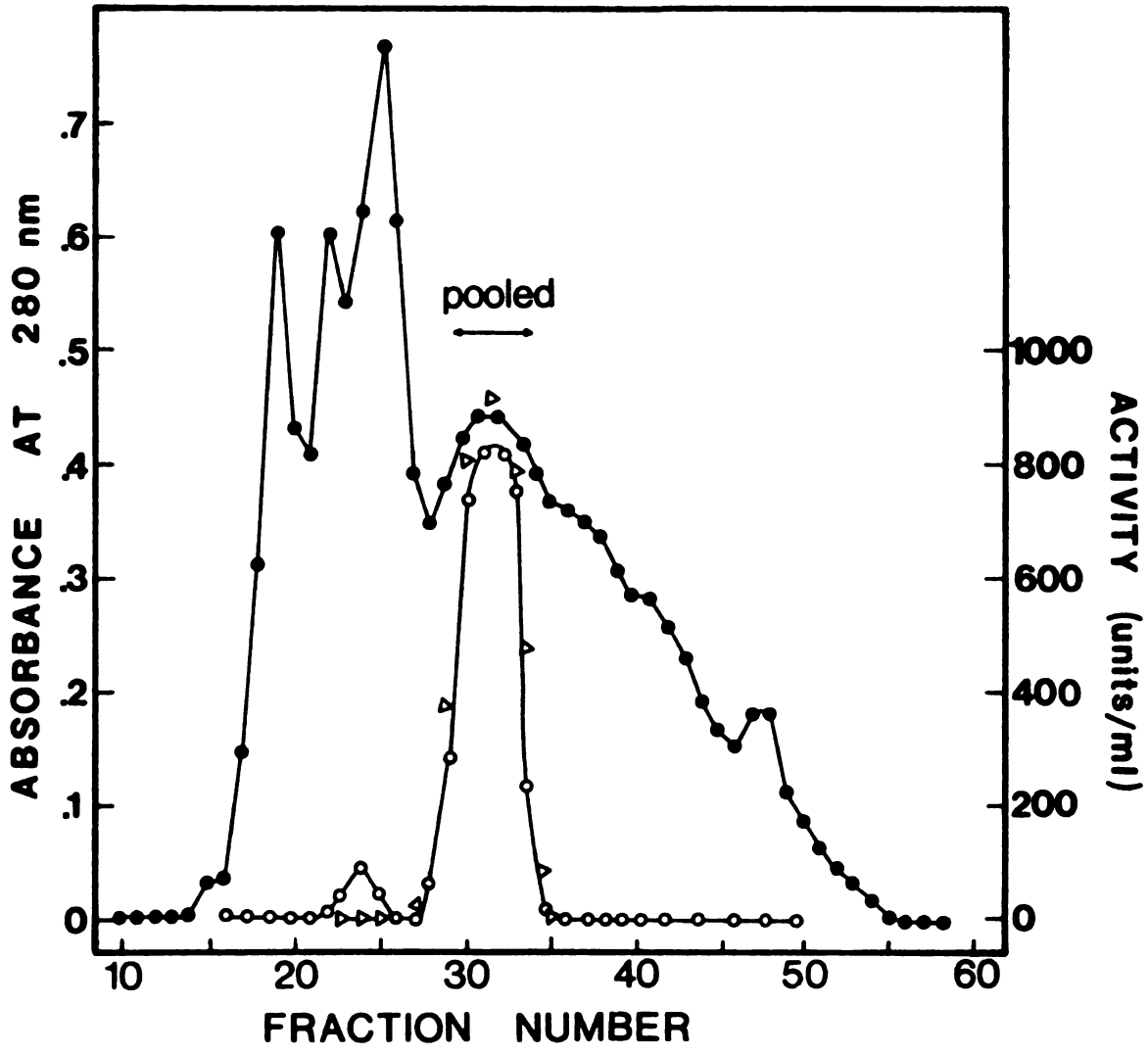

Fig. 6. Chromatography of pea SOD on Sephadex G-100. The 35 to 70% $(NH_4)_2SO_4$ precipitate was redissolved in and dialyzed against 0.1 M KCl, 0.005 M potassium phosphate, and 0.01 mM EDTA, pH 7.8. One ml of this solution was applied on a column (2x90cm) equilibrated with the same buffer. Fractions of 5.0 ml were collected and assayed for SOD activity (o—o) and absorbance at 280 nm (●—●). The enzyme eluted as a major and a minor peak. When 1% mercaptoethanol was included in the eluting buffer, and an identical sample run on the same column, the minor peak was eliminated (Δ—Δ).

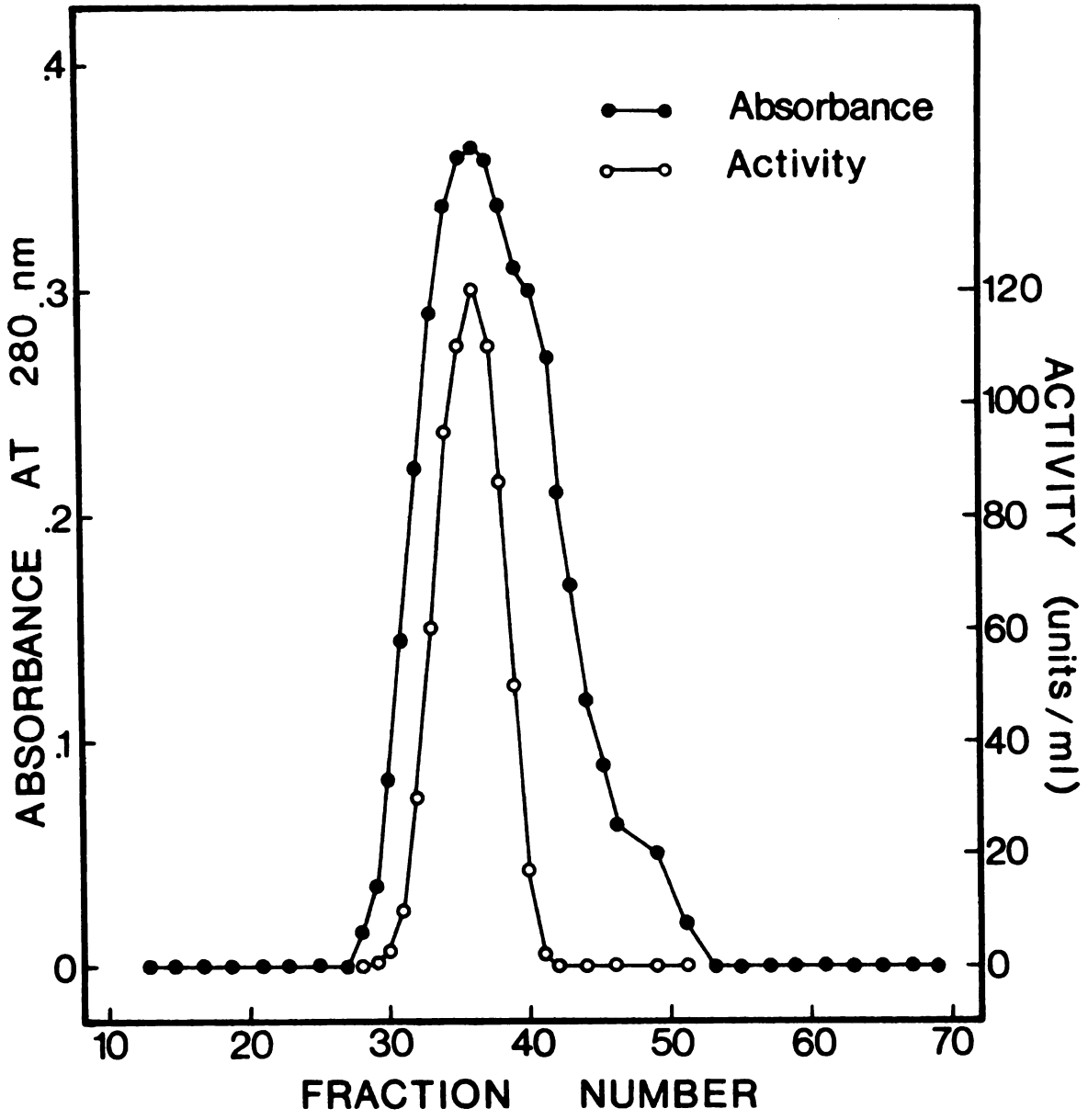

Fig. 7. Chromatography of pea SOD on Bio-gel P-30 (2nd). The column (1x60 cm) was equilibrated and the enzyme eluted with the same buffer described in Figure 6. The enzyme collected from a first run on this column was concentrated to 0.5 ml, dialyzed, and rechromatographed.

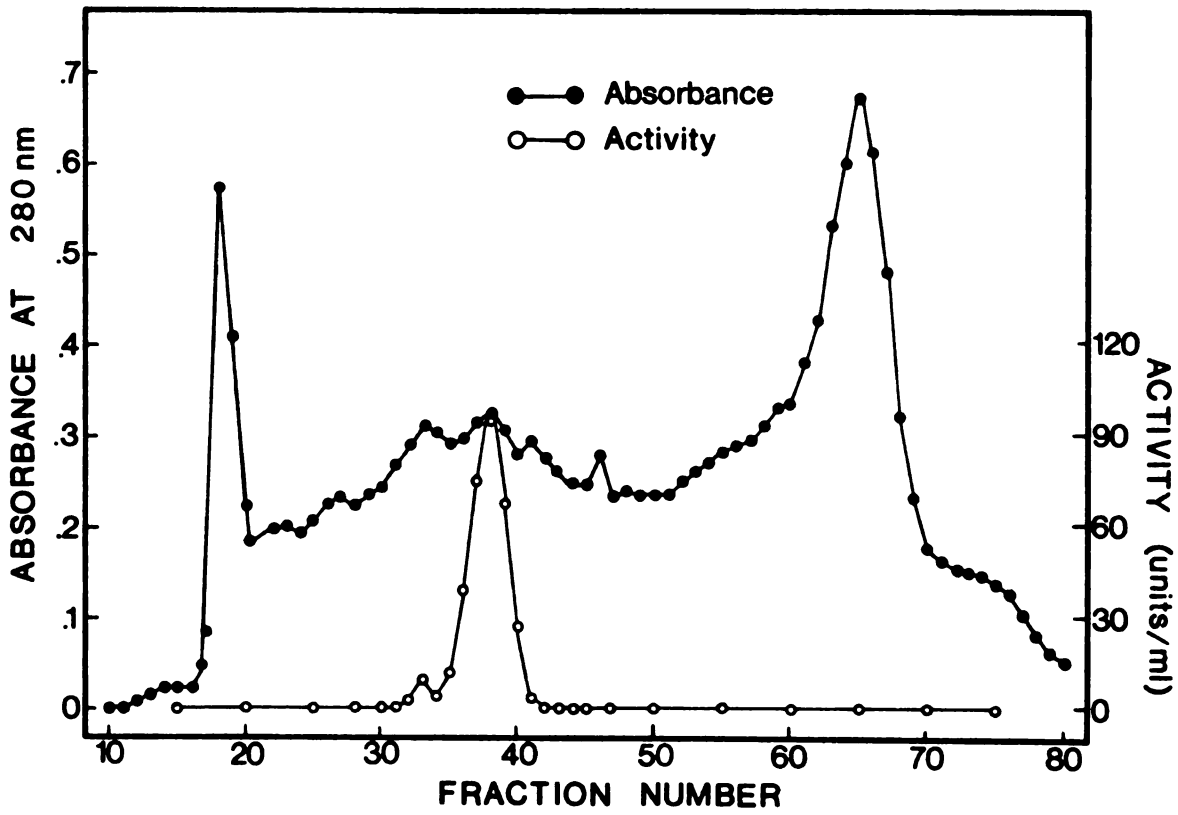

Fig. 8. Chromatography of corn SOD on Sephadex G-100. The column and buffer were the same described in Figure 6. The 45 to 95 % $(NH_4)_2SO_4$ precipitate was redissolved in and dialyzed against the buffer before loading the column. Fractions are 4.0 ml each.

minor peak represents a polymeric form of the enzyme, and that aging in the cold promoted polymerization. During the course of this study, storage of pea or corn enzyme resulted in additional faint bands on gels. This indicates that the plant enzyme polymerizes similarly to the chicken enzyme. The polymerization apparently does not inactivate the enzyme. The major peaks eluted from the Sephadex column represent the bulk of the enzyme which did not undergo any alteration during purification.

The enzyme from each species eluting as the major peak from Sephadex is apparently homogeneous with regard to mol wt, heterogeneous with regard to electrophoretic properties. This supports the view that the SOD bands correspond to isozymes of SOD. The major peak obtained with pea enzyme contained all three SOD bands found in the crude extract. These bands could be eliminated with cyanide, indicating they are due to isozymes of cupro-zinc SOD. The major peak obtained with corn enzyme contained five out of the seven SOD bands found in the crude extract. All of these bands could be eliminated with cyanide and, thus, are isozymes of cupro-zinc SOD. Pea and corn cupro-zinc enzyme have the same mol wt (approximately 30000) as indicated by similar elution volumes from the Sephadex columns (Figs. 6 and 8).

The two SOD bands of corn that were not present in the major peak were SOD 2 and 5. The SOD 2 band was a cyanide-sensitive protein and was shown to be an artifact due to peroxidase (Fig. 10). The SOD 5 band was a cyanide resistant, chloroform-ethanol sensitive protein, which may be a manganese-containing SOD. The manganese enzyme has a considerably higher mol wt than the cupro-zinc enzyme (4), and therefore, it would not copurify with the major peak.

The enzyme from oats was not purified. It was shown, however, that

one of the three SOD bands of oats was an artifact due to peroxidase (Fig. 10). The other two bands had the same relative mobility on gels as two of the pea bands, and therefore, they may also correspond to isozymes of cupro-zinc SOD.

Seedling Enzyme as Percentage of the Water-soluble Protein. The specific activity in photochemical units/mg protein was found to be 14 and 2039 for the crude and the purified pea enzyme, respectively (Table 7). The photochemical unit as defined in the previous study is equivalent to 3.03 standard units (10). Therefore, the specific activity in standard units/mg protein is 42 and 6178 for the crude and the purified pea enzyme, respectively. All specific activities in the following discussion are in standard units/mg protein.

The enzyme from pea seeds was also purified by Sawada *et al.* (11). They determined a specific activity for the pure enzyme of 6400, which is similar to the specific activity found in this study. However, their specific activity for the crude enzyme was 9.9, considerably lower than determined in this study. Sawada *et al.* used the xanthine/xanthine oxidase assay system (10) for their assays. Evidence was obtained in the course of this study that impurities in crude extracts depress the enzyme activity when determined by this assay system (see section on interferences). This may explain the low specific activity of the crude enzyme as determined by Sawada *et al.*

The purified pea enzyme was tested for purity by electrophoresis. One weak band not corresponding to any SOD-active protein was localized on the gels. The actual specific activity of the enzyme, therefore, is expected to be somewhat higher than the above value. Asada *et al.* have purified spinach leaf SOD to a crystalline state (2). The specific

activity of this enzyme was 9320. Their method of protein determination (absorption at 258 nm) was different from the Lowry procedure used in this study and by Sawada *et al.*, thus, a direct comparison of the values may not be relevant. However, it is reasonable to assume that the specific activity of SOD in higher plant species is within the range of 6178 to 9320.

The specific activity of the crude enzyme in roots and shoots of seedlings has already been determined (Table 5). This data was used to estimate the percentage of water-soluble protein accounted for by SOD, assuming specific activities for the pure enzyme in the range 6178 to 9320 (Table 9). Superoxide dismutase accounts for 0.9 to 3.1% of the water-soluble protein.

Interferences with Enzyme Activity in Crude Extract Assays. Experiments were conducted to study interference of impurities in the crude extracts with the enzyme activity. Thus, a basis could be provided for accessing the reliability of the data presented in the previous section.

The effect of chemical treatments on the SOD activity of crude extracts was studied. Complete loss of activity was achieved, when 3 mM KCN was included in the assay mixture (Table 10). Chloroform-ethanol treatment of the extracts caused an approximately 5% loss of activity only in the case of corn. Cupro-zinc SOD is inactivated by cyanide and manganese SOD by the chloroform-ethanol treatment (13). Even though complete loss of activity was obtained with potassium cyanide, other enzymes may interfere with the assay, since cyanide is not a specific inhibitor of SOD. However, the results from this experiment indicate that there is no interference by small mol wt impurities. Dialysis of the crude extracts confirmed this conclusion.

Catalase and peroxidase are other enzymes which might interfere with the photochemical assay. Catalase at concentration as high as

Table 9. *Seedling SOD as Percentage of Water-soluble Protein.*

Species	Part	SOD (% of water-soluble protein)		
		Minimum[1]	Maximum[2]	Average
Corn	Shoot	2.3	3.4	2.85
Corn	Root	1.3	2.0	1.65
Oats	Shoot	1.7	2.5	2.10
Oats	Root	2.5	3.7	3.10
Peas	Shoot	0.7	1.1	0.90
Peas	Root	1.3	2.0	1.65

[1] Assuming specific activity for SOD 9320 units/mg protein, as reported for enzyme purified to crystalline state from spinach leaves (2).

[2] Assuming specific activity for SOD 6178 units/mg protein, as determined in the present study for pea seed enzyme.

Table 10. *Effect of Potassium Cyanide and Chloroform-ethanol Treatments on SOD Activity of Crude Extracts.*

Extract	Treatment	SOD Activity (units/ml)	% Inhibition
Corn	None	50.0	...
Corn	Chloroform-ethanol	42.3	5.4
Corn	KCN 1 mM	10.0	80.0
Corn	KCN 3 mM	0.0	100.0
Oats	None	62.3	...
Oats	Chloroform-ethanol	62.1	0.0
Oats	KCN 3 mM	0.0	100.0
Peas	None	31.5	...
Peas	Chloroform-ethanol	31.5	0.0
Peas	KCN 3 mM	0.0	100.0

150 µg/3 ml did not mimic SOD in retarding the accumulation of blue formazan. Horse radish peroxidase, however, did retard the accumulation of blue formazan, thus exhibiting an SOD-like activity (Fig. 9). This activity was equivalent to 40.6 photochemical units/mg of peroxidase and was not due to contamination of this enzyme with SOD. In the photochemical assay system used in this study, SOD inhibits the reduction of NBT to blue formazan. Peroxidase is not likely to inhibit the reduction of NBT but is likely to catalyze the oxidation of the blue formazan formed. Hydrogen peroxide, which is required for the action of the peroxidase, is produced by the assay system. In absence of SOD, H_2O_2 is produced at low concentrations from the spontaneous dismutation of $O_2^{\cdot -}$ (8). Higher concentrations of H_2O_2 are produced in the presence of SOD from both spontaneous and enzymatic dismutation of $O_2^{\cdot -}$ (10). When purified pea SOD and horse radish peroxidase were both included in the assay mixture, the SOD-like activity of the peroxidase was doubled (Fig. 9). Increasing the SOD concentration did not further increase the activity of the peroxidase. This suggests that low H_2O_2 concentration was limiting the action of peroxidase in absence but not in the presence of SOD.

Attempts were made to estimate the extent to which peroxidase in the crude extracts could interfere with the SOD assays (Table 11). Peroxidase and SOD activities were both determined in crude extracts of corn, oats and peas. Peroxidase activity varied from 263 to 9022 units/ml. As estimated from Figure 9, peroxidase at these concentrations would account for less than 2.5 SOD units/ml, which is probably not statistically significant. It was further observed that peroxidase could be easily inactivated by heating the crude extracts in a boiling water bath; SOD was relatively heat-stable. The peroxidase of the crude extracts was

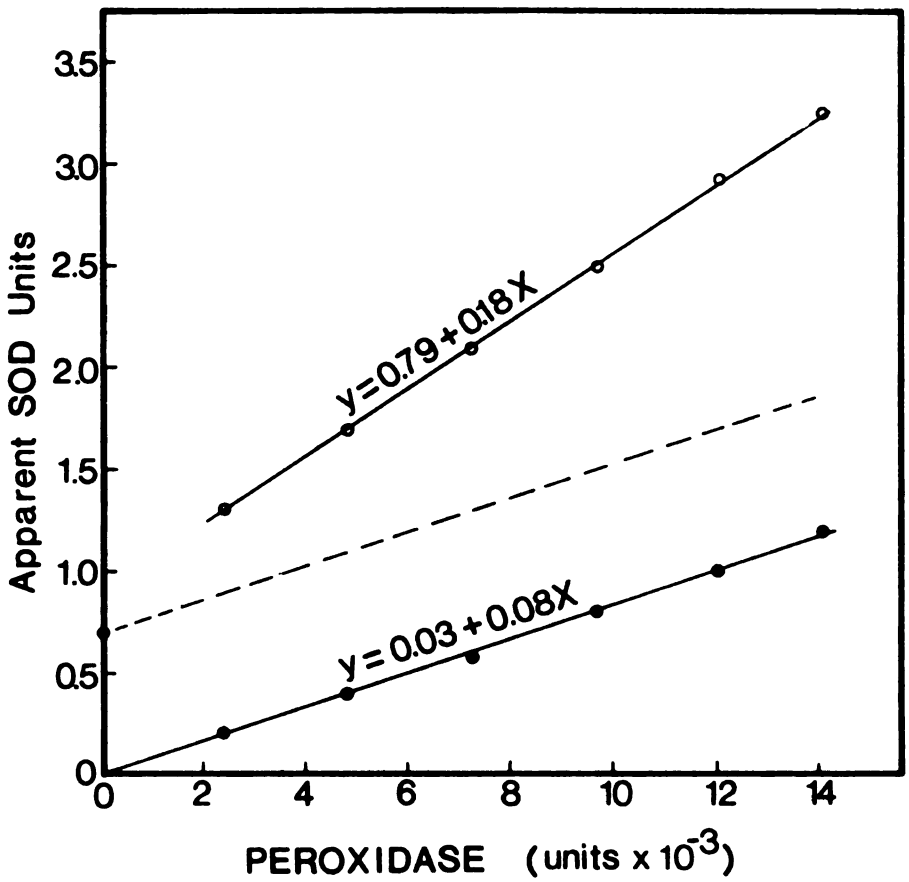

Fig. 9. Superoxide dismutase-like activity of horse radish peroxidase. The photochemical assay system for SOD was used. Reaction mixtures contained the indicated amounts of peroxidase (●—●), or the indicated amounts of peroxidase plus 0.7 unit of purified pea SOD (○—○). The synergistic effect of SOD on the SOD-like activity of peroxidase is apparent by the difference in the slope of the predicted (○- - -) and observed (○—○) lines.

completely inactivated after heating for 1.5 min. Inactivation of the peroxidase was not accompanied by any loss of SOD activity in the corn and oat extracts. Some loss of SOD activity which was observed in the case of the pea extract may not be due to the inactivation of peroxidase, since pea SOD seems to be less heat-stable than corn and oat enzyme. After heating for 20 min, corn, oat and pea extracts retained 100, 30 and 20% of the SOD activity respectively. It may be concluded from these results that interference by peroxidase is negligible, and corrections in Table 9 need not be made.

Table 11. *Effect of Heating in a Boiling Water Bath on the SOD and Peroxidase Activity of Crude Extracts.*

Species	Heating Time (min)	SOD Activity (units/ml)	Peroxidase Activity (units/ml)
Corn	0.0	65.7	263
Corn	1.5	69.0	00
Corn	20.0	66.1	...
Oats	0.0	67.0	3579
Oats	1.5	66.8	0
Oats	3.0	52.0	...
Oats	20.0	19.8	...
Peas	0.0	21.0	9022
Peas	0.5	19.5	6315
Peas	1.5	17.0	0
Peas	20.0	4.1	...

The heat-stability of SOD, which was observed when the crude extracts were heated, was not observed when purified enzyme was heated. Corn and pea SOD partially purified with $(NH_4)_2SO_4$ and acetone fractionation were completely inactivated by heating at 75 C for 2 to 3 min. Addition of corn crude extract to the purified corn and pea enzyme before heating provided partial protection. These results may suggest that impurities in the crude extracts, and especially in the corn crude extract, protect SOD from heat inactivation. In the case of corn crude extract, this may also explain the resistance of the enzyme to precipitation by $(NH_4)_2SO_4$ or acetone (Table 8).

In another experiment, purified pea enzyme was mixed with crude extracts at various proportions and assayed. The SOD activity of the mixtures was equal to the sum of the activities, when the photochemical assay system was used; but, it was less than the sum of the activities, when the xanthine/xanthine oxidase assay system (10) was used. This observation provides additional evidence that the photochemical assay system is reliable for the determination of SOD concentration in crude extracts.

Electrophoresis resolves the SOD of crude extracts into a number of dintinct bands. The possibility that some of these bands may be due to peroxidase was examined (Fig. 10). Two out of 10 SOD bands obtained from the three species, namely SOD 2 of corn and SOD 10 of oats, coincided with peroxidase bands indicating that these two bands may be artifacts due to peroxidase. This also may be the reason purified corn enzyme does not contain the SOD 2 protein. It is evident that peroxidase interfered more during electrophoresis than during assays. Localization of SOD on gels was performed at pH 7.8, whereas assays were at pH 10.2.

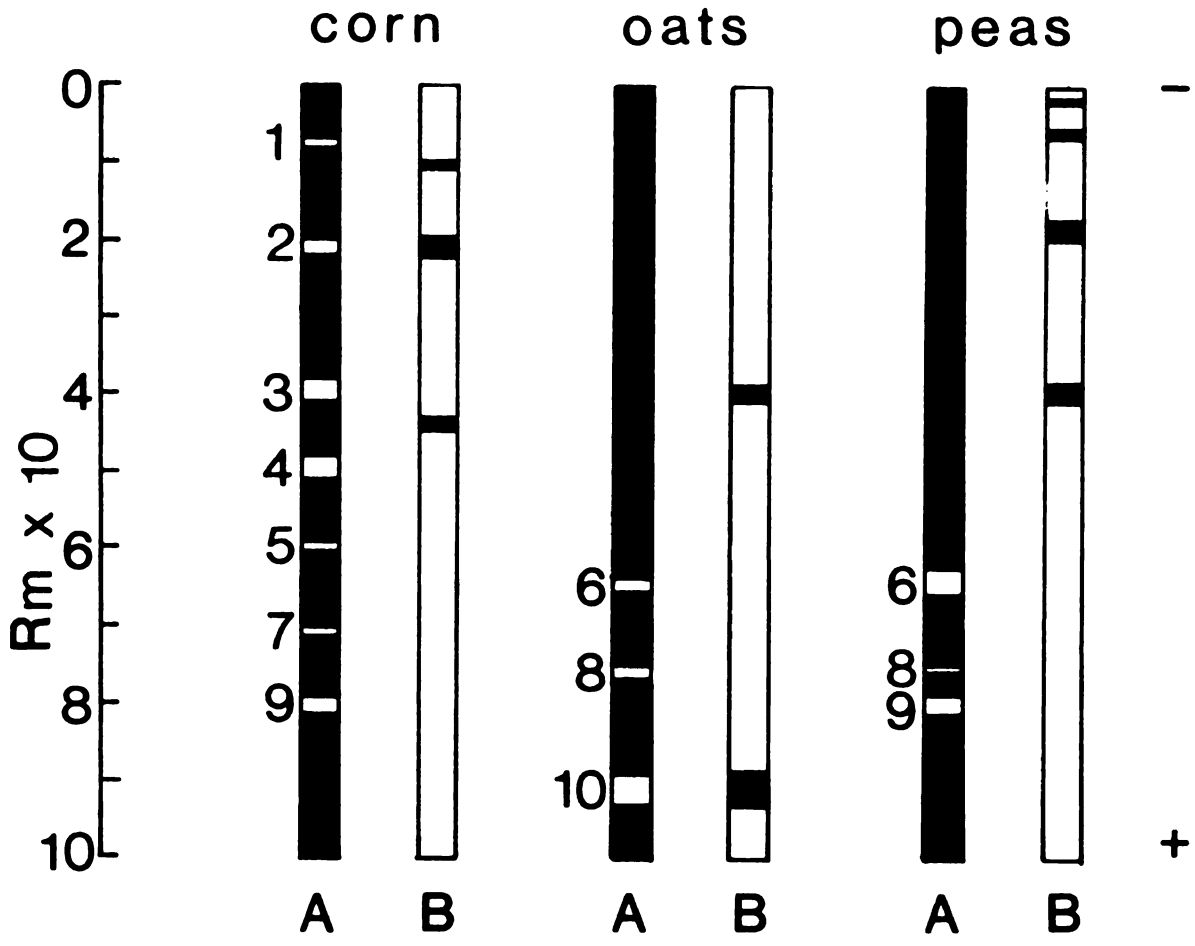

Fig. 10. Electrophoretic comparison of superoxide dismutases with peroxidases. Crude extracts from shoots of 10-day-old seedlings were applied on gels. For each species, one gel was stained for SOD (gels A), and one gel for peroxidase (gels B). Superoxide dismutase bands are numbered as before (Figs. 3 and 4).

The optimum pH for peroxidase is 7.0 (3), which may explain the above observation.

Changes in Specific Activity of SOD. Evidence for the inducibility of SOD has been presented for bacteria (5,6) and blue-green algae (1). In this study, SOD specific activity was shown to increase during greening of pea plumules and germination of oats.

Pea and oat plumules excised from seedlings grown in the dark were either kept in the dark or transferred to the light. After 48 hr, the SOD specific activity of the green plumules was compared with that of the etiolated ones (Table 12). Green pea plumules had developed a specific

Table 12. *Growth, Water-soluble Protein, and SOD Activity of Plumules.*

Plumules were excised from 7-day-old seedlings grown in the dark, placed in petri dishes containing 1% sucrose, and kept at 25 C in the dark (E) or under cool white light (G) for 48 hr.

Species	Etiolated (E) or Green (G)	Fresh wt (mg/plumule)	SOD (units/ g fresh wt)	Protein (mg/ g fresh wt)	SOD units/ mg protein
Oats	E	36.5	712.0	12.2	59.3
Oats	G	36.8	992.7*	16.9*	58.7
Peas	E	39.6	883.7	23.9	37.4
Peas	G	67.0*	901.0	17.3*	52.2*

* F value for difference between the means of the species is significant at the 0.05 level.

activity which was approximately 40% higher than that in the etiolated pea plumules. Green and etiolated oat plumules had developed the same specific activity. A different growth response to light by the two species seems to be associated with these results. Over the 48-hr period, pea plumules grew faster in the light (hook opening, plumular expansion) than in the dark. Oat plumules grew at the same rate in the dark and light. On a fresh weight basis, green oat plumules accumulated more SOD than etiolated oat plumules; but they also accumulated proportionally more water-soluble protein and thus the specific activity did not increase. Associated with increased growth rate of pea plumules in the light is a decrease of water-soluble protein but not of SOD. This explains the increased specific activity of pea plumules with greening.

Results from a kinetic experiment with pea plumules indicated that the increase of SOD preceded that of growth (Fig. 11). Although, on a per plumule basis, water-soluble protein decreased with time at the same rate both in the dark and light, SOD increased. However, the increase of SOD was faster in the light. Growth in the light was accelerated after a lag period of 12 hr. The rapid increase in SOD preceded the acceleration of growth.

The development of the SOD specific activity in oats and peas was also examined during the first days of termination (Fig. 12). On the first day, the specific activity was approximately the same in oats and peas. The specific activity in oats rapidly increased and remained at a high level. In peas, it remained at the initial low level. From experiments with 10-day-old seedlings, it was observed that the SOD specific activity was considerably lower in peas than in oats. The above results confirm this observation and indicate that the difference between the two

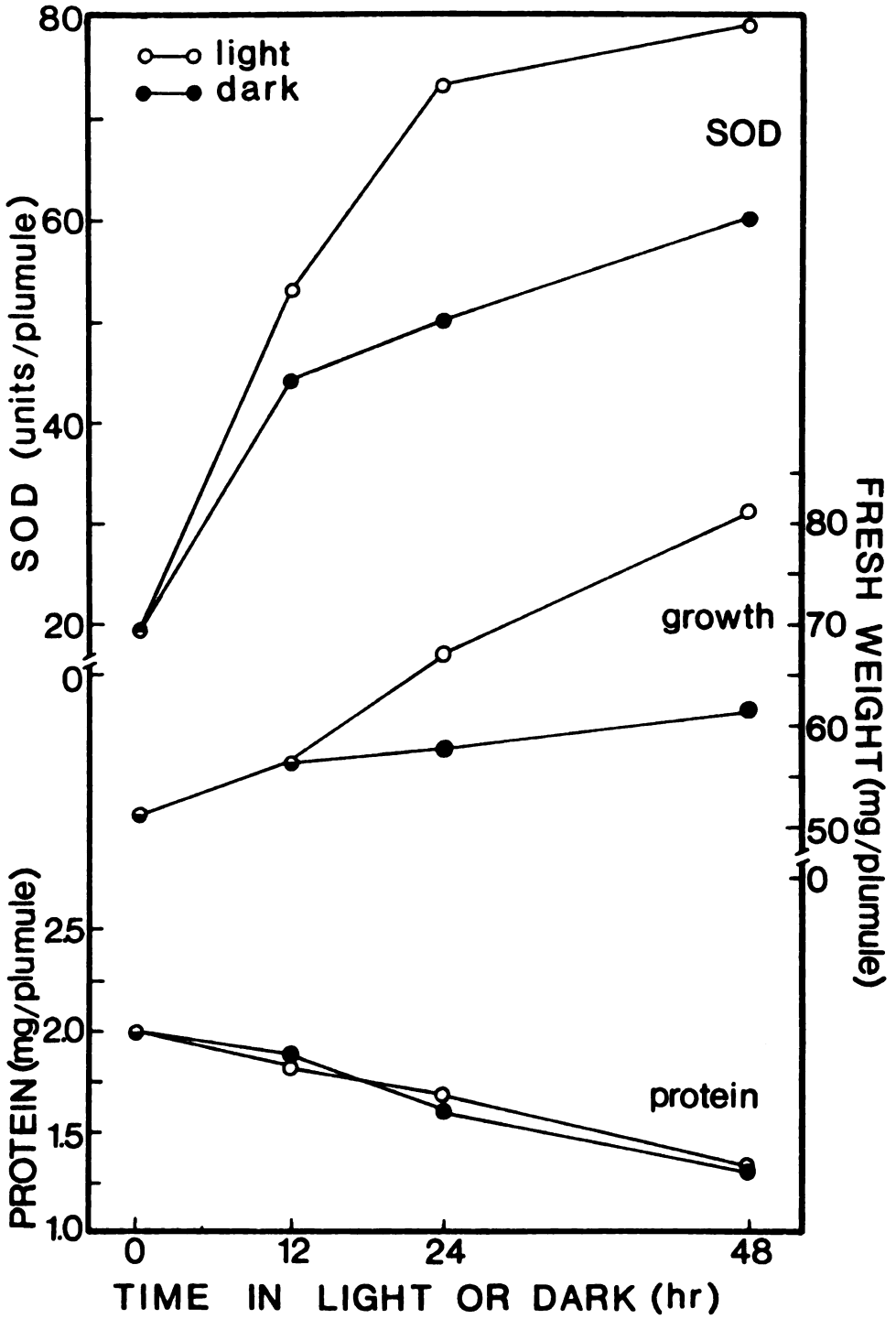

Fig. 11. Development of SOD activity with growth and water-soluble protein content of excised pea plumules. Plumules were excised from 7-day-old seedlings grown in the dark, placed in petri dishes containing 1 % sucrose solution and kept in either dark or light conditions.

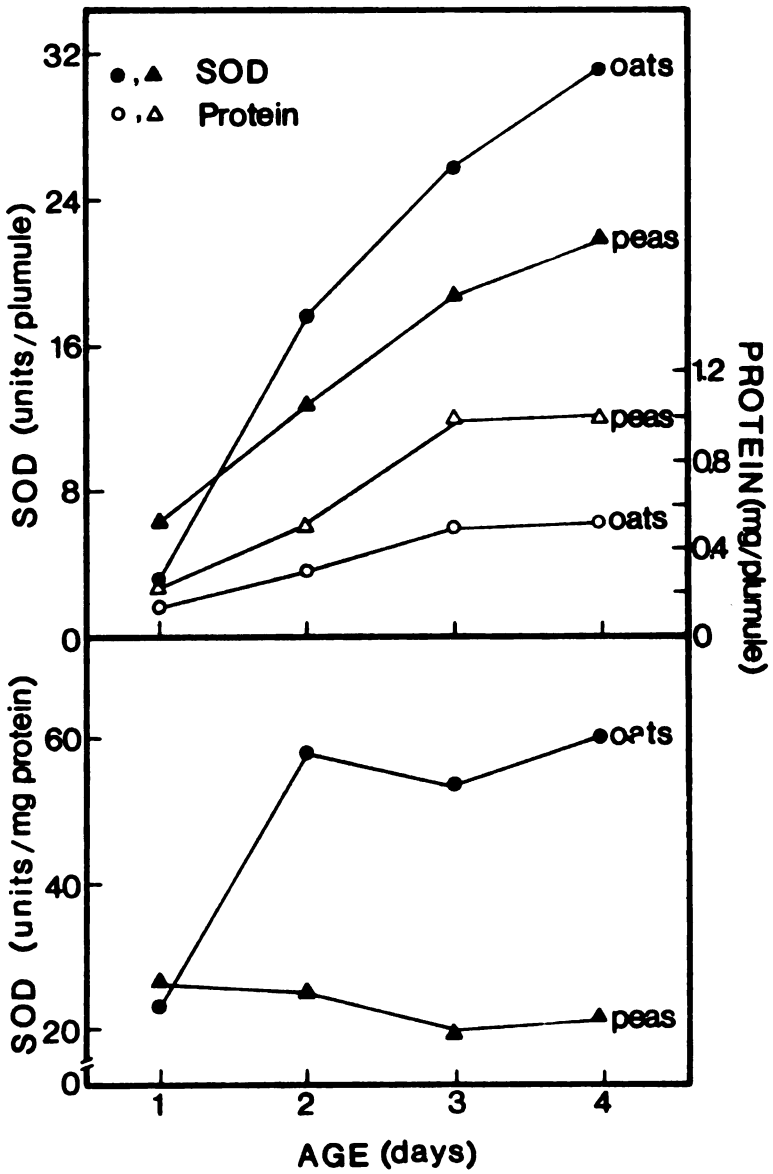

Fig. 12. Development of SOD activity during germination of oats and peas. Seeds were germinated in the dark, on moist filter paper in petri dishes.

species is established since early during germination.

The physiological significance of the above described changes in SOD specific activity cannot be assessed at the present time. These changes, however, may support the reality of previously described variations in SOD content from organ to organ and species to species.

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SECTION THREE

IN VITRO PRODUCTION OF SUPEROXIDE RADICAL FROM PARAQUAT AND ITS INTERACTION WITH MONURON AND DIURON

IN VITRO PRODUCTION OF SUPEROXIDE RADICAL FROM PARAQUAT AND ITS INTERACTION WITH MONURON AND DIURON

ABSTRACT

The ability of herbicides to produce superoxide radical as well as their ability to react with this radical was examined through their effect on the superoxide-induced reduction of *p*-nitro blue tetrazolium chloride. Paraquat enhanced and diuron inhibited the reduction of *p*-nitro blue tetrazolium chloride. Paraquat was reduced photochemically (riboflavin/methionine) or enzymatically (xanthine/xanthine oxidase) and produced superoxide radical upon reoxidation. Diuron and monuron interacted with photochemically produced superoxide radical, but not with enzymatically produced superoxide radical. The product of the monuron/superoxide interaction was a demethylated, dechlorinated water-soluble compound containing phenolic hydroxyl group(s), and was not toxic to oats. The enzyme superoxide dismutase prevented the formation of this product. Other herbicides (atrazine, metribuzin, terbacil, 2,4-D, CDEC, diphenamid) had little effect on the *p*-nitro blue tetrazolium chloride reduction.

INTRODUCTION

The occurrence of superoxide free radical ($O_2^{\cdot -}$) in organisms is suggested by at least two independent pieces of evidence. First, the wide distribution of the enzyme SOD[1] (14), which scavenges the free radical and is thus thought to constitute the basis of a defense mechanism against its deleterious action (8,10). Secondly, the documented production of $O_2^{\cdot -}$ from a variety of chemical and enzymatic reactions of biological origin. Reduced ferredoxins (15), flavins and quinones (16) produce $O_2^{\cdot -}$ upon reoxidation. The aerobic action of xanthine oxidase (14), flavoenzymes (13), NADPH-cytochrome *c* reductase (2), and other enzymes gives rise to $O_2^{\cdot -}$. Production of $O_2^{\cdot -}$ by illuminated chloroplasts (1) and by leucocytes (4) has also been demonstrated.

Rapid killing of cells subjected to $O_2^{\cdot -}$ and other detrimental effects to biological systems associated with $O_2^{\cdot -}$ or its transient decay products (singlet oxygen, hydroxyl radical) (8) has prompted investigators to examine the possibility that the toxic action of certain bactericides and herbicides is due to their ability to produce $O_2^{\cdot -}$. Steptonigrin was shown to produce $O_2^{\cdot -}$ (23) and its toxicity to *Escherichia coli* could be reduced by inducing the SOD activity of the organism (9). Production of $O_2^{\cdot -}$ from diquat (21) and paraquat (7) was also shown *in vitro*. Paraquat-induced toxicity to rats could be reduced by administering SOD intravenously (3).

The possible involvement of $O_2^{\cdot -}$ in biological transformations of pesticides should also be considered. Superoxide radical is both a

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[1] Abbreviations: SOD: superoxide dismutase; NBT: *p*-nitro blue tetrazolium chloride; FMN: riboflavin 5'-phosphate sodium.

reducing and an oxidizing agent (10), and the hydroxyl radical arising from $O_2^{\cdot -}$ is one of the most potent oxidants (8). Evidence for involvement of $O_2^{\cdot -}$ in the sulfoxidation of thioethers was obtained with ethionamide (20). Hydroxylation of aromatic compounds catalyzed by enzymes from *Aspergillus niger* was shown to involve $O_2^{\cdot -}$ (19). *s*-Triazine herbicides were dealkylated by a hydroxyl-radical generating system to products identical to those isolated from various biological systems (18).

Direct proof of $O_2^{\cdot -}$ involvement in action or metabolism of herbicides from *in vivo* experiments is not easy to obtain at the present time due to lack of a reliable technique for detecting the radical in plant tissues. However, reliable information may be obtained from *in vitro* experiments utilizing model systems developed in recent years. In the present study, the ability of herbicides from various chemical classes to either produce $O_2^{\cdot -}$ or to react with $O_2^{\cdot -}$ was tested by using such model systems. It became evident from these studies that paraquat is able to produce $O_2^{\cdot -}$, whereas monuron and diuron readily react with $O_2^{\cdot -}$. The mechanism of $O_2^{\cdot -}$ production from paraquat and the interaction of monuron and diuron with $O_2^{\cdot -}$ were further studied.

MATERIALS AND METHODS

Non-radioactive herbicides[2] were analytical grade and recrystallized from appropriate solvents. Carbonyl-^{14}C-labeled monuron and diuron

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[2] Chemical names of herbicides: paraquat: 1,1'-dimethyl-4,4'-bipyridylium dichloride; 2,4-D: (2,4-dichlorophenoxy) acetic acid; CDEC: 2-chloroallyl diethyldithiocarbamate; diuron: 3-(3,4-dichlorophenyl)-1,1-dimethylurea; monuron: 3-(*p*-chlorophenyl)-1,1-dimethylurea; terbacil: 3-*tert*-butyl-5-chloro-6-methyluracil; atrazine: 2-chloro-4-(ethylamino)-6-(isopropylamino)-*s*-triazine; metribuzin: 4-amino-6-*tert*-butyl-3-(methylthio)-*as*-triazin-5(4*H*)one; diphenamid: *N*,*N*'-dimethyl-2,2'-diphenylacetamide.

were obtained from E.I. du Pont de Nemours and Company and purified by TLC. The Rf values of the radioactive herbicides were identical with those of authentic monuron and diuron in two solvent systems. Xanthine and xanthine oxidase (from milk) were purchased from Sigma Chemical Corporation, NBT from Aldrich Chemical Company, Inc. Catalase (from beef liver) was obtained from Nutritional Biochemicals Corporation and was chromatographed on Sephadex G-100 to remove contaminant SOD. Superoxide dismutase was purified from pea seeds and corn seedlings as described elsewhere (section two). All other chemicals were of analytical reagent grade and the solvents of pesticide grade.

The systems utilized to produce $O_2^{\cdot -}$ were based on the autoxidation of photoreduced flavins (5), the oxidation of xanthine by xanthine oxidase (14), and the autoxidation of reduced phenazine methosulfate (17). These systems were used as described in the literature, unless otherwise indicated. All experiments were conducted at room temperature. Photoreactions were routinely performed in glass test tubes (Kimax, 1.7 x 14.5 cm) using cool white light from a circular lamp (Sylvania, FC12T 10-CW-RS) at 25 C as previously described (see section one). Ultra violet and visible absorption spectra were obtained with a Beckman DB-G grating spectrophotometer equipped with a Sargent Model SR recorder. Infra red spectra were measured with a Perkin Elmer Model 337 IR spectrophotometer and KBr discs of the compounds. Radioactivity was measured with a Packard 3003 Tri-Carb Scintillation Spectrophotometer using internal spiking with ^{14}C-toluene to determine DPM.

Formation of water-soluble compounds from carbonyl-^{14}C-labeled monuron and diuron were quantitatively followed by extracting the reaction mixtures with an equal volume of methylene chloride. The two

phases were separated after centrifugation and the radioactivity determined in each phase by transferring 0.2 ml to counting vials containing 15 ml of a scintillation solution consisting of 666 ml toluene, 333 ml Triton-X-100, 4g PPO and 50 mg dimethyl-POPOP. Corrections were made for monuron and diuron remaining in the water phase under the various experimental conditions. Formation of methylene chloride-soluble compounds was quantitated by TLC analysis of the methylene chloride phase. Three ml of this phase were transferred into a screw-cap test tube, the solvent was evaporated under vacuum, and the residue redissolved in 0.5 ml acetone. An 0.2-ml alquot of the acetone was spotted on pre-coated TLC plates (Silica G-25 UV254, Brinkmann Instruments, Inc.) and overspotted with 5 μg of non-radioactive monuron or diuron. The plate was developed three times in the same direction to a 15-cm solvent front with benzene-acetone (3:1 v/v). Monuron or diuron was localized under ultra violet light and the spot carefully scraped with a razor blade and transferred into a vial containing 15 ml of scintillation liquid (600 ml toluene, 400 ml methyl-cellosolve, 33.3 ml water, 4 g BBOT and 80 g naphthalene).

Preparative separation of the products from monuron was carried out according to the following procedure. The reaction mixture consisted of 500 ml of a 50 μM solution of monuron (12300 DPM/ml) in distilled water, 2.8 mg riboflavin, 16.8 mg EDTA, 258.7 mg K_2HPO_4 and 456 mg KH_2PO_4 (pH 6.5). The mixture was placed in a 1-liter round bottom flask (Pyrex) and illuminated for 2 hr under constant mechanical stirring in a box lined with aluminum foil and equipped with a circular fluorescent lamp. The average light intensity on the outside surface of the flask was 4850 luxes. The reaction mixture was extracted twice with 500 ml

of methylene chloride. Sixty-two % of the original radioactivity was recovered in the water phase and 30% in the methylene chloride phase.

The methylene chloride phase was dried with anhydrous calcium chloride, the solvent evaporated under vacuum, and the residue redissolved in 1.0 ml of acetone. An 0.25-ml aliquot of the acetone was chromatographed on a TLC plate as in the quantitative experiments. The radiochromatogram was obtained by scraping off the gel in 1-cm strips and measuring radioactivity.

The water phase was freeze-dried and the residue repeatedly extracted with acetone and ethanol. After the solvents were removed by evaporation under vacuum, the residue was redissolved in 1.5 ml of distilled water. The recovery of the water-soluble radioactivity was approximately 78%. This solution was applied in 0.5-ml portions on a sephadex G-10 column (1.5 x 60 cm). The column was eluted with distilled water, fractions of 4 ml were collected and assayed for radioactivity. The fractions corresponding to the major radioactive peak (92% of total radioactivity in the effluent) were tested for purity by measuring absorbance at 230 nm. The absorption and the radioactivity peaks coincided, indicating that no contaminant was copurified with the major water-soluble product. The combined fractions were used in toxicity tests after dilution or in identification tests after freeze-drying.

RESULTS AND DISCUSSION

Effect of Herbicides on the Reduction of NBT. Superoxide radical is generated during reoxidation of photoreduced riboflavin (5), or oxidation of xanthine by xanthine oxidase (14). The production of $O_2^{\cdot -}$

by either system can be measured spectrophotometrically by following the reduction of NBT, since $O_2^{\cdot -}$ reduces NBT to blue formazan (5). Information on the ability of a herbicide to produce $O_2^{\cdot -}$ or to react with $O_2^{\cdot -}$ may be indirectly obtained from the effect it will have on the NBT reduction.

The NBT reduction by $O_2^{\cdot -}$ generated from photoreduced riboflavin was measured in presence of various herbicides (Table 13). Atrazine, metribuzin, diphenamid, CDEC, 2,4-D and terbacil had little effect on the rate of NBT reduction. Paraquat markedly enhanced and diuron markedly inhibited the NBT reduction.

The effect of paraquat and diuron on the NBT reduction by the xanthine/xanthine oxidase system was also examined. Paraquat enhanced the NBT reduction by this system (Fig. 13). This enhancement could be prevented with SOD. Paraquat itself did not reduce NBT. Clearly, the effect of paraquat on NBT reduction is due to increased production of $O_2^{\cdot -}$. Diuron, on the other hand, did not affect the NBT reduction by the xanthine/xanthine oxidase system, although it inhibited NBT reduction by photoreduced riboflavin. A possible explanation for this discrepancy is presented in the section on diuron.

Production of $O_2^{\cdot -}$ from Paraquat. It has been well documented by using isolated chloroplasts that paraquat and related compounds are capable of accepting a single electron and becoming reduced to the corresponding free radicals (6,12,24). The xanthine/xanthine oxidase system used in this study also reduced paraquat as indicated by the characteristic spectral change under anaerobic incubation (Fig. 14). Similarly, accumulation of the paraquat free radical was evident from development of intense violet color upon illumination of a solution containing paraquat, riboflavin,

Table 13. *Effect of Herbicides on the Photoreduction of NBT in the Presence of Riboflavin and Methionine.*

Reaction mixtures contained 1.5 μM riboflavin, 0.01 M methionine 60 μM NBT, and 0.1 M sodium carbonate pH 10.2. Herbicides were dissolved in ethanol (paraquat in distilled water) and added so that the final concentration was 0.1 mM for the herbicide and 1% for the ethanol. Ethanol at 1% did not affect the NBT reduction.

Herbicide	Rate of NBT Reduction[1] (ΔA_{560}/min)	% of Control
None	0.025	...
Paraquat	0.042	168
2,4-D	0.021	84
CDEC	0.030	120
Diuron	0.012	48
Terbacil	0.022	88
Atrazine	0.027	108
Metribuzin	0.028	112
Diphenamid	0.028	112

[1] LSD: 0.004 at 5%, 0.006 at 1%.

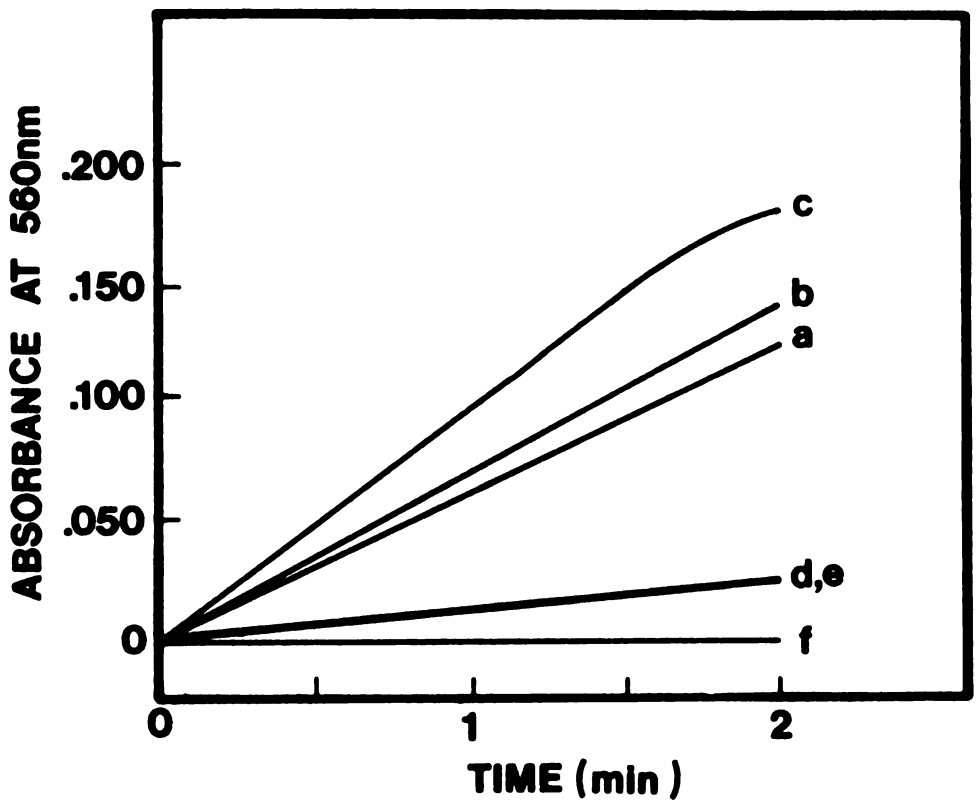

Fig. 13. Effect of paraquat on the reduction of NBT by $O_2^{\cdot -}$ generated from xanthine/xanthine oxidase. The standard reaction mixture contained 0.1 mM EDTA, 20 μM xanthine, 60 μM NBT, 0.05 M potassium phosphate, pH 7.8, and 0.05 ml of a xanthine oxidase solution. Modifications of the standard mixture: (a) none; (b) plus 0.3 mM paraquat; (c) plus 3 mM paraquat; (d) plus 30 units of SOD; (e) plus 3 mM paraquat and 30 units of SOD; (f) minus the xanthine oxidase and plus 3 mM paraquat. Changes of absorbance at 560 nm were recorded with a Sargent Model SR recorder.

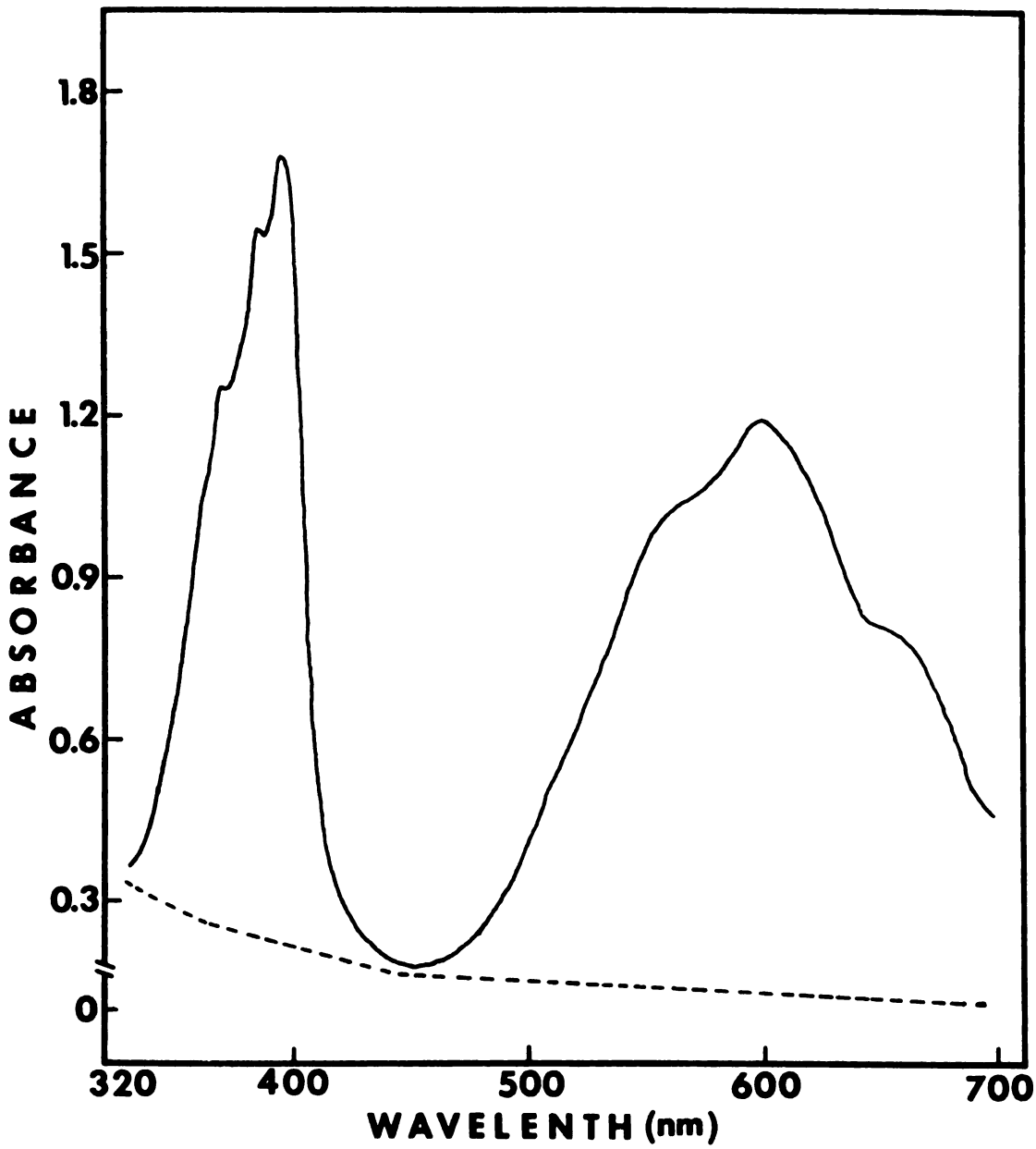

Fig. 14. Reduction of paraquat by xanthine/xanthine oxidase as indicated by the spectral change under anaerobic conditions. The cuvette contained the standard reaction mixture (Fig. 13), except that NBT was omitted and paraquat was added at 6 mM. The cuvette was equilibrated with air (-----) or nitrogen (———). Spectra were recorded 5 min after addition of the xanthine oxidase.

and methionine in absence of air.

Because of its low redox potential, the paraquat radical is rapidly reoxidized in presence of oxygen. It was recently shown by Farrington *et al.* that $O_2^{\cdot -}$ is produced upon reoxidation of the paraquat free radical (7). The enhancement of NBT reduction by paraquat in connection with the fact that SOD prevents this enhancement are in agreement with the finding of Farrington *et al.* that $O_2^{\cdot -}$ is produced from paraquat. Further support is provided by the fact that paraquat can be reduced by the two $O_2^{\cdot -}$ generating systems used in this study. The two systems and NBT can probably be used as convenient assays for testing suspected production of $O_2^{\cdot -}$ from other pesticides.

Interaction of Diuron and Monuron with $O_2^{\cdot -}$. An aqueous solution of carbonyl-^{14}C-labeled diuron, riboflavin and methionine was exposed to light. At time intervals, aliquots were extracted with methylene chloride to remove the diuron. The amount of radioactivity in the water phase increased with time of illumination, indicating that diuron was converted to water-soluble compounds (Fig. 15). Superoxide dismutase inhibited the formation of water-soluble compounds by at least 70 % (Fig. 16). The yield of water-soluble compounds from diuron was maximum at pH 6.0, and the inhibition by SOD was maximum at pH 8.0 to 9.0 (Fig. 17). Experiments with carbonyl-^{14}C-labeled monuron yielded the same results. Conversion of diuron and monuron to water-soluble compounds results from an interaction of the herbicides with $O_2^{\cdot -}$, since SOD is effective in preventing this conversion.

Diuron reacted with $O_2^{\cdot -}$ produced photochemically from flavins, but not with $O_2^{\cdot -}$ produced from other systems (Table 14). The photochemical system was still effective in converting diuron to water-soluble compounds

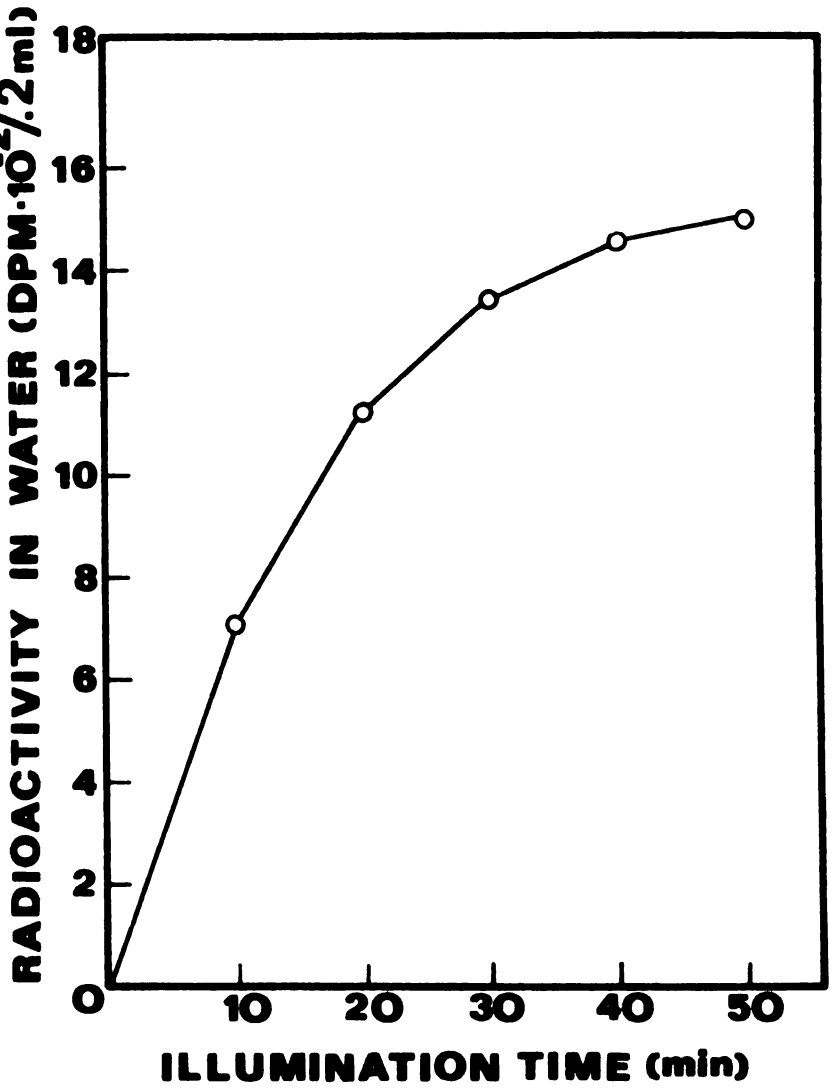

Fig. 15. Radioactivity remaining in the water phase after extraction with methylene chloride as a function of the illumination time. Reaction mixtures (final volume 4.0 ml) containing 1.5 μM riboflavin, 0.01 M methionine, 15 μM carbonyl-^{14}C-labeled diuron, and 0.1 M potassium phosphate, pH 6.0, were illuminated, extracted with 4.0 ml of methylene chloride, and the radioactivity in 0.2 ml of the water phase determined.

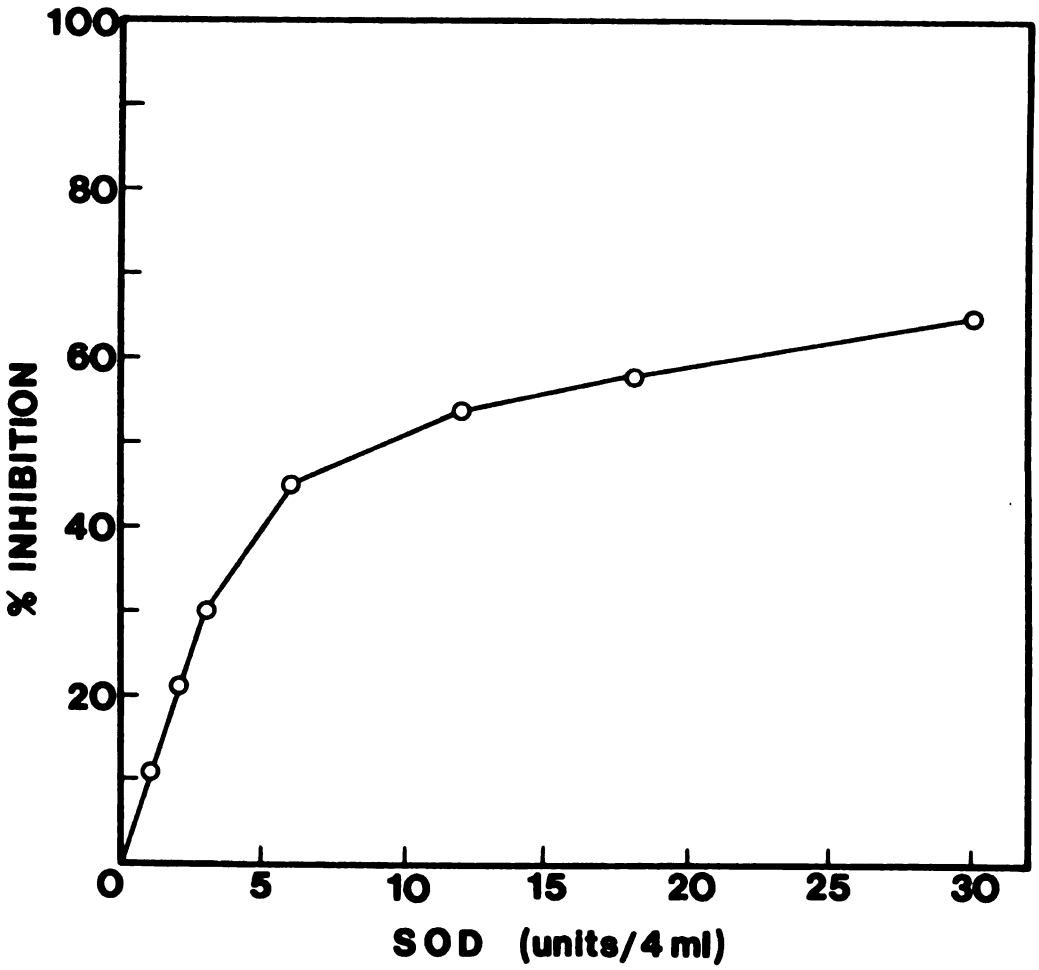

Fig. 16. Inhibition of the formation of water-soluble products from diuron by SOD. Reaction mixtures as in Figure 15 containing the indicated amounts of SOD were illuminated for 7 min.

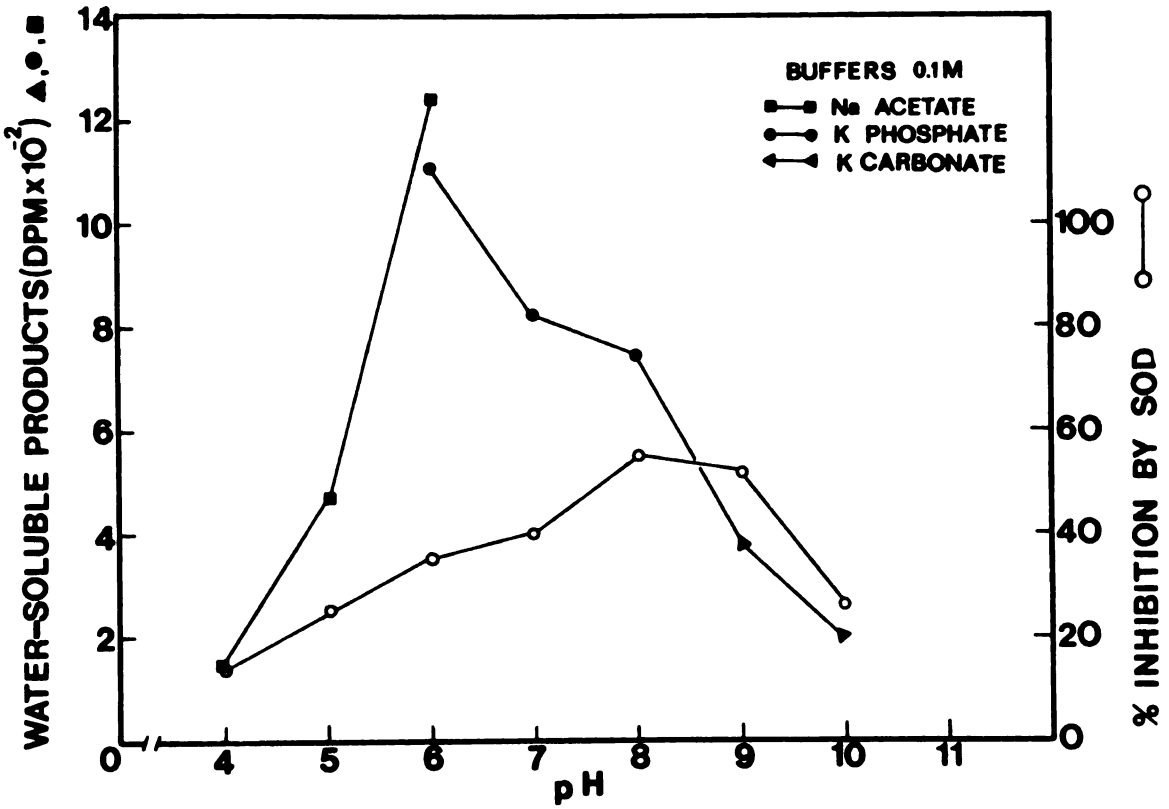

Fig. 17. Formation of water-soluble products from diuron and inhibition by SOD as a function of pH. Reaction mixtures containing riboflavin, methionine, and diuron as in Figure 15, and the indicated buffers were illuminated for 7 min. Superoxide dismutase was added at 3.0 units/4 ml.

Table 14. *Conversion of Diuron to Water-soluble Products by Various $O_2^{\cdot -}$ Generating Systems.*

Reaction mixtures contained in a final volume of 4 ml: carbonyl-^{14}C-diuron 45 nmoles; riboflavin, FMN, xanthine, or phenazine methosulfate 45 nmoles; methionine, EDTA, or NADH 50 μmoles; potassium phosphate, pH 6.5, 150 μmoles. Catalase was added at 30 units and SOD at 6 units. Incubation time was 1 hr.

$O_2^{\cdot -}$ Generating Systems and Modifications	Diuron Converted to Water-soluble Products (nmoles/4 ml)
Riboflavin + Methionine + Light	27
minus riboflavin, methionine, or light	0,0, or 0
plus pea SOD	10
plus boiled pea SOD	28
plus corn SOD	11
plus boiled corn SOD	27
plus catalase	27
Riboflavin + EDTA + Light	30
minus riboflavin	0
plus pea SOD	16
plus catalase	30
FMN + EDTA + Light	31
minus EDTA	0
plus pea SOD	13
plus catalase	30
Xanthine + EDTA + Xanthine Oxidase	0
Phenazine methosulfate + NADH	0

when riboflavin was substituted by FMN and methionine by EDTA. The xanthine/xanthine oxidase (14) and phenazine methosulfate/NADH (17) systems were not effective, although they produced $O_2^{\cdot -}$. Conversion of diuron to water-soluble compounds was possible only when a complete photochemical system was employed, and it could be inhibited only by SOD, which indicates that $O_2^{\cdot -}$ is undoubtedly responsible for the conversion. Catalase had no effect, which excludes the possibility that hydrogen peroxide or hydroxyl radical produced from $O_2^{\cdot -}$ are involved. Interaction of diuron with photochemically produced $O_2^{\cdot -}$ but not with $O_2^{\cdot -}$ produced from xanthine/xanthine oxidase may explain why diuron inhibited NBT reduction by the former but not by the latter system.

Monuron has been reported to inhibit the photooxidation of EDTA and other substances catalyzed by flavins, but not the oxidation catalyzed by other dyes (11). Inhibition of the oxidations by monuron was however monitored as a reduction in oxygen consumption and was found to be more pronounced at low oxygen concentrations. Interaction of monuron with $O_2^{\cdot -}$ produced from the flavins may be indicated by these results. Such an interaction would most likely convert $O_2^{\cdot -}$ back to molecular oxygen and would appear as a net reduction in oxygen consumption.

Interaction of monuron and diuron with $O_2^{\cdot -}$ produced from photoreduced flavins but not with $O_2^{\cdot -}$ produced from other systems may suggest that the herbicides are not sensitive to $O_2^{\cdot -}$, but they are specifically sensitized by flavins. Carbonyl-^{14}C-labeled monuron reacted in the riboflavin/EDTA/light system. The radioactive products were partitioned between water and methylene chloride and analyzed by chromatography (Figs. 18 and 19). One major water-soluble product and several minor products soluble in either water or methylene chloride were

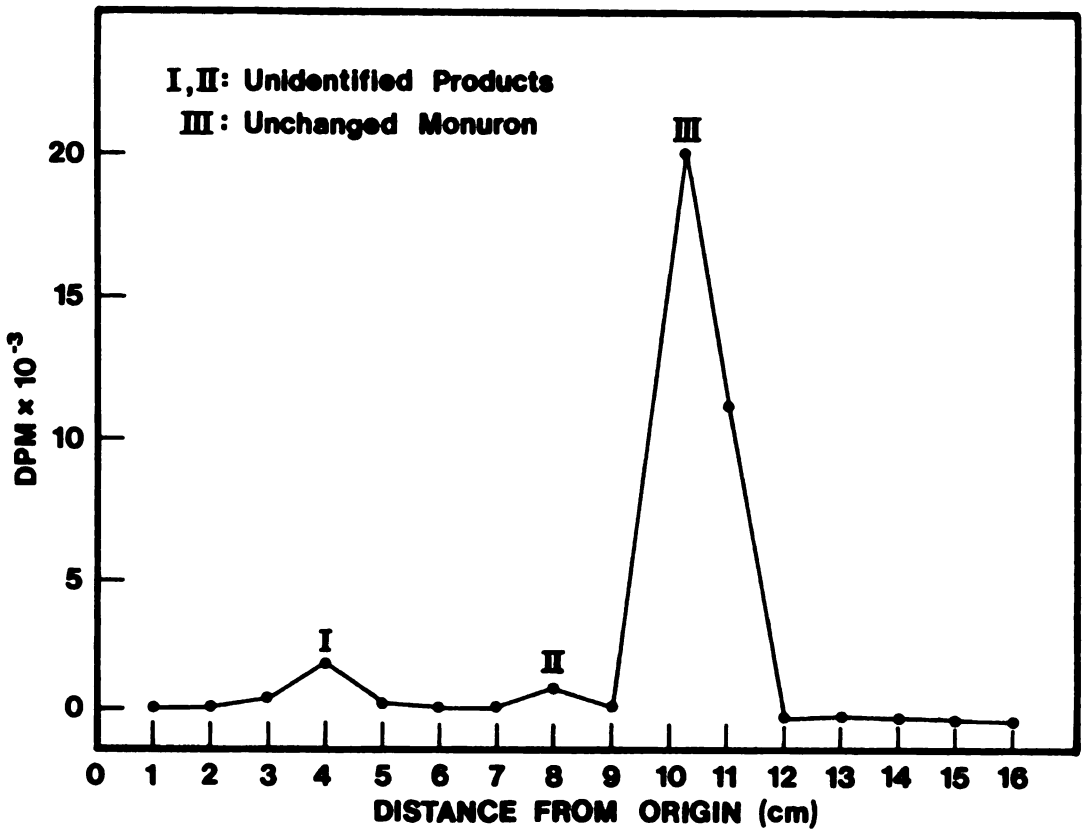

Fig. 18. Radioactive products from the monuron/$O_2^{\cdot -}$ interaction and unchanged monuron recovered in the methylene chloride phase. Reaction mixture containing carbonyl-^{14}C-monuron, riboflavin, and EDTA was illuminated and then extracted with methylene chloride. The methylene chloride phase was concentrated and spotted on silica-gel TLC plates. The radiochromatogram was obtained from a developed plate by scraping off the gel in 1-cm strips and measuring radioactivity. See "Materials and Methods" for more details.

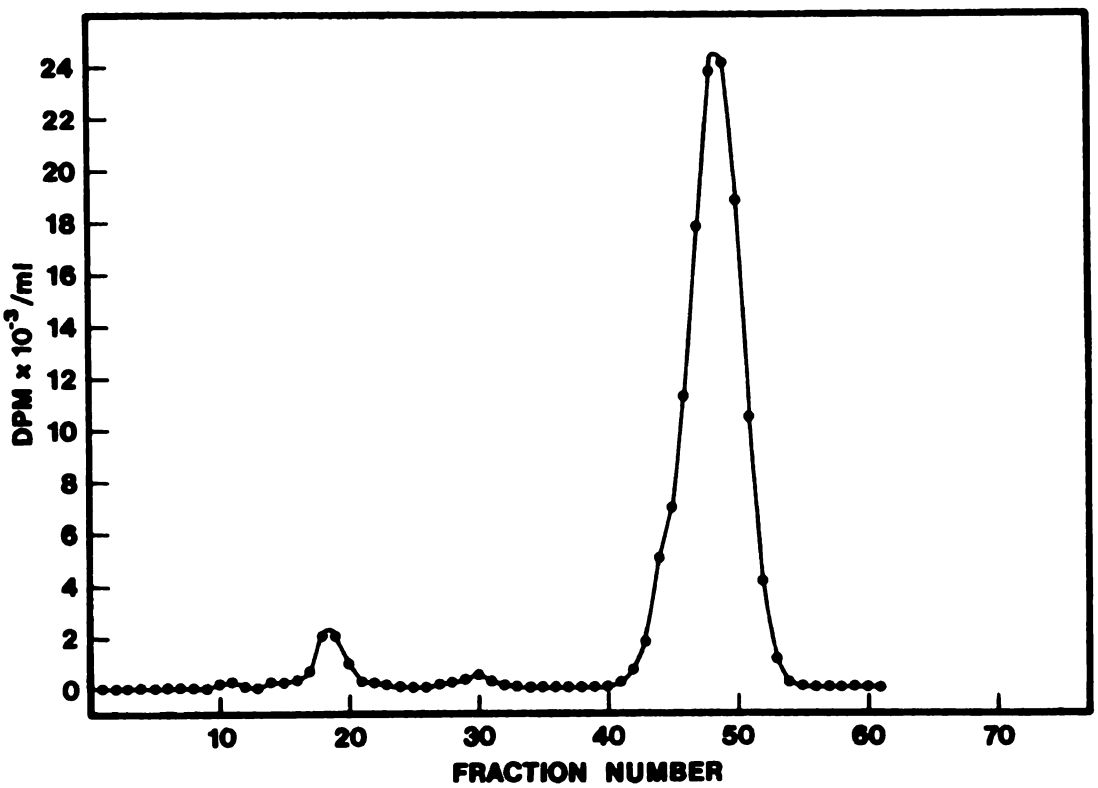

Fig. 19. Radioactive products from the monuron/$O_2^{\cdot -}$ interaction recovered in the water phase. The water phase from the reaction mixture extracted with methylene chloride (Fig. 18) was concentrated and chromatographed on a Sephadex G-10 column (1.5 x 60 cm). Fractions of 4.0 ml were collected and assayed for radioactivity.

obtained. No liberation of $^{14}CO_2$ could be detected during the course of the reaction, which indicates that hydrolysis of the amide bonds of monuron did not occur. Thus, non-radioactive products of monuron not detected by this procedure can be only methyl groups and/or chlorine. When the formation of the water-soluble products was completely prevented by using SOD, the radioactivity corresponding to these products was recovered as unchanged monuron, and no other methylene chloride-soluble products were observed. This indicates that methylene chloride-soluble products are side-reaction products rather than photochemically produced intermediates from which the major water-soluble product is formed by the subsequent action of $O_2^{\cdot -}$. Therefore, these structural changes of the original monuron molecule do not appear likely to precede the monuron/$O_2^{\cdot -}$ interaction.

Sweetser has reported that riboflavin or FMN converts monuron to an unidentified inactive product of high molecular weight (22). In the present study, illumination of monuron for 1 hr in presence of riboflavin alone resulted in 50% change of monuron to a methylene chloride-soluble product. This product was not formed when monuron was illuminated in presence of riboflavin and a reductant (methionine or EDTA), nor when the $O_2^{\cdot -}$ was intercepted by SOD. The flavin-monuron photoreaction described by Sweetser is not, thus, the precursor for the monuron/$O_2^{\cdot -}$ interaction either.

Identification of the major water-soluble product from monuron was attempted. The purified product was a yellowish solid with melting and boiling points above 350 C. Due to its high boiling point the mass spectrum of this compound could not be obtained. The UV spectrum resembled that of monuron and *p*-chlorophenylurea (E_2, K, and B aromatic bands),

although the K-band was slightly shifted to a shorter wavelength and the B-band intensified. The UV spectrum indicated that riboflavin was not incorporated into the product.

The compound was soluble in aqueous HCl, NaOH and $HaHCO_3$ without undergoing hydrolysis. The infra red spectrum of the product was simpler than that of monuron and *p*-chlorophenylurea. The product was dechlorinated (no C-Cl band at around 1090 cm^{-1}) and hydroxylated (C-O band at 1245 cm^{-1}). Bathochromic shift (shift to the red) and intensification of the UV bands with changing the solvent from water to aqueous NaOH (pH 13.0) further indicated phenolic character of the product. The N-H stretching bands (around 3300 cm^{-1}) resembled those of *p*-chlorophenylurea rather than of monuron, and thus the product may be demethylated.

The product and monuron were compared for toxicity on 8-day-old seedlings of oats. Both chemicals in 10 μM solutions were applied to roots (nutrient solution) or to foliage (dipping twice). No toxicity symptoms or reduction of dry weight was observed with the product 10 days after application. Monuron reduced the dry weight by 61% when applied to the roots. Neither monuron nor the product reduced the dry weight of the seedlings when applied to the foliage.

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SUMMARY AND CONCLUSIONS

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The photochemical assay system for SOD generating $O_2^{\cdot -}$ from riboflavin/methionine upon illumination and reducing NBT to blue formazan is reliable for determination of the enzyme in crude extracts from plant tissues. The only impurity in the crude extracts which may interfere with this assay is peroxidase. Peroxidase, however, is heat-labile, in contrast to the heat-stable SOD, and thus the interference can be avoided by heating the crude extracts before assaying. One unit of SOD has been defined as the amount that causes 50% inhibition of the NBT reduction, and thus far the enzyme has been quantitated on basis of % inhibition. The kinetics of the assay reaction indicated that % inhibition and enzyme units are not linear. More accurate determination of the enzyme can be made from the ratio of NBT reduction in absence *versus* presence of SOD, and defining 1 unit as the amount of enzyme for which this ratio equals 2. An equation for calculating the enzyme units from this ratio was derived from the kinetics of the reaction and was confirmed with a variety of crude extracts.

Large quantities of SOD were found in shoots, roots, and seeds of corn, oats, and peas. On a dry weight basis shoots contain more enzyme than roots and both shoots and roots more enzyme than seeds. In seeds, the enzyme is present in both the embryo and the storage tissue. In 10-day-old seedlings, SOD accounts for 0.9 to 3.1% of the water-soluble protein. Quantitative differences exist between species and between

organs within a species. Furthermore, development of the SOD activity during seedling growth follows a different pattern in oats than in peas. The enzyme activity increases with age in oats, but not in peas. Conversely, the activity increases with greening in peas, but not in oats.

The extractable SOD of the plant species studied is a copper-zinc enzyme. Manganese-enzyme accounting for less than 5% of the activity may be present in corn. A family of isozymes of the copper-zinc SOD was observed. The isozymic pattern varied with species. Variations in the isozymic pattern were also observed between organs within a species and were explained by the organelle specificity of the isozymes.

The occurrence of SOD in plants indicates that $O_2^{\cdot -}$ is formed in plant tissues. Variations in quantity and in isozymic pattern of SOD with species, organ, and stage of seedling growth may reflect similar variations in the formation of the radical.

Possible involvement of the $O_2^{\cdot -}$ in herbicidal action and metabolism was studied by *in vitro* experiments. The ability of herbicides to either produce $O_2^{\cdot -}$ or to react with $O_2^{\cdot -}$ was examined by using model systems. Paraquat was reduced photochemically or enzymatically and produced $O_2^{\cdot -}$ upon reoxidation. Monuron and diuron were converted to non-toxic water-soluble compounds by photochemically produced $O_2^{\cdot -}$. Formation of $O_2^{\cdot -}$ within plants as a result of herbicide treatment, if it is in excess of the concentration which can be scavenged by the endogenous SOD, may be related to the toxicity of the herbicides. Interaction of herbicides with $O_2^{\cdot -}$ in plants, on the other hand, may be a mechanism of herbicide metabolism. Much more research is needed, however, before conclusions can be made.