INTERACTIONS BETWEEN ASCORBATE AND IRON IN DNA DAMAGE

by

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Interaction between Ascorbate and Iron in DNA Damage

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ABSTRACT

Being a dietary antioxidant, ascorbate (vitamin C) plays an important role in protecting our body against reactive oxygen species attacks. Anti-oxidant actions are, however, only one aspect of ascorbate. In the presence of non protein-bound or "decompartmentalized" iron, ascorbate can be a pro-oxidant. The presence of metals, metal chelating agents, the presence of other anti-oxidants, and the concentration of ascorbate in the tissue also affect whether ascorbate acts as an anti-oxidant or a pro-oxidant. The combined influence of these factors on the actions of ascorbate is not clearly understood.

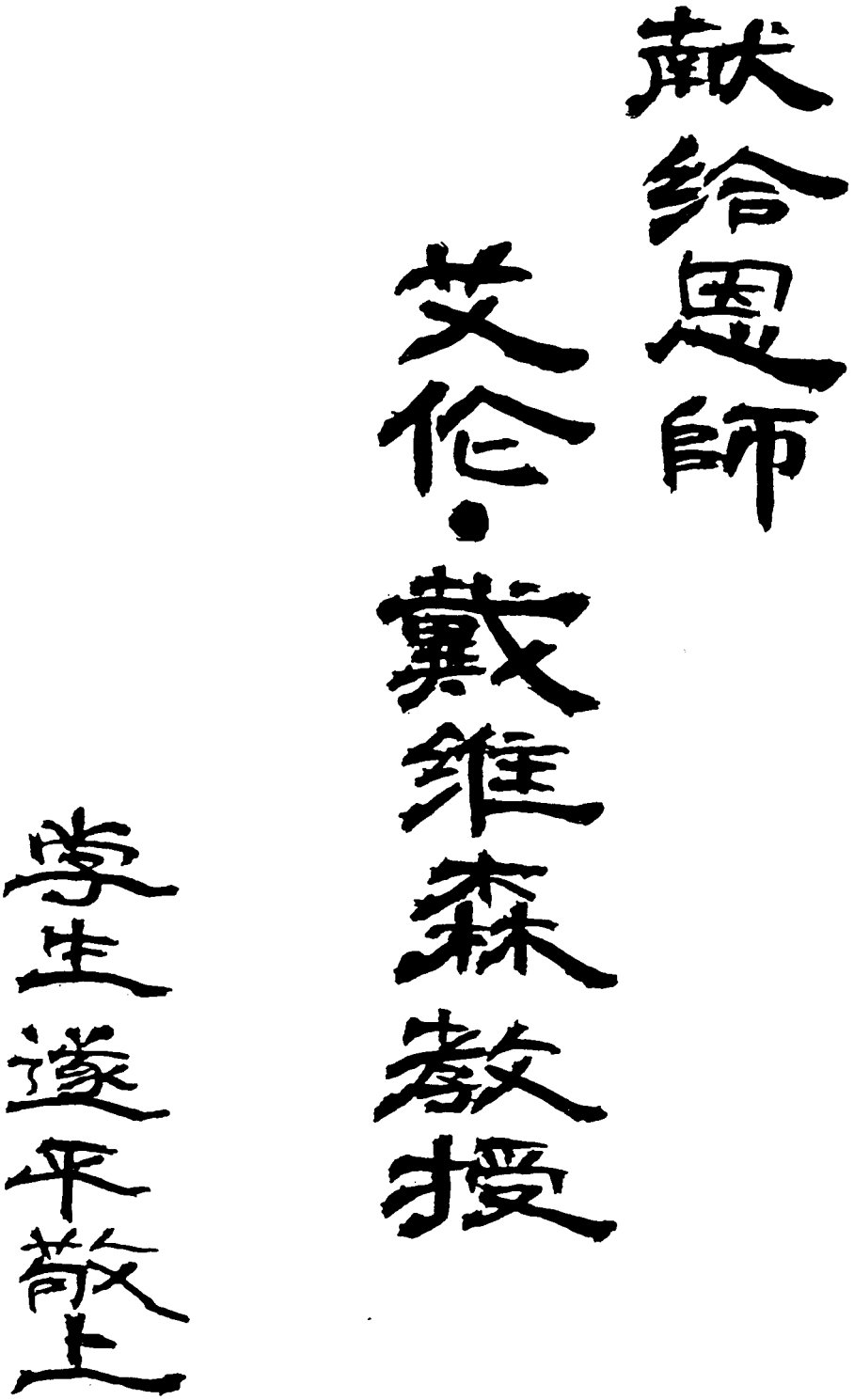
The present study examined the interaction of ascorbate and Fe(III) in DNA damage using supercoiled DNA as a test system. We found Fe(III) alone (up to 320 μM) did not induce significant damage of DNA, but it enhanced the damage of DNA by ascorbate. The metal chelators desferrioxamine (100 μM), ADP (800 μM) and EDTA (500 μM) protected against metal/ascorbate mediated damage to DNA. Among the free radical scavengers tested, catalase (10 U/ml) inhibited the DNA damage by metal/ascorbate, while neither superoxide dismutase (20 U/ml) nor formate nor mannitol (up to 200 μM) protected. The DNA damage by ascorbate or ascorbate plus iron is presumed to occur via metal mediated site-specific mechanisms, and hydrogen peroxide is involved in mediating the damage.

The study also examined the action of Fe(III) on the rates of reaction of ascorbate with oxygen. The effects metal and ascorbate on DNA damage could not be explained on the basis of modified reactivity of ascorbate with oxygen. Iron mediated damage by ascorbate to DNA, resides more in the subsequent reaction of reactive oxygen species and their direction

toward sensitive targets, than in the generation of active species in the ascorbate/oxygen reaction.

DEDICATION

This thesis is dedicated to my Senior Supervisor, Professor Allan Davison who guides me throughout my degree: from stumbling for survival to struggling for success.



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I would like to extend my warmest gratitude to the members of my graduate committee, Dr. Siu Sing Tsang and Dr. Miriam Rosin for their guidance in my experimental phase, assistance and valuable editorial comments in thesis evolution. I much appreciate my coworkers in the Bioenergetic Lab., particularly Anna Li, Brian Bandy, Jim Moon, Luoping Zhang and Eunice Rousseau for their academic help, moral support and friendship over the years. I also thank my parents for their love and support, and my relatives in Canada, for their encouragement and help. Finally, and most important, my greatest debt goes to my wife and my daughter. Their love, patience and tolerance make my dream come true.

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PREFACE

This thesis consists of three parts: a general introduction, a main section in which we present the experimental results and their interpretation, and a general discussion which includes future directions.

In the general introduction, we provide background information regarding oxygen and reactive oxygen species relevant to the iron-mediated reduction of oxygen by ascorbate, and their roles in tissue injury. This section also discusses the defense systems against active oxygen species in biological systems. Thus, the introduction will serve to set the goals of current study in a clear perspective.

This thesis was prompted by the paradoxical roles of ascorbate in iron overloaded patients. There is a dilemma as to whether the decreased ascorbate pool in these patients should be brought back to normal values by supplementation, or further diminished by ascorbate restriction. We therefore discuss the ambivalent roles of ascorbate in patients with hereditary hemochromatosis or thalassemia.

The main section, the data section is the core of the thesis and takes the form of a manuscript to be submitted for publication. The focus of the manuscript is an attempt to define conditions under which ascorbate is beneficial and when it is harmful, and to define the roles of iron in the "crossover" effect ascorbate from protection to damaging actions. Because the most severe consequence facing hemochromatosis patients is cancer, we selected DNA damage as our experimental target system. The manuscript presents the impact of iron on anti-oxidant and pro-oxidant actions of ascorbate at the molecular level, specifically, the

impact of iron on the effects of ascorbate on DNA. It also describes how other factors affect these interactions.

Following initial suggestions from Drs. Tsang and Davison, I took the initiative in the detailed design of the study which I presented as my thesis proposal. I did all the experimental work for this section, processed and graphed the data, undertook the statistic and analysis and wrote the first draft of this manuscript.

In the general, concluding, discussion we consider possible underlying mechanisms and the broader implications of our findings. In this section we also highlight the unsolved questions and suggest future directions for studies in this area.

General Introduction

Oxygen and reactive oxygen species

a. We cannot survive without oxygen

In our everyday life we sometimes fail to recognize how important oxygen is even though we breathe it in and out every minute. Oxygen is so abundant in our surroundings that we take its existence for granted. We can survive without any food for a few weeks and without water for a few days, but without oxygen we will die within minutes. In the course of billions of years of evolution, nature chose oxygen to achieve an energy revolution among living things in their metabolic processes. Except for a few types of organisms that have adapted to living anaerobically (without oxygen), almost all animals and plants need oxygen for efficient energy metabolism. The benefit of using oxygen is obvious when we calculate how many ATP (the currency of energy in biological systems) are produced anaerobically and aerobically from burning one molecule of glucose. Without oxygen, one glucose can only produce two ATP through the anaerobic pathway while in the presence of oxygen, one glucose can produce 36 or 38 ATP via the aerobic pathway. However, everything has two sides and under some circumstances oxygen can cause problems.

b. Molecular oxygen in its ground state is a triplet molecule

The reaction of most biological molecules with ground state molecular oxygen is kinetically unfavorable.[1] According to molecular orbital theory, in an oxygen molecule (Fig. 1),[2] twelve valence electrons of two oxygen atoms compose the molecular orbitals: four

electrons from the 2s subshell of adjacent two oxygen atoms form two sigma orbitals, one pro-bonding (σ_{2s}) and one anti-bonding (σ^*_{2s}), while two electrons from the 2p subshell combine to make another sigma bond (pro-bonding σ_{2p}); Four electrons make two pi bonds (pro-bonding π_{2p}), leaving the last two electron in two pi orbitals (anti-bonding π^*_{2p}).

The last two electrons, which reside in anti-bonding orbitals, are unpaired electrons in that each occupies a molecular orbital by itself. According to Hund's rule, they have the same spin state, i.e. they have parallel spins, or in other words they have the same spin quantum number. The spin quantum value of an electron can be either + ½ or - ½ (the sign indicates the spin direction). Since these two electrons spin in the same direction, the sum (S) of the spin quantum values of two electrons with the same spin state is 1. According to the formula 2S +1, the spin multiplicity of oxygen is 3. That is why oxygen in its ground state is referred to as a triplet molecule. Furthermore, because the two electrons in its outer orbit are unpaired, oxygen is also a free radical. The formal definition of a free radical is any species capable of independent existence that contains one or more unpaired electrons.[3]

Despite being a free radical, oxygen is very stable in its ground state. The two electrons with the same spin state in the outer orbital of oxygen provide a kinetic barrier to its reactions with most biological molecules. If oxygen is to react to form a covalent bond by obtaining two electrons from compounds, these two electrons must also be in the same spin state to fit into the two half occupied π^* orbitals. In the case of an electron pair, this is highly unlikely because, according Pauli's principle, the electrons in the outer shell orbital of most molecules usually have opposite spins. Several biological molecules have been reported to react directly with oxygen, but the reactions are too slow ($k < 10^{-5}$ M^{-1} s^{-1})[1] to be

physiologically significant. For the oxygen to react with potential electron donors, the kinetic barrier must first be overcome. Oxygen must be "activated" before it can be an effective oxidant.

c. Singlet oxygen

Oxygen can be activated in several ways. Among the biologically important processes are (1) activation to singlet state; or (2) relieving the kinetic barrier by transition metals. In the first case, oxygen in its ground state absorbs energy (usually from light) and is transformed to an excited state. In excited states, the configurations of two electrons in anti-bonding orbital(s) of oxygen are different from that of its ground state. They can occupy two or one anti-bounding orbitals (π^*_{2p}). In either case, the two electrons of singlet oxygen have anti-parallel spins (Fig. 2).

Since the two electrons have anti-parallel spins, their spin quanta cancel each other and the spin multiplicity becomes 1, based on the formula 2S +1 (S is zero in this case). That is why oxygen in its excited state is often called singlet oxygen. If two electrons occupy two π^*_{2p}, the singlet oxygen is denoted as $^1\Sigma g$ (Fig. 2a). If two electrons occupy only one π^*_{2p}, the singlet oxygen is denoted as $^1\Delta g$. In both forms of singlet oxygen, the spin restriction is removed. However, only $^1\Delta g$ (Fig. 2b) has biological relevance because $^1\Sigma g$ has higher energy (37.5 kcal per mole) than that of $^1\Delta g$ (23.4 kcal per mole).[1] Consequently it is so unstable that it has only a transient existence, decaying rapidly to the state of $^1\Delta g$.

Singlet oxygen can be generated chemically in the laboratory by the thermal decomposition of endoperoxide 3, 3'-(1,4-naphthylidene) dipropionate ($NDPO_2$),[4] or by

mixing hydrogen peroxide and hypochlorite (OCl^-). You can buy $Na^+ OCl^-$ in the Safeway store as "Chlorox", but do not try this at home:

$$OCl^- + H_2O_2 \Leftrightarrow Cl^- + H_2O + {}^1O_2 \text{ (singlet)} \qquad (1)$$

Singlet oxygen reacts rapidly with compounds containing carbon-carbon double bond, producing endoperoxides and hydroperoxides. These reactions are of importance in lipid peroxidations. Singlet oxygen can directly damage proteins by oxidizing methionine, tryptophan, histidine, or cysteine residues.[3]

d. The role of transition metals in reactions of oxygen

As mentioned before, certain transition metal ions can relieve the kinetic barrier to the transfer of electrons to oxygen. Transition metals are those having partially filled *d* or *f* subshell orbitals. They are "transition" elements because they lie between the "main groups", and are all metals. They have access to a variety of valence and spin states, and this allows them to undergo facile changes in oxidation state. Thus, by accepting and donation a single electron, transition metals can bypass the spin restrictions of molecular oxygen. For example,[5] in the presence of iron, oxygen will undergo reduction and produce superoxide ($O_2\bullet^-$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($\bullet OH$):

$$Fe^{2+} + O_2 \Leftrightarrow Fe^{3+} + O_2\bullet^- \qquad (2)$$

$$Fe^{2+} + O_2\bullet^- \Leftrightarrow Fe^{3+} + H_2O_2 \qquad (3)$$

$$Fe^{2+} + H_2O_2 \Leftrightarrow Fe^{3+} + OH^- + \bullet OH \qquad (4)$$

$$Fe^{2+} + \bullet OH \Leftrightarrow Fe^{3+} + OH^- \quad (5)$$

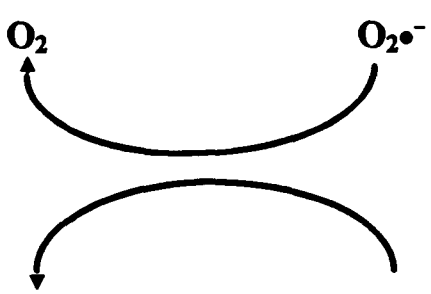
If we reverse the reaction (2), the net result of (2) and (4) will be:

$$Fe^{3+} + O_2\bullet^- \Leftrightarrow Fe^{2+} + O_2 \quad (2)$$

$$Fe^{2+} + H_2O_2 \Leftrightarrow Fe^{3+} + OH^- + \bullet OH \quad (4)$$

$$\text{Net: } O_2\bullet^- + H_2O_2 \Leftrightarrow O_2 + OH^- + \bullet OH \quad (6)$$

Reaction (4) is also called the Fenton Reaction and (6) is referred to as the Haber-Weiss reaction or a superoxide-driven Fenton reaction. The reason why the Haber-Weiss reaction is also called a superoxide-driven Fenton reaction is self evident when we redraw it the following way:



$$Fe^{2+} + H_2O_2 \Rightarrow Fe^{3+} + OH^- + \bullet OH$$

Clearly, superoxide will push the reaction toward the written direction by removing the product (Fe^{3+}) and adding the reactant (Fe^{2+}). In other words, superoxide recycles the

reactant on the left side of reaction by reducing product Fe^{3+} back to Fe^{2+}. The above reaction will continue if superoxide or another reducing agent, such as ascorbate, is available in the system. This also implies that the Haber-Weiss reaction will occur at a much faster rate in the presence of transition metals like iron.

e. Reactive oxygen species and free radicals

Singlet oxygen, and the products of the above reactions, such as $O_2\bullet^-$, H_2O_2, and •OH are called reactive oxygen species because they are usually generated as intermediates in the redox process of oxygen. Among them, $O_2\bullet^-$ and •OH are free radicals for they contain unpaired electrons in their outer orbitals. The unpaired electrons make them very reactive. They seek electrons from electron rich compounds, such as lipids, proteins, and DNA, to pair with their *unpaired* electrons so that they achieve a more stable state. Furthermore, once a single free radical is generated, it can initiate a chain reaction which is propagated and the whole process will continue until the termination reactions dominate and bring the reaction to an end:

Initiation:

$$M^n + H_2O_2 \Leftrightarrow M^{(n+1)} + OH^- + \bullet OH$$

Propagation (by H• abstraction):

$$\bullet OH + RH \Leftrightarrow H_2O + R\bullet$$

$$R\bullet + H_2O \Leftrightarrow \bullet OH + RH$$

Termination:

$$\bullet OH + \bullet OH \Leftrightarrow H_2O_2$$

$$R\bullet + R\bullet \Leftrightarrow R\text{-}R$$

$$R\bullet + \bullet OH \Leftrightarrow R\text{-}OH$$

Free radical reactions provide useful biological weapons in infected cells or tissues. For example, during phagocytosis superoxide is released, then the hydrogen peroxide formed from superoxide through dismutation can quickly kill some strains of bacteria. Hydroxyl radicals generated from superoxide and hydrogen peroxide through the Haber-Weiss reaction can also kill bacteria.[3] In neutrophils, hydrogen peroxide, in the presence of myeloperoxidase, reacts with chloride to produce a powerful oxidant (hypochlorous acid) which plays a critical role in the destruction of invading pathogens.[6] The antimalarial response in host organisms also depends in part on phagocyte-derived oxidants.[7]

The role of free radicals in disease and aging

The adverse biological effects of free radicals have been well documented over the past two decades. Free radicals are involved in cancer,[8],[9],[10] atherosclerosis,[11],[12] inflammation,[13],[14] ischaemia/reperfusion injury,[15],[16] hemochromatosis,[17],[18] autoimmune disease, exercise-induced oxidant damage, lung damage, and adult respiration distress syndrome.[3] Free radicals also play an important role in aging processes.[19]] They attack electron rich molecules, and can injure cells and tissues.[20]

Hydroxyl radicals generated from the Fenton reaction or other pathways are reactive enough to attack almost any biomolecules they encounter near the site where they are

produced. Although superoxide is relatively non-reactive in an aqueous solution, it can form hydroxyl radicals through the Haber-Weiss reaction. Hydrogen peroxide is not a free radical, but is a reagent of the Fenton reaction and is able to generate hydroxyl radicals when reduced forms of any of several transition metals are available in the system. These species act together causing DNA damage, oxidation of essential residues of polypeptide or protein, and lipid peroxidation. These actions in turn lead to altered gene expression, protein dysfunction, and membrane damage. Thus, functions of cells and enzymes are disrupted in response to "oxidative stress" tissue injury (Fig. 3).

Protection against free radical toxicity in biological systems

a. Enzymes

Facing the continuous bombardment of free radical attack, cells have developed defense systems to prevent associated damage. Cells produce anti-oxidant enzymes (including superoxide dismutase, catalase, and glutathione peroxidase) to deal with free radicals generated from biological process.

Superoxide dismutase can remove superoxide radical ions from a system by disproportioning them into hydrogen peroxide and oxygen:

$$O_2\bullet^- + O_2\bullet^- \longrightarrow H_2O_2 + O_2 \qquad (7)$$

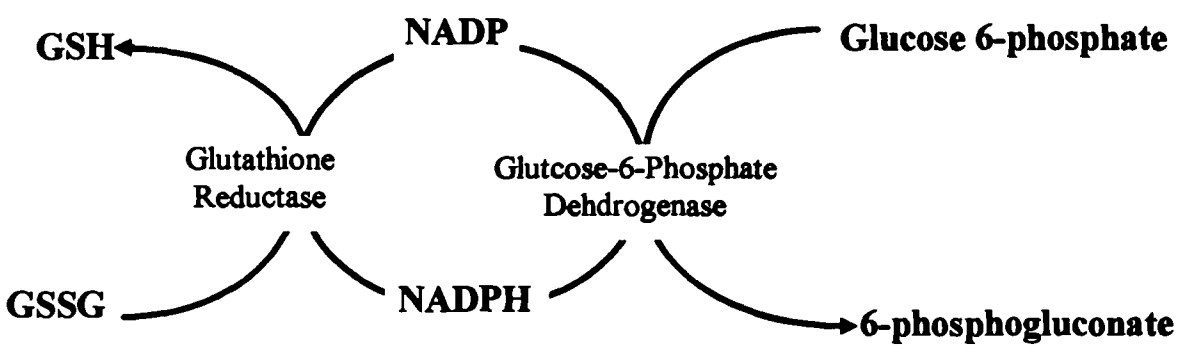
The hydrogen peroxide generated from the above reaction can be removed by catalase through the following reaction:

$$2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2 \qquad (8)$$

Glutathione peroxidase also plays an important role in removing hydrogen peroxide:

$$2GSH + H_2O_2 \xrightarrow{\text{glutathine peroxidase}} GSSG + 2H_2O \quad (9)$$

The oxidized glutathione (GSSG) can be re-reduced to GSH at the expense of NADPH recycled primarily through the oxidative pentose phosphate pathway:



b. Low molecular weight anti-oxidants

Some small molecules, such as ascorbic acid (vitamin C), α-tocopherol (vitamin E), β-carotene, and uric acid, also protect against free radical damage.

One of these anti-oxidants, ascorbic acid is an important essential nutrient for humans. Lack of ascorbic acid in the human diet induces a severe disease called scurvy, a deadly killer among sailors in earlier times. Since ascorbic acid exists in a anionic form at physiological

media, it is most accurately termed ascorbate where referring to the dissolved substance at neutral pH (Fig. 4).

Biologically, the most important chemical property of ascorbate is its ability to act as an electron donor. It is a powerful water soluble anti-oxidant and can react rapidly with superoxide and hydroxyl radicals. In such reactions, ascorbate itself is oxidized to the semidehydroascorbate (or ascorbyl) radical (Fig. 5a). The semidehydroascorbate, which is stable enough to be radical, is relatively harmless. It can either undergo disproportion to ascorbate and dehydroascorbate or be further oxidized to dehydroascorbate (Fig. 5b). Dehydroascorbate and semidehydroascorbate can be reduced back to ascorbate at the expense of reduced glutathione under the enzyme dehydroascorbate reductase:[3]

$$\textbf{dehydroascorbate} + \textbf{2GSH} \xrightarrow[\text{reductase}]{\text{dehydroascorbate}} \textbf{GSSG} + \textbf{ascorbate} \qquad (10)$$

$$\textbf{2 semidehydroascorbate} + \textbf{2GSH} \xrightarrow[\text{reductase}]{\text{dehydroascorbate}} \textbf{GSSG} + \textbf{2 ascorbate} \qquad (11)$$

In addition to its anti-oxidant effect in aqueous environments, ascorbate can function synergistically with the lipophilic anti-oxidant α-tocopherol (vitamin E) to protect cell membranes from free radical damage.[21] Furthermore, ascorbate scavenges a range of oxidants including hypochlorous acid (HOCl), singlet O_2, superoxide ($O_2\bullet^-$), hydroxyl radical (•OH), thiyl radicals (S•), sulphenyl radicals R-S•), and nitroxide radicals ($NO_2\bullet$).[22, 23]

Balz Frei and coworkers showed that ascorbate could completely protect blood plasma from detectable peroxidative damage induced by aqueous peroxyl radicals. On the

basis of their data they suggested increasing the recommended dietary allowance (RDA) for ascorbate from the current 60 mg to 150 mg to maximize its beneficial effects.[24] The late Linus Pauling, a two-time winner of Nobel prize, encouraged consumption of even larger amounts of supplementary ascorbate. He recommended that for most people the optimum daily intake was somewhere between 250 mg and 10 g.[25]

However, the matter does not rest there. Ascorbate occasionally has clinically deleterious actions. Supplementary vitamin C is counter-indicated in patients with hemochromatosis or β-thalassemia. In purified chemical systems, ascorbate can readily be shown to damage cells and cellular constituents. A mechanism for the cytotoxicity of ascorbate is readily available. Since ascorbate is a reducing agent, it can reduce Fe^{3+} to Fe^{2+}, increasing the likehood that it will become decompartmentalized in these iron-overloaded patients. This sometimes causes problems because in the presence of hydrogen peroxide the reduced iron can produce hydroxyl radicals through the Fenton reaction. Of lesser importance in treated patients, reducing Fe^{3+} to Fe^{2+} helps gastrointestinal tract absorb iron, which is no good for those who already have too much iron in their bodies. Another mechanism for ascorbate induced toxicity is its ability to inactivate catalase.[26] Since the cytotoxicity of ascorbate is oxidant mediated, anti-oxidants might be predicted to protect these patients against toxic actions of ascorbate.

α-Tocopherol and β-carotene are the primary dietary fat soluble anti-oxidants. α-tocopherol plays an important role in preventing oxidant injury to polyunsaturated fatty acid and thiol-rich protein constituents of cellular membranes and the cytoskeleton.[27] β-carotene, a less effective radical scavenger, is nevertheless one of the most efficient quenchers of singlet

oxygen and plays a crucial role in protecting against UV light photoxicity. Its anticarcinogenic effect is also well documented although with some controversies.[28] Uric acid, the end product of purine metabolism, is also an important anti-oxidant in biological fluids. It can scavenge singlet oxygen, peroxyl radicals, and hydroxyl radicals.[29]

c. Proteins

The role of transition metals in free radical generation has already been discussed. Fortunately, under normal conditions, redox active transition metals in our body are tightly bound by proteins. For example, iron in our body is bound as hemoglobin, myoglobin, some enzymes such as catalase, iron-transferrin, and ferritin. Copper is bound by ceruloplasmin and several enzymes. Albumin, some small peptides, and histidine also sequestrate these metals. The bound transition metals do not participate in catalyzing the free radical reactions. Nevertheless, if these metals are released from their binding proteins ("decompartmentalized"), as in iron over-loaded diseases, they can trigger free radical reactions and cause clinical problems.

Hemochromatosis

a. Abnormal accumulation of iron in hemochromatosis

In human body approximately two thirds of irons in our body is bound in functional form as a prosthetic group of hemoglobin, myolglobin, and some enzymes. The other one third of iron is nonfunctional, but also bound ("compartmentalized") to transport or storage proteins like transferrin and ferritin. Hemoglobin and myoglobin carry oxygen in our bodies. They utilize iron as an oxygen binding site. Iron acts as a coenzyme for cytochromes,

peroxidase, and catalase. Transferrin is the transporting protein for iron which carries it to its destination when needed. Ferritin is a stored form of iron. In the body of a healthy person, only 20-30% of ferritin is saturated, so the concentration of non-protein-bound iron is zero.[30] In iron overloaded diseases, such as hemochromatosis, things are different. Due to the continued, excessive absorption of dietary iron, ferritin becomes more saturated. Under a range of stresses the excess iron in these people can be released. The decompartmentalized iron can participate in free radical reactions that generate oxidant stress and induce tissue damage.[31]

b. Do patients with hemochromatosis need more ascorbic acid?

There is evidence of oxidant stress in patients with hemochromatosis. For example, levels of ascorbate in plasma of hemochromatosis is 51.3 μmol/l, a little more than half the normal 89.1.[32] This observation rouses the question: do patients with hemochromatosis need to take more ascorbate to compensate for increase its anti-oxidant demand?

In other oxidant stress conditions such as smoking, the ascorbate level of serum is also lower than in normal subjects.[33] Smokers are advised to increase their dietary intake of ascorbate to fight the oxidant stress. The RDA of ascorbate for smokers was recently increased from 60 to 100 mg. A study by Schectman *et al.* has shown that even this amount might not be adequate. Smokers might need to consume more than 200 mg ascorbate to achieve the same level of ascorbate found in non-smokers consuming the RDA (60 mg).[34] Since serum concentrations of ascorbate are lower in hemochromatosis, these patients may also need to increase their dietary intake of ascorbate to increase its serum level to fight the stress. Neinhuis, however, disagreed. He suggested that the lower level of ascorbate in the

iron overloaded population represents a defensive mechanism.[35] These people might benefit from lower intakes of ascorbate. His hypothesis is that lower levels of ascorbate diminish the release of iron from reticuloendothelial cells into the blood, and thus diminish free radical generation in the Fenton reaction.

Another argument against increasing ascorbate intake in patients with hemochromatosis is that ascorbate can accelerate iron uptake from the gastrointestinal tract in patients with hemochromatosis who are already iron overloaded. However, the force of this argument is diminished in treated patients.[36] The iron loss from phlebotomy dwarfs all possible levels of iron absorption. Consequently, any change in the rate of absorption of iron is inconsequential. It is more plausible that in hemochromatosis ascorbate acts as a pro-oxidant as a result of the presence of non-protein-bound iron because it readily produces cytotoxic oxygen species.

Minetti *et al.* have shown that after iron concentrations reach or exceed the plasma latent iron-binding capacity, the production of ascorbate free radicals increases.[37] On this basis, the damaging actions of ascorbate can be explained by its ability to reduce Fe^{3+} to Fe^{2+}, resulting in formation of hydroxyl radicals through the Fenton reaction.

The accumulation of Fenton reactive hydrogen peroxide is also enhanced by the presence of ascorbate. Ascorbate reacts "directly" with oxygen to produce dehydroascorbate and hydrogen peroxide. Any peroxide so formed is more likely to persist because of the ability of ascorbate to inhibit catalase. Ascorbate inactivates catalase and this is accelerated by the presence of copper.[26],[38],[39]

Due to the ambivalent effects of ascorbate, sound recommendations regarding this nutrient for patients with hemochromatosis are not available. These patients presumably need

more anti-oxidants to relieve the oxidant stress induced by iron overload. On the other hand ascorbate supplements reportedly aggravate the symptoms of this disease,[35][40][41] possibly because in the presence of iron ascorbate acts as a pro-oxidant. Thus, whether patients with hemochromatosis should increase or decrease their ascorbate intake is a urgent issue waiting to be answered.

c. The impact of ascorbate on hemochromatosis

The impact of ascorbate on hemochromatosis is currently controversial. Hershko *et al.* take the stance that ascorbate has adverse effects on hemochromatosis.[42] Halliwell and others have shown that incubating ferritin with ascorbate induces the reductive release of Fe^{2+} from the protein and that the reduced Fe^{2+} catalyzes the free radical reactions.[3] Hoffman *et al.*, however, have shown that ascorbate delays iron release from ferritin by retarding ferritin degradation.[43] On this basis, ascorbate should be beneficial in hemochromatosis because iron in ferritin can leave the body when epithelial cells containing ferritin are shed from the gastrointestinal tract. In addition, ascorbate can increase urinary iron excretion induced by desferrioxamine, again suggesting a beneficial role in hemochromatosis.[44][45]

The model that emerges for tissue injury in hemochromatosis is one in which free iron concentrations sporadically reach the threshold level for damage, in particular tissues (liver, pancreas, articular cartilage or heart). This event triggers a cascade of responses in which (1) ascorbate or other cellular reductants "cross-over" from being protective to becoming damaging, (2) catalase inhibition (initially reversible by one-electron reduction) removes one barrier to hydrogen peroxide accumulation, (3) hydrogen peroxide irreversibly denatures hemoproteins, including catalase and myoglobin, (4) in the presence of hydrogen peroxide

irreversible inhibition of catalase further predisposes to hydrogen peroxide accumulation, yielding a dramatic increase in Fenton reactive iron. In this model we see hemochromatosis pathology advancing in a series of sporadic, local crises, self-perpetuating by the above positive feedback mechanisms

The effect of reaction conditions on the reactivity of ascorbate

Several lines of approach confirm that reaction conditions modulate the extent to which ascorbate acts as an anti-oxidant or as a pro-oxidant. These conditions include the order of addition of reagents, ascorbate concentration, metal chelators, and the presence of other anti-oxidants. In the redox cycling autoxidable neurotoxin, 6-hydroxydopamine, the order in which agents are added determines whether ascorbate acts as an anti-oxidant or as a pro-oxidant in damaging catacholaminergic nerve terminals.[46] When ascorbate was added to the medium before dopamine, it scavenged superoxide radicals and suppressed oxygen consumption by 92 to 96%. When ascorbate was added to the medium after dopamine, however, the ascorbate facilitated redox cycling of a pre-melanin oxidation product derived from dopamine, and amplified both oxygen consumption (by 640%) and production of hydrogen peroxide.

Ascorbate at low concentrations increased lipid peroxidation; while at high concentration, it inhibited. For example, Rees and Slater have shown that at micromolar levels ascorbate increases lipid peroxidation by both carbon tetrachloride and cumene hydroperoxide, but at millimolar levels ascorbate diminishes lipid peroxidation, or even protects.[47] Rousseau from our laboratory also has observed the ascorbate "cross-over" effect in her studies of lipid peroxidation in homogenates of retina.[48]

Sees and Slater pointed out that at low concentrations ascorbate acts mainly as a pro-oxidant by reducing the transition metals in the system, resulting in the production of the strongly oxidizing hydroxyl radicals and increase of lipid peroxidation. At high concentrations, due to its ability to form relative stable semi-dehydroascorbate radical in the scavenging reaction that removes the reactive radicals, the anti-oxidant actions of ascorbate become dominant. At millimolar levels, ascorbate can directly scavenge •OH.[3],[47] These actions are chain terminating since they reduce some of the lipid peroxyl radicals to hydroperoxide.

This cross-over effect of ascorbate may also relate to the Fe^{3+}/Fe^{2+} ratio which is an important factor in lipid peroxidation. Aust *et al.* have found that both Fe^{3+} and Fe^{2+} are needed for lipid peroxidation to occur. When the Fe^{3+}/Fe^{2+} ratio is equal, the maximal rate is achieved.[49],[50] They argue that at low concentrations ascorbate reduces some Fe^{3+} to Fe^{2+} and maintain an Fe^{3+}/Fe^{2+} ratio appropriate for lipid peroxidation. At high concentrations, ascorbate disturbs the optimum Fe^{3+}/Fe^{2+} ratio by reducing all Fe^{3+} to Fe^{2+}, resulting in inhibiting lipid peroxidation.

As mentioned before, the direct reaction of oxygen with ascorbate is spin forbidden. Buettner's study shows that without catalytic metal ions, ascorbate is very stable at pH 7.[51] Transition metals, such as iron and copper, can relieve the spin restriction of dioxygen, releasing ascorbate oxidation, and triggering free radical reactions. Metal chelators inhibit the autoxidation of ascorbate catalyzed by metals and protect oxidant sensitive tissue constitutes.[52]

Desferrioxamine is a powerful metal chelator. It can be used to remove excess iron from the bodies of patients with hemochromatosis. Because ascorbate increases the urinary iron excretion induced by desferrioxamine, it has been used together with desferrioxamine in

treating hemochromatosis. There is another risk, however. Like ascorbate, desferrioxamine, can also act as a paradoxical pro-oxidant.[53] For example, desferrioxamine can stimulate generation of hydrogen peroxide from Fe^{2+}, causing damage.

The objective of the thesis

The overall goal of this study is an initial attempt to determine the conditions under which ascorbate is beneficial and when it is harmful. In order to answer this question we pursued several detailed objectives: (1) Does the ascorbate alone damage DNA or are both ascorbate and iron needed to induce the damage? (2) If iron is needed, how does it alter the effects of ascorbate? (3) When ascorbate (alone or in the presence of iron) causes damage, what are the mechanisms? (4) Is the concentration of ascorbate significant in determining its action? In other words, we are looking for a rational understanding of reaction conditions under which ascorbate is maximally effective as an anti-oxidant.

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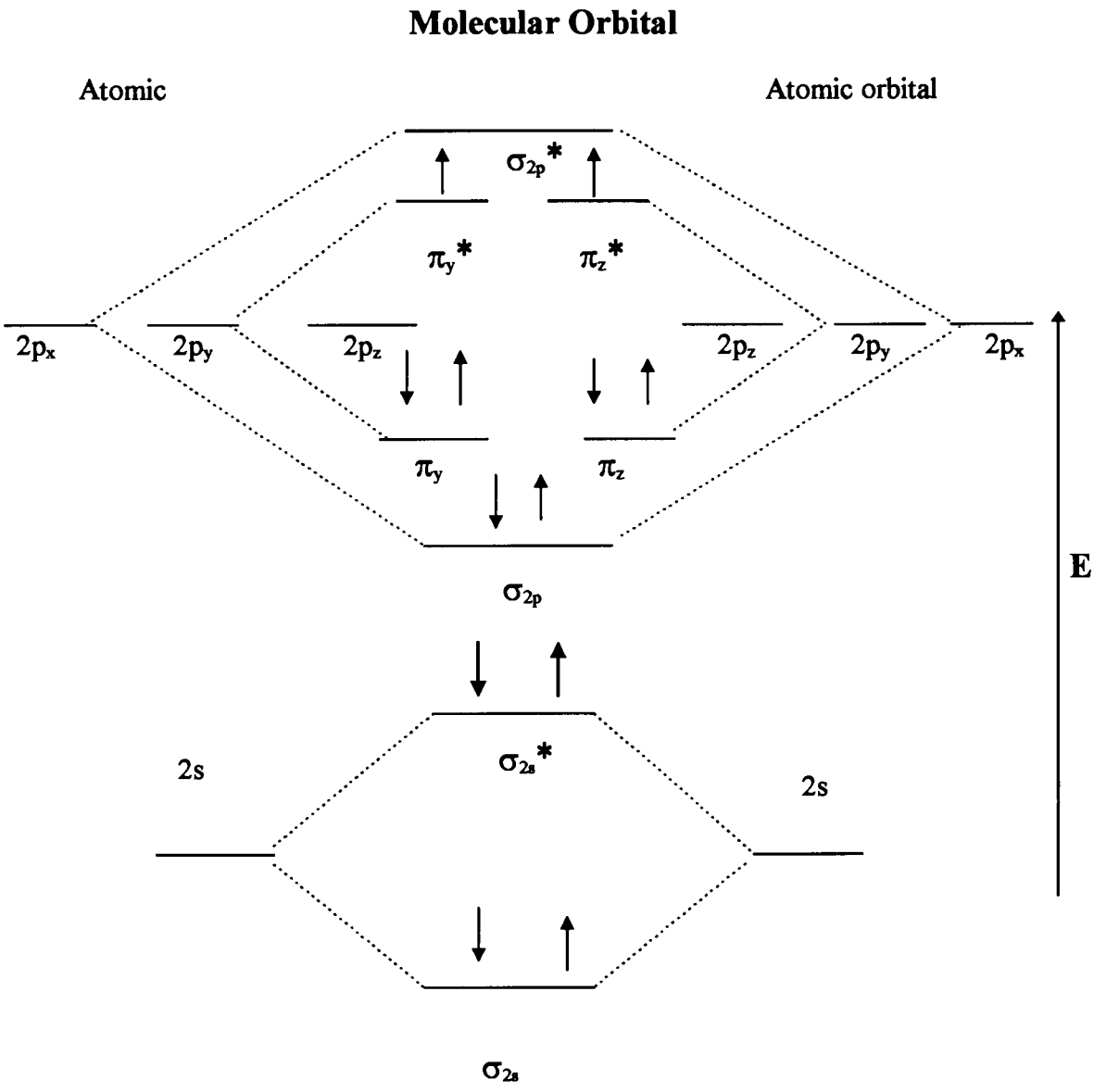


Fig. 1 Molecular orbital diagram of ground state oxygen

(Adapted from Stanle RR and Marjior HN, 1990)*

* Stanley, R. R., and Marjorie, H.N. eds. *Chemistry*, West publishing Company, 1990

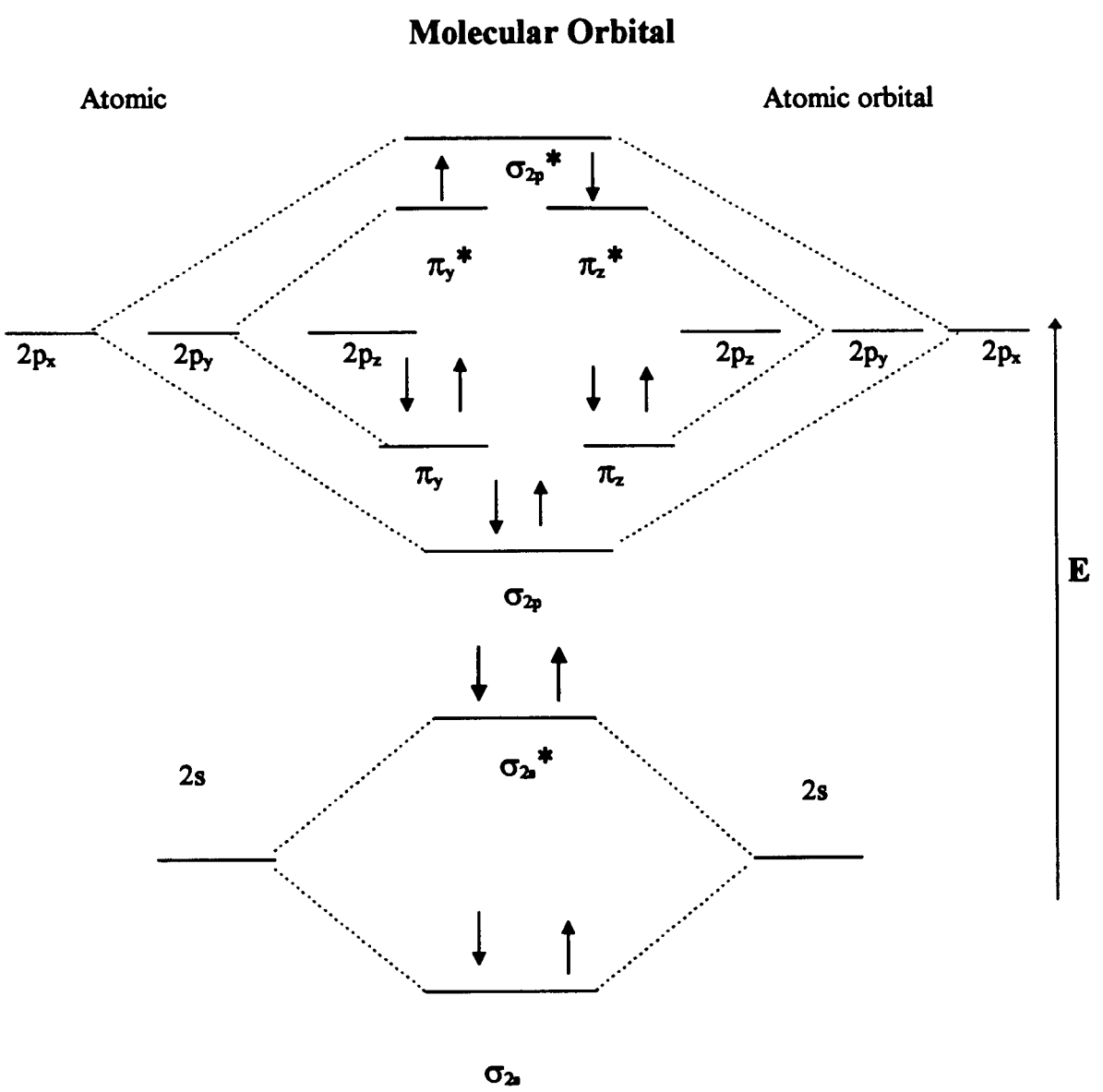


Fig. 2a Singlet State Oxygen ($^1\Sigma g$)

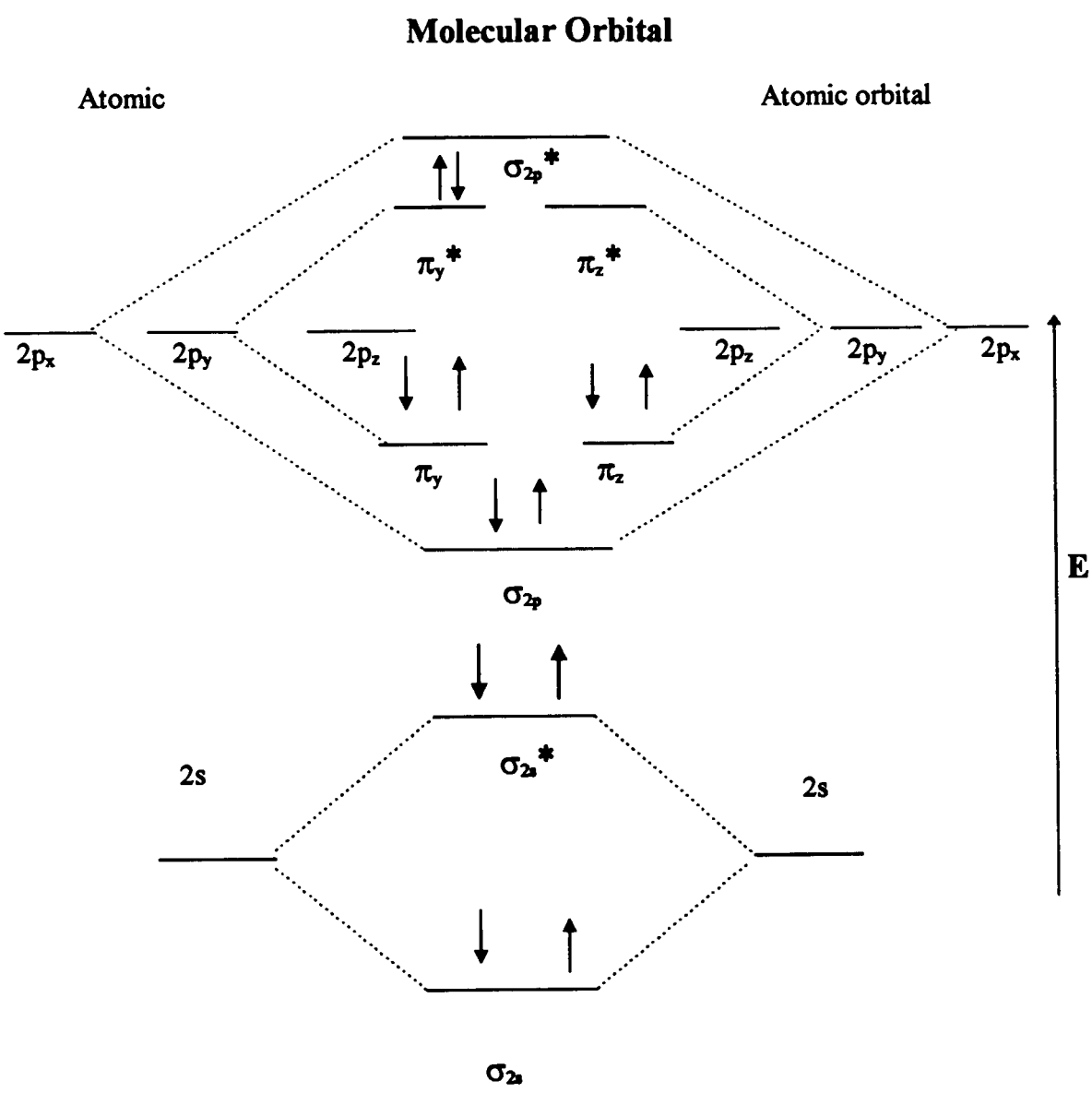


Fig. 2b Singlet State Oxygen ($^1\Delta g$)

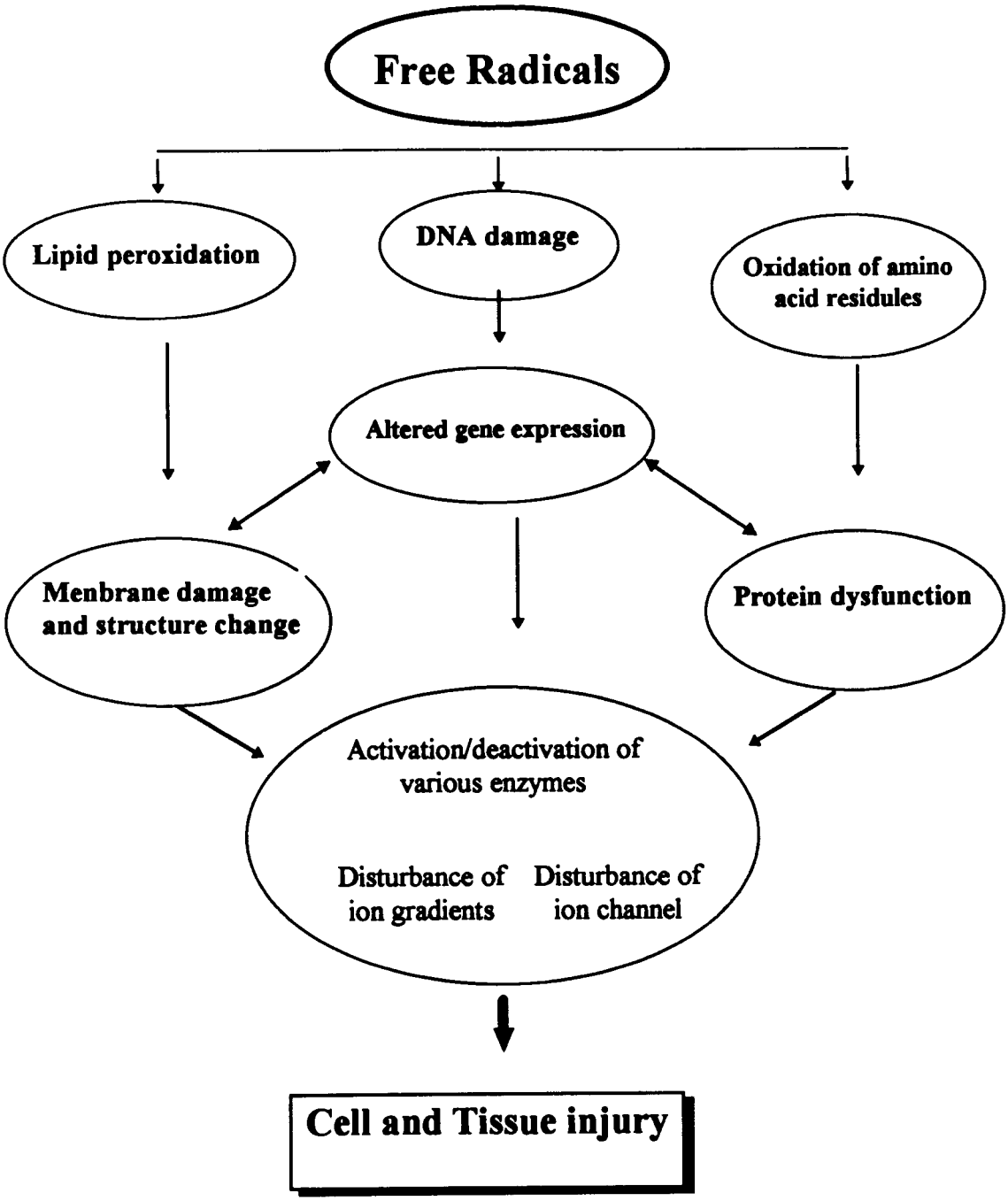


Fig 3 Mechanism of free radical toxicity

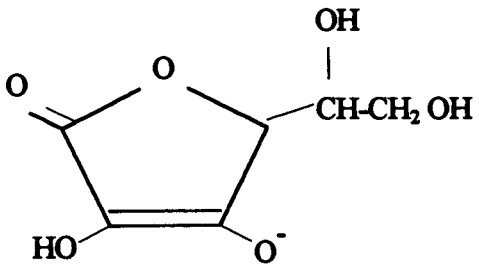
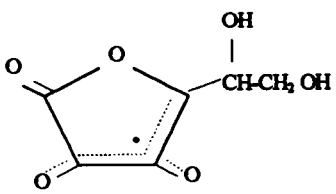
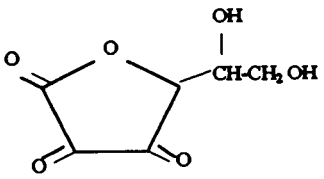


Fig. 4 Structure of Ascorbic Acid (AH^-)



a

semidehydroascorbate radical (A^-)



b

dehydroascorbate (A)

Fig. 5 Products of oxidation of ascorbate

INTERACTIONS BETWEEN ASCORBATE AND IRON IN DNA DAMAGE

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Abstract

The dual role of ascorbate as both an anti-oxidant and a pro-oxidant is well documented, but the conditions that determine when ascorbate serves as an anti-oxidant and when its pro-oxidant effects become dominant have not yet been fully defined. The present study emphasizes the impact of iron on the anti-oxidant and pro-oxidant properties of ascorbate in the nicking of supercoiled PM2 phage DNA.

At concentrations up to 320 μM, Fe(III) did not induce significant damage of DNA, but it enhanced the damage of DNA by ascorbate. The metal chelators desferrioxamine (100 μM), ADP (800 μM) and EDTA (500 μM) protected. Among the free radical scavengers tested, catalase (10 U/ml) inhibited DNA damage by ascorbate or ascorbate plus Fe(III), while neither superoxide dismutase (20 U/ml) nor formate nor mannitol (up to 200 μM) affected this damage. The DNA damage by ascorbate or ascorbate plus Fe(III) is presumed to occur via metal-mediated site-specific mechanisms with hydrogen peroxide mediating the damage.

Introduction

Ascorbate (vitamin C) is a water soluble anti-oxidant that reacts rapidly with superoxide and hydroxyl radicals.[1] It also scavenges other oxidants including hypochlorous acid (HOCl), singlet O_2, superoxide ($O_2\bullet^-$), hydroxyl radicals ($\bullet OH$), thiyl radicals ($S\bullet$), sulphenyl radicals ($R\text{-}S\bullet$), and the nitroxide radicals ($NO_2\bullet$).[2],[3] In addition to anti-oxidant effects in aqueous environments, ascorbate is synergistic with the lipophilic anti-oxidant α-tocopherol (vitamin E) in protecting cell membranes against free radical damage.[4] Paradoxically, ascorbate can act as a pro-oxidant in the presence of transition metals. For example, the toxic and clastogenic effects of vitamin supplements correlate closely with the amounts of ascorbate and iron in these preparation.[5] The phenomenon of a substance's action being changed from an anti-oxidant to a pro-oxidant (or vice-versa) has been called the "cross-over effect".[6]

The genotoxicity induced by iron in the presence of ascorbate may be relevant to the high incidence of cancer (hepatocellular carcinoma) in patients with hereditary hemochromatosis. In these patients some of the symptoms of iron toxicity are known to be exacerbated by ascorbate. To provide a more detailed understanding of the genotoxicity of iron/ascorbate we sought to investigate mechanisms of the impact of iron on the clastogenicity of ascorbate toward DNA *in vitro*. Specifically, we added anti-oxidant enzymes, synthetic metal chelators, and selected free radical scavengers to the reaction mixture, intending to provide an initial characterization of the mechanism. Since Fe(II) can be auto-oxidized in phosphate buffer solutions, we chose Fe(III) in our experiments to allow a clearer interpretation of the actions of ascorbate, as the sole reductant present.

Supercoiled DNA from bacteriophage PM2 is a sensitive and commonly used target test system for oxidative DNA scission. Damage to supercoiled DNA is readily visualized using agarose gel electrophoresis. In electrophoresis on gel various species of DNA move at different rates according to their shape, size, and electric charge. Thus, supercoiled circular DNA (being more compact) migrates faster than linear and nicked DNA. After staining with 0.5 μg/ml ethidium bromide for 20-40 minutes, the respective DNA bands can be visualized under UV-light and quantified using densitometric scanning of the photographic positives.

The anti-oxidant enzymes used in current study included superoxide dismutase and catalase. Metal chelators used were desferrioxamine, EDTA, and ADP. Hydroxyl radical scavengers used in the study included formate and mannitol.

Materials and Methods

a. Reagents

Ferric chloride and disodium ethylenediamine-tetraacetate (EDTA) were purchased from Fisher Scientific (Fair Lawn, N. J.). L-ascorbate, superoxide dismutase (4050 U/mg), Formic acid (sodium salt), D-mannitol, and adenosine diphosphate (ADP) were ordered from Sigma Chemical Co. (St. Louis, MO). Catalase (65, 000 U/mg) came from DDI Pharmaceutical Inc. (Dorval, PQ) and desferrioxamine from Ciba-Geigy Pharmaceutic Co. (Summit, NJ).

All the solutions and buffer were prepared with deionized distilled water. The buffer was treated with Chelex 100 and then filtered with polypropylene Econo-Columns from Bio-Rad Laboratories (catalog number 731-1110, columns sizes: 0.7 X 4 cm). Solution of ferric chloride and ascorbate were freshly prepared prior to every experiment. Heat denatured catalase was prepared by heating the enzyme at 100°C for 30 minutes while the hydrogen peroxide denatured superoxide dismutase was inactivated by treating it with 10 mM hydrogen peroxide in 0.1 M sodium bicarbonate at pH 9.5, 23°C for 1.5 hour.

b. Preparation of PM2 bacteriophage DNA

Pseudomonas Bal-31 bacteria in 1000 ml Bal-broth medium [20 ml of 10 mM Tris-HCl (pH 7.5), 24 gm magnesium sulfate ($MgSO_4.7H_2O$), 52 gm sodium chloride, 16 gm bacto-nutrient broth, 20 ml 1 M calcium chloride and 7 ml 20% potassium chloride per liter of deionized water] were cultured at 29°C with vigorous stirring. When the optical density of culture reached 0.4 (about 3×10^8 of bacteria per milliliter), the bacteria were infected with

PM2 phage at a ratio of bacterium to phage 1:10. Then the culture was incubated at 29°C overnight. The phage was purified by CsCl gradient centrifugation as described by Espejo and Canelo[7] and modified by Tsang.[8] Phage particles were collected, dialyzed extensively against 1.5 liter of buffer [0.02 M Tris-HCl (pH 7.5), 0.1 M NaCl, 1 mM EDTA] at 4°C, and then lysed by adding 10% sodium dodecyl sulfate. The phage DNA was extracted in the aqueous phase by phenol.

The concentration of DNA was determined by measuring the absorbance at 260 nm. and calculated by the following equation: (optical density reading at 260 nm – optical density reading at 310 nm) x 50/volume of DNA sample (ml). This equation assumes that 1 optical density corresponds to 50 μg of DNA. A typical bacterial culture yielded approximately 400 μg of PM2 DNA per liter of culture.

c. Detection of DNA breakage by agarose gel electrophoresis

All reactions were carried out in potassium phosphate buffer (pH 7.4, 50 mM*) under ambient oxygen pressure. The total volume of reaction mixture was 10 μl. Briefly, after incubating DNA with reagents in an Eppendorf tube, 2 μl of dye containing 0.05% bromophenol blue and 6% glycerol was added. Then 6 μl of the mixture from each sample was loaded into a agarose gel well, and fractionated by electrophoresis at 60 volts in 0.7% (w/v) agarose gels in TAE buffer [0.04 M Tris-acetate (pH 7.4), and 0.001 M EDTA] for 90 to 120 minutes. The gel was stained with 0.5 μg/ml ethidium bromide for 20 minutes. The

* When DNA was incubated with high concentration (up to 25 mM) of ascorbate, the concentration of buffer was increased to 200 mM in order to buffer the acidity of ascorbate.

DNA bands were visualized under illumination by UV-light. A Polaroid MP-4 camera and Polaroid 57 or 55 (with negative) professional films were used for the photograph.

d. Quantification of DNA Breakage by Densitometry Scanning

The percentage of supercoiled, nicked circular and linear DNA was quantified using densitometric scanning of the photographic positives.

e. Analysis of Oxidation of Ascorbate by Spectrophotometric Assay

Ascorbate stock solution was prepared with argon-saturated deionized distilled water and sealed under a slight positive pressure of argon. Aliquots were removed through a rubber septum with a Hamilton gas-tight syringe. Ascorbate oxidation was followed spectrophotometrically in 50 mM air-saturated potassium phosphate buffer (pH7.4), by adding ascorbate stock solution to the buffer to give an initial concentration of 100 μM. The rate of oxidation of ascorbate was determined from the loss of absorbance at 265 nm (E_{265} = 15000 M^{-1} cm^{-1}).

f. Statistical Analysis

The level of confidence required for significance was selected in advance to be $p < 0.05$. Each experiment was replicated two or three times, but some internal controls, like the effect of ascorbate on DNA in the absence and presence of iron, were repeated daily through the experimental period, to provide an accurate estimate of variability and a consistent basis for making comparisons across experiments. Conclusions of most of experimental results were based on replications of the entire experimental protocol, and inferences were drawn where there was semiquantitative consistence between replicates. Given the absence of a widely accepted method for deriving the total number of DNA "hits"

from the proportion of supercoiled , nicked, and linear DNA, rigorous statistical comparisons of total damage were not appropriate. Confidence limits were derived for the graphs, to allow the reader to assess the reliability of the data. Statistical calculation was only conducted when it was appropriate. The variances for the internal controls of the effect of ascorbate at concentration 0, 12.5 μM, 25 μM, and 50 μM on DNA in the absence and presence of 80 μM Fe(III) were 0.40%, 0.23%, 0.87%, 0.11%, and 0.79%, 0.55%, 0.06%, 0%, respectively. These variances were obtained using ANOVA (two-factor with replication).

Results:

a. Ascorbate induced DNA damage in a dose dependent manner

DNA damage is shown as a function of ascorbate concentration in Fig. 1a, in which it can be seen that the damage was dose dependent. When ascorbate concentration reached 400 μM, almost all supercoiled DNA was nicked to circular DNA. Linear DNA was not the dominant form of damage when compared with nicked circular DNA. It was evident only at concentrations of ascorbate over 50 μM, and even here, linear DNA accounted for only 5% of total DNA at the highest concentrations of ascorbate tested (400 μM). If the horizontal axis is plotted as a logarithmic scale, it shows that DNA damage was a linear function of the logarithm of ascorbate concentration (Fig. 1b).

b. Ferric chloride enhanced DNA breakage by ascorbate

At all concentrations tested (20 to 320 μM) Fe(III) alone did not damage DNA (Fig. 2), but it dramatically enhanced DNA damage by ascorbate (Fig. 3). In the presence of 80 μM Fe(III), almost 90 % of DNA became nicked at only 25 μM ascorbate. Above 25 μM ascorbate, the nicked form of DNA began to decrease, being gradually replaced by linear DNA, which accounted about half of total DNA damage when ascorbate concentration reached 400 μM. At a fixed concentration of ascorbate (12.5 μM), nicking was dependent on Fe(III) concentration within a test range of 0-10 μM (Fig. 4), above which adding more iron ion did not cause further DNA damage (i.e. it did not convert the nicked circular DNA to linear DNA). It is clear from the data in Fig. 3 and Fig. 4 that the effects of iron reflect and are

limited by the availability of stoichiometric amounts of reducing equivalents from ascorbate. It is also clear that the effects of iron reflect mainly *added* Fe(III). Extrapolation suggests that effects of adventitious metals in the reaction mixture were no greater than would have been produced by 0.3 μM Fe(III).

c. Effect of iron on the crossover point

When DNA was incubated with ascorbate at millimolar levels in the absence of iron, damage increased with increasing ascorbate, reaching a plateau at ascorbate concentration over 3 mM. The presence of iron increased DNA damage at all ascorbate levels. For example, in the presence of 80 μM iron and 3.125 mM ascorbate all DNA degraded to small fragments (Fig. 6). When ascorbate levels exceeded the crossover concentration of 3.125 mM, linear and nicked DNA reappeared in increasing amounts as the concentration of ascorbate increased. Concomitantly the degraded DNA product decreased until, when concentrations of ascorbate reached 25 mM, nicked DNA became the dominant form, accounting for more than 50% of total DNA. This represents substantial DNA damage, but much less than at the point of maximal genotoxicity.

d. Metal chelators protect DNA but by different mechanisms

Desferrioxamine at 100 μM completely inhibited DNA damage by ascorbate alone over the concentration range 0-100 μM (Fig. 7a, c). In the presence of added iron, inhibition by desferrioxamine was incomplete, i.e. 20% of the supercoiled DNA was converted to nicked circular DNA when 80 μM iron was present in the reaction mixture with 100 μM ascorbate (Fig. 7b, d). Protection by desferrioxamine was dose dependent up to a maximum at 50 μM

(Fig. 8). EDTA did not affect damage by ascorbate plus Fe(III), but strongly inhibited DNA damage by ascorbate alone ($p < 0.05$, Figs. 9, 10). For example, at 50-100 μM ascorbate, formation of nicked circular DNA decreased about 30% when 500 μM EDTA was added to the incubate. In contrast, ADP (800 μM) did not protect DNA against damage by ascorbate alone (Fig. 11), but inhibited DNA damage by iron/ascorbate by 10-15% ($p < 0.05$, Fig. 12).

e. Effects of scavengers of oxygen-derived active species

Catalase strongly inhibited DNA damage induced by ascorbate, or by ascorbate plus Fe(III). When DNA was incubated with 25 μM ascorbate alone, catalase decreased the production of nicked circular DNA from 70% to less than 30%. In the presence of 80 μM iron, nicked circular and linear DNA increased from 80% to 70% and 20% to less than 5%, respectively, when catalase was added into the system, indicating the protection by catalase (Fig. 13). The apparent protection by heat-denatured catalase to the genotoxicity of ascorbate or ascorbate plus Fe(III) was not statistically significant (Fig. 14). Neither native nor hydrogen peroxide-inactivated superoxide dismutase are particularly effective protective agents. If they protect at all their actions do not compare with those of active catalase or desferrioxamine in protecting DNA against damage by ascorbate plus Fe(III). Paradoxically, they increased DNA damage by ascorbate alone (Figs. 15, 16). Formate and mannitol too failed to protect DNA against damage by ascorbate plus Fe(III) (Figs. 17, 18).

f. The effect of Fe(III) on the oxidation of ascorbate

Although Fe(III) did not change the rate of oxidation of ascorbate, it increased the absorbance of ascorbate at 265 nm. This increased absorbance is dose dependent and presumably reflects formation of an iron-ascorbate complex (Fig. 19).

Discussion:

a. Ascorbate acts as a pro-oxidant facilitating the Fenton reaction in the presence of metals

The current data are consistent with the postulates that without metals or without hydrogen peroxide there is no biological damage. The metal chelator desferrioxamine completely inhibited damage, no doubt by blocking redox cycling of metals such as copper and iron, and by removing them from the target DNA (Figs. 7, 8). As discussed later, catalase also protected strongly, no doubt by pre-empting Fenton-type reactions through the removal of hydrogen peroxide. Clearly, ascorbate alone does not break DNA even with presence of oxygen. Rather the oxidative genotoxicity of ascorbate is contingent upon trace of "adventitious" metals. The DNA damage caused by ascorbate "alone" (Figs. 1, 4, 5, 7a, 10a, 13a, 14a, 15a, 16a) reflects interactions of both DNA and ascorbate with "adventitious" metals present as contaminants in the reaction mixture. Untreated phosphate buffer solutions contain approximately 0.13 μM copper and 0.7 μM iron.[9] Even using deionized buffer treated with Chelex 100, it is impossible to completely remove all traces of metals from aqueous solutions.

b. Metals are required for the "autoxidation" of ascorbate and ascorbate mediated damage

The dependence of oxidative damage on metals is consistent with the conclusions of Buettner *et al.* that "in the absence of catalytic metals ascorbate did not auto-oxidize at

pH 7''. Oxidation of ascorbate requires the presence of a metal catalyst.[9],[10],[11] This is because its reaction with oxygen in air saturated solution is spin forbidden, precluding production of free radicals in its oxidation. Some studies suggest that ascorbate can undergo at least some autoxidation by directly reacting with oxygen to produce free radicals.[12],[13] Nevertheless, based the preponderance of current and previous data, including results from our laboratory,[14] we could contend that true autoxidation of ascorbate is too slow ($k < 6 \times 10^{-7}$ s^{-1} in air-saturated phosphate buffer pH 7)[9],[10] to cause appreciable damage.

c. Site directed Fenton reactions mediate DNA damage by ascorbate

Taken together with other studies, the current data suggest that DNA damage observed reflects the actions of free radicals generated through a site-directed ascorbate-driven Fenton reaction. On this basis, ascorbate has a dual role: (1) serving as an electron source for production of hydrogen peroxide; (2) reducing the DNA-bound oxidized metal to the reduced metal. Hydrogen peroxide and reduced metals are essential ingredients of the Fenton reaction.

d. Ascorbate is less damaging to DNA at high concentrations

As to the diminished genotoxicity of ascorbate at high concentrations (Fig. 6), there are a few plausible explanations: (1) The disturbance of Fe^{3+}/Fe^{2+} ratio by high concentrations of ascorbate, as has been proposed by Aust *et al.* in ascorbate driven lipid peroxidation; (2) At high concentrations, ascorbate competitively binds the iron, removing it from target site on the DNA, pre-empting metal-mediated site-specific DNA damage;[13] (3) Ascorbate acts not only as a radical generator, but also as a radical scavenger. High concentrations tip the

balance in favour of scavenging;[15],[16] (4) High concentrations of ascorbate diminish oxygen concentrations below those required for maximal toxicity. Any or all of these might contribute to the diminished genotoxicity of ascorbate at high concentrations. To distinguish between these possibilities detailed studies of the kinetic and saturation properties of the system are indicated.

e. Removal of metals from target by ligand

After considerable experimentation we selected a concentration of Fe(III) of 80 μM for our studies. Under these reaction conditions precipitation of iron in the incubate was not a concern. EDTA protected against DNA damage by ascorbate "alone" and ADP protected against DNA damage by ascorbate plus iron, respectively. On the basis of a site-specific Fenton mechanism, the protection by EDTA or ADP most plausibly reflects removal of the metal from the DNA target site.

f. Effect of ligand on reduction potentials of copper

Buettner has shown that without added metals, the most important trace metal catalyzing the oxidation of ascorbate is copper [Cu(II) is 80 times more effective than Fe(III)[9]]. Thus, the DNA damage caused by ascorbate "alone" is presumably due to the interaction of ascorbate and copper. EDTA has a higher affinity for Cu(II) than Cu(I),[17] sufficiently that when EDTA binds copper, the reduction potential of the complex is below the appropriate range for its redox cycling. Reduction of the EDTA•copper complex by ascorbate is, therefore, thermodynamically unfavorable. Buettner's finding that copper mediates ascorbate oxidation is consistent with the observation that EDTA greatly decreased the

oxidation of ascorbate, both in buffer without Chelex treatment and in Chelex treated buffer with added copper.[10]

g. EDTA iron interactions

Another plausible mechanism for protection by EDTA in DNA damage induced by ascorbate "alone" is the [EDTA]/[metal] ratio. When the [EDTA]/[metal] ratio is high (as when incubating DNA with ascorbate "alone") EDTA inhibits the recycling of metal and thus decreases the DNA damage.[18]

This explanation is not tenable for the situation in the presence of iron and ascorbate. Although, EDTA binds Fe^{3+} far more strongly than Fe^{2+} (Ka = 10^{25} for Fe^{3+} versus 10^{14} for Fe^{2+}), this increase the cytotoxic capacity of iron.[19] When EDTA binds iron, it lowers the reduction potential of iron from 0.77 to 0.12 V,[19],[20] bringing it to the range appropriate for redox cycling of the EDTA•iron complex. Especially when the ratio of [EDTA]/[iron] is lower, as when DNA was incubated with ascorbate and iron, EDTA enhances redox cycling of the iron, resulting in increased production and reduction of hydrogen peroxide. This helps explain why EDTA did not decrease DNA breakage induced by the interaction of ascorbate with added Fe(III). One might have expected that EDTA should increase DNA damage because EDTA both increases the ability of iron to generate free radicals, and accelerates ascorbate oxidation by iron.[10] There are two possible explanations for the failure of EDTA to increase DNA damage by ascorbate plus Fe(III): (1) 80 μM Fe(III) had already achieved maximal DNA damage (Figs. 9, 10), and the increased redox cycling induce no additional DNA damage; (2) EDTA removes Fe(III) from DNA and prevents the "site-specific" Fenton mechanism, regardless of enhanced redox cycling for iron and ascorbate oxidation.

h. Effect of ADP on ascorbate-mediated DNA damage

ADP suppresses both ascorbate autoxidation and ascorbate-mediated hydroxyl radical production.[21] Nevertheless, ADP did not inhibit DNA damage by ascorbate "alone". This is consistent with the failure of ADP to inhibit lipofusin-like fluorophore formation by preventing iron catalyzed ascorbate oxidation.[22] ADP slows the air oxidation of Fe(II), leading to increased production of hydroxyl radicals from hydrogen peroxide in Fenton reactions.[23],[24] ADP, however, protected DNA against damage by ascorbate plus Fe(III). This protection may be due to removal of iron from DNA by ADP. Because of its similar structure to the bases of DNA, ADP likely competes with DNA binding sites for the possession of iron ions, and in this way, like EDTA, ADP can protect DNA by pre-empting site-specific mechanisms in DNA damage.

i. Hydrogen peroxide is required for DNA damage induced by ascorbate or ascorbate and Fe(III)

Since catalase so strongly inhibited DNA breakage by ascorbate alone or ascorbate plus Fe(III) (Fig. 13), hydrogen peroxide is clearly a crucial species mediating DNA damage in the current system. Hydrogen peroxide itself is not a free radical, and being uncharged it readily migrates to the site where the Fe(II) [produced from Fe(III) by ascorbate] binds to the DNA. Here it readily produces 'crypto' hydroxyl radicals through the site-directed Fenton reaction.[25] Such hydroxyl radicals are extremely active species that can damage DNA at the site where they are produced.

j. The role of superoxide in DNA damage cannot be ruled out

The role of superoxide in DNA damage by ascorbate or ascorbate plus Fe(III) cannot be ruled out, even though superoxide dismutase failed to inhibit these reactions. The role of superoxide in the of autoxidation of ascorbate is controversial. Scarpa *et al.* contended that superoxide ion was an active intermediate in the autoxidation of ascorbate by molecular oxygen since superoxide dismutase at concentration higher than 10^{-7} M decreased the oxidation rate by 50%.[26] In contrast, Halliwell and Foyer could not find evidence of superoxide production in autoxidation of ascorbate.[27] The discrepancy may arise in differences in reaction conditions. Skov has shown that pH profoundly affects the rate of oxidation of ascorbate.[28] Scarpa used buffer of pH 7.4, while Halliwell used buffer of pH 8.8. The rate of oxidation of ascorbate was higher at pH 8.0 than that at pH 7.4.[29] Increasing pH may not only change the rate of oxidation of ascorbate but also change its pathway, e.g. shifting it from a superoxide-dependent pathway to a superoxide-independent mechanism.

Differences in pH can not explain the difference between Scarpa's results and those from our laboratory. Scarpa's result indicated that aposuperoxide dismutase or bovine serum albumin had no effect on the ascorbate oxidation, while we have shown that denatured superoxide dismutase decreases the oxidation of ascorbate.[14] The pH of phosphate buffer in our study was 7.0, similar to that used by Scarpa.

The slight enhancement of ascorbate-induced DNA breakage by superoxide dismutase may be due to traces of copper in the protein rather than the activity of enzyme, since the hydrogen peroxide-inactivated superoxide dismutase provided the same effect as active enzyme (Figs. 15, 16). When exogenous Fe(III) was added to the system the effect of traces

of copper from superoxide dismutase become inconsequential, so that neither native superoxide dismutase, nor hydrogen peroxide inactivated enzyme, increased DNA damage by ascorbate plus Fe(III).

The one salient conclusion from the failure of superoxide dismutase to protect, is that if superoxide participates, it is kinetically inaccessible. In other words if it participates, it does so after the rate determining step, or within a solvent shell via "inner-sphere" reaction mechanisms.[30]

k. DNA damage by ascorbate or ascorbate plus Fe(III) occurs by metal-mediated site-specific mechanisms

For reasons similar to those discussed for superoxide dismutase, the failure of hydroxyl radical scavengers, such formate and mannitol, to protect DNA against damage by ascorbate or ascorbate plus Fe(III) (Fig. 24, 25) does not rule out participation of hydroxyl radicals in DNA damage. Rather this observation provides further support for a metal-mediated site-specific Fenton mechanism.[31] In such a mechanism, the concentrations of hydroxyl radical scavengers used do not access the target sites.[13] Hydroxyl radicals produced through site-specific mechanisms damage DNA only at the site where they are generated. They are too confined and too short-lived to cause damage away from the site they are produced, or even, in the current situation to be accessible to scavengers. Moreover, there are alternative mechanisms for the participation of hydroxyl radicals. For example, hydrogen peroxide and Fe(II) in Fenton reaction can form Fe (IV) (ferryl) species:[32],[33]

$$Fe^{2+} + H_2O_2 \Rightarrow FeOH^{2+} \text{ (or } FeO^{2+} + H^+) + OH^-$$

Fe(IV) is not sensitive to hydroxyl radical scavengers (including formate and mannitol),[34],[35] and is believed to be as reactive as hydroxyl radicals.[36] It may also damage DNA.

In conclusion then, ascorbate can act as a pro-oxidant in the presence of metals to damage DNA *in vitro*. Iron plays an important role in determining the "cross-over" point at when ascorbate shift from damaging to being harmless or protective. DNA damage by ascorbate or ascorbate plus Fe(III) is mediated by site-specific mechanisms. H_2O_2 is required for DNA damage by ascorbate or ascorbate plus Fe(III).

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LEGENDS TO FIGURES*

Figure 1. DNA damage induced by ascorbate alone. DNA (final concentration of 0.0415 μg/μl) was first incubated with the indicated concentrations of ascorbate at 37°C for 30 minutes in potassium phosphate buffer (50 mM, pH 7.4). Then the sample was run in 0.7% of agarose gel at 60 V for 1.5 h and quantified densitometrically. Both Fig. A and Fig. B were plotted from the same experimental data except that in Fig. B the horizontal axis was logarithmic.

Figure 2. The effect of Fe(III) on DNA damage. Reaction conditions were as the same as in Fig. 1 except that DNA was incubated with a range of concentrations of Fe(III) rather than ascorbate.

Figure 3. The effect of Fe(III) on DNA damage induced by ascorbate. Reaction conditions were as the same as in Fig. 1 except that DNA was incubated with the indicated concentrations of ascorbate in the presence of 80 μM Fe(III). Both Fig. A and Fig. B were plotted from the same experimental data except that in Fig. B the horizontal axis was logarithmic.

Figure 4. The effect of graded concentrations of Fe(III) on DNA damage induced by ascorbate. Reaction conditions were as the same as in Fig. 1 except that DNA was incubated

* Graphs represent a typical experiment chosen from 2-3 replicates.

with the indicated concentrations of Fe(III) in the presence of 12.5 μM ascorbate. Since DNA was incubated with Fe(III) in the presence of 12.5 μM ascorbate, the supercoiled DNA at zero of iron concentration was the result of DNA incubation with 12.5 μM ascorbate.

Figure 5. DNA damage induced by high concentrations of ascorbate. Reaction conditions were as the same as in Fig. 1 except that DNA was incubated with ascorbate at millimolar levels instead of at micromolar levels and the concentration of buffer was 200 mM instead of 50 mM.

Figure 6. The effect of Fe(III) on DNA damage induced by high concentrations of ascorbate. Reaction conditions were as the same as in Fig. 5 except that DNA was incubated with ascorbate at millimolar concentrations in the presence of 80 μM Fe(III). Both Fig. A and Fig. B were plotted from the same experimental data except that in Fig. A the data were plotted in linear style while in Fig. B the data were plotted as a bar graph.

Figure 7. The effect of desferrioxamine on DNA damage induced by ascorbate and Fe(III). Reaction conditions were as the same as in Fig. 3 except that in reactions 100 μM desferrioxamine was added to the reaction mixture before DNA was incubated with the indicated concentrations of ascorbate in the absence and presence of 80 μM Fe(III).

Figure 8. The effect of concentrations of desferrioxamine on DNA damage induced by ascorbate and Fe(III). Reaction conditions were as the same as in Fig. 4 except that in

reactions the indicated concentrations of desferrioxamine were added to the reaction mixture before DNA was incubated with 12.5 μM ascorbate in the presence of 80 μM Fe(III).

Figure 9. The effect of EDTA on DNA damage. Reaction conditions were as the same as in Fig. 3 except that 500 μM ethyenediaminotetraacetic acid (EDTA) was added to the reaction mixture before DNA was incubated with 25 μM ascorbate in the absence and presence of 80 μM Fe(III). Standard deviations were less than 4%.

Figure 10. The effect of EDTA on DNA damage induced by ascorbate and Fe(III). Reaction conditions were as the same as in Fig. 3 except that in reactions 500 μM ethyenediaminotetraacetic acid (EDTA) was added to the reaction mixture before DNA was incubated with graded of concentrations of ascorbate in the absence and presence of 80 μM Fe(III).

Figure 11. The effect of ADP on DNA damage. Reaction conditions were as the same as in Fig. 3 except that 800 μM adenosine diphosphate (ADP) was added to the reaction mixture before DNA was incubated with 25 μM ascorbate in the absence and presence of 80 μM Fe(III). As in experiment in Fig. 9, standard deviations were less than 4%.

Figure 12. The effect of ADP on DNA damage induced by ascorbate and Fe(III). Reaction conditions were as the same as in Fig. 4 except that in reactions 800 μM adenosine

diphosphate (ADP) was added to the reaction mixture before DNA was incubated with a range of concentrations of Fe(III) in the presence of 12.5 μM ascorbate.

Figure 13. The effect of active catalase on DNA damage induced by ascorbate and Fe(III). Reaction conditions were as the same as in Fig. 7 except that in reactions 10 U/ml active catalase, instead of 100 μM desferrioxamine, was added to the reaction mixture before DNA was incubated with a range of concentrations of ascorbate in the absence and presence of 80 μM Fe(III).

Figure 14. The effect of denatured catalase on DNA damage induced by ascorbate and Fe(III). Reaction conditions were as the same as in Fig. 13 except that in reactions 10 U/ml heat denatured catalase, instead of active catalase, was added to the reaction mixture before DNA was incubated with a range of concentrations of ascorbate in the absence and presence of 80 μM Fe(III).

Figure 15. The effect of active SOD on DNA damage induced by ascorbate and Fe(III). Reaction conditions were as the same as in Fig. 7 except that in reactions 20 U/ml active superoxide dismutase (SOD), instead of 100 μM desferrioxamine, was added to the reaction mixture before DNA was incubated with a range of concentrations of ascorbate in the absence and presence of 80 μM Fe(III).

Figure 16. The effect of denatured SOD on DNA damage induced by ascorbate and Fe(III). Reaction conditions were as the same as in Fig. 15 except that in reactions 20 U/ml

hydrogen peroxide inactivated SOD, instead of active SOD, was added to the reaction mixture before DNA was incubated with a range of concentrations of ascorbate in the absence and presence of 80 μM Fe(III).

Figure 17. The effect of formate on DNA damage induced by ascorbate and Fe(III). Reaction conditions were similar as in Fig. 3 except that in reactions the indicated concentrations of formate was added to the reaction mixture before DNA was incubated with 25 μM ascorbate in the presence of 80 μM Fe(III).

Figure 18. The effect of mannitol on DNA damage induced by ascorbate and Fe(III). Reaction conditions were similar as in Fig. 3 except that in reactions the indicated concentrations of mannitol was added to the reaction mixture before DNA was incubated with 25 μM ascorbate in the presence of 80 μM Fe(III).

Figure 19. The effect of Fe(III) on oxidation of ascorbate. This curve shows the progress of the reaction when ascorbate (final concentration 100 μM) was allowed to react with oxygen in the presence of the indicated concentrations of Fe(III). The reaction was initiated by mixing the appropriated aliquot of ascorbate with sufficient Fe(III) to provide the indicated concentrations of iron, in a spectrophotometer cuvette. Progress of the reaction was followed at 265 nm..

Fig. 1 (a)
DNA Damage Induced by Ascorbate Alone

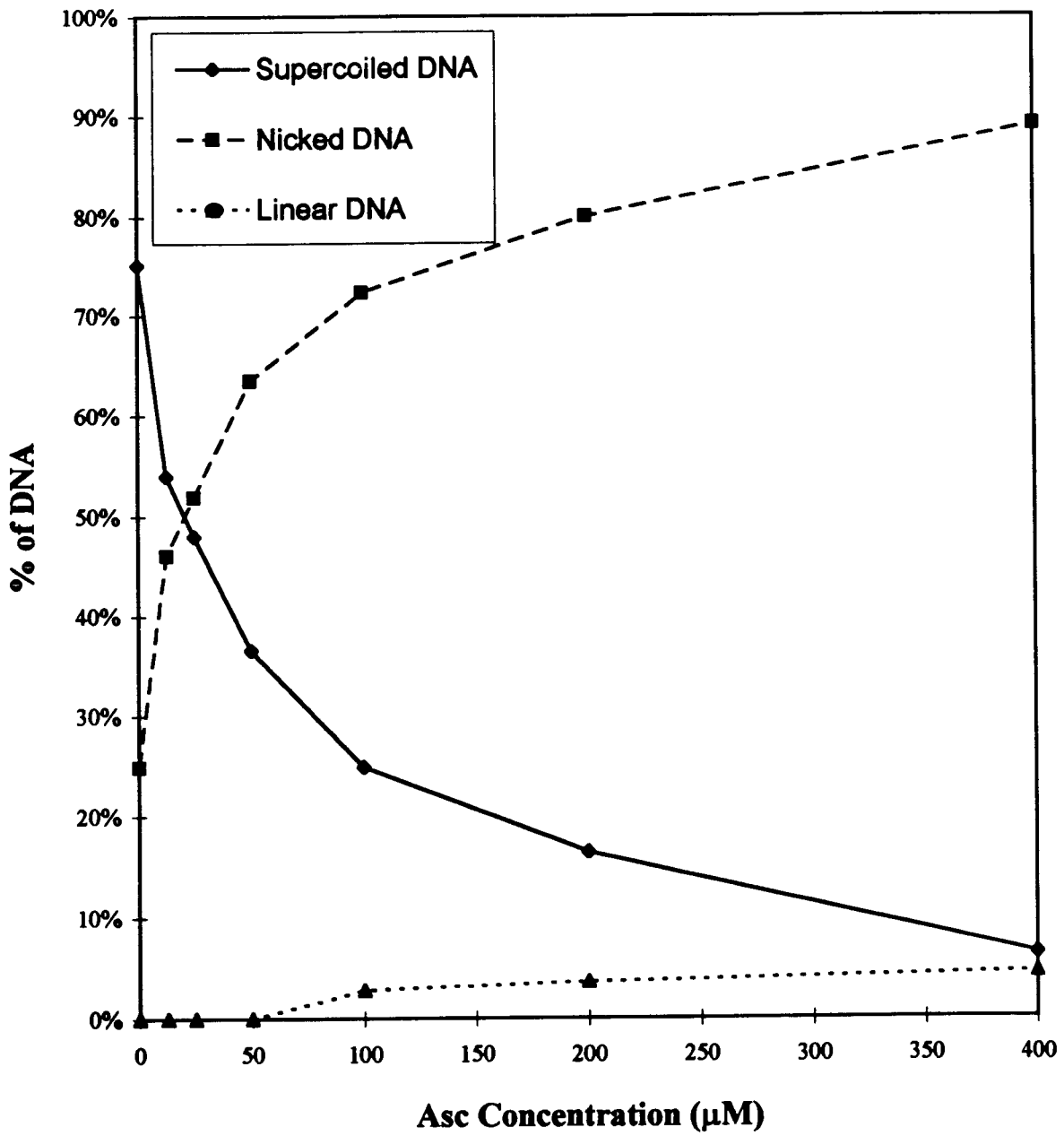


Fig. 1 (b)
DNA Damage Induced by Ascorbate Alone

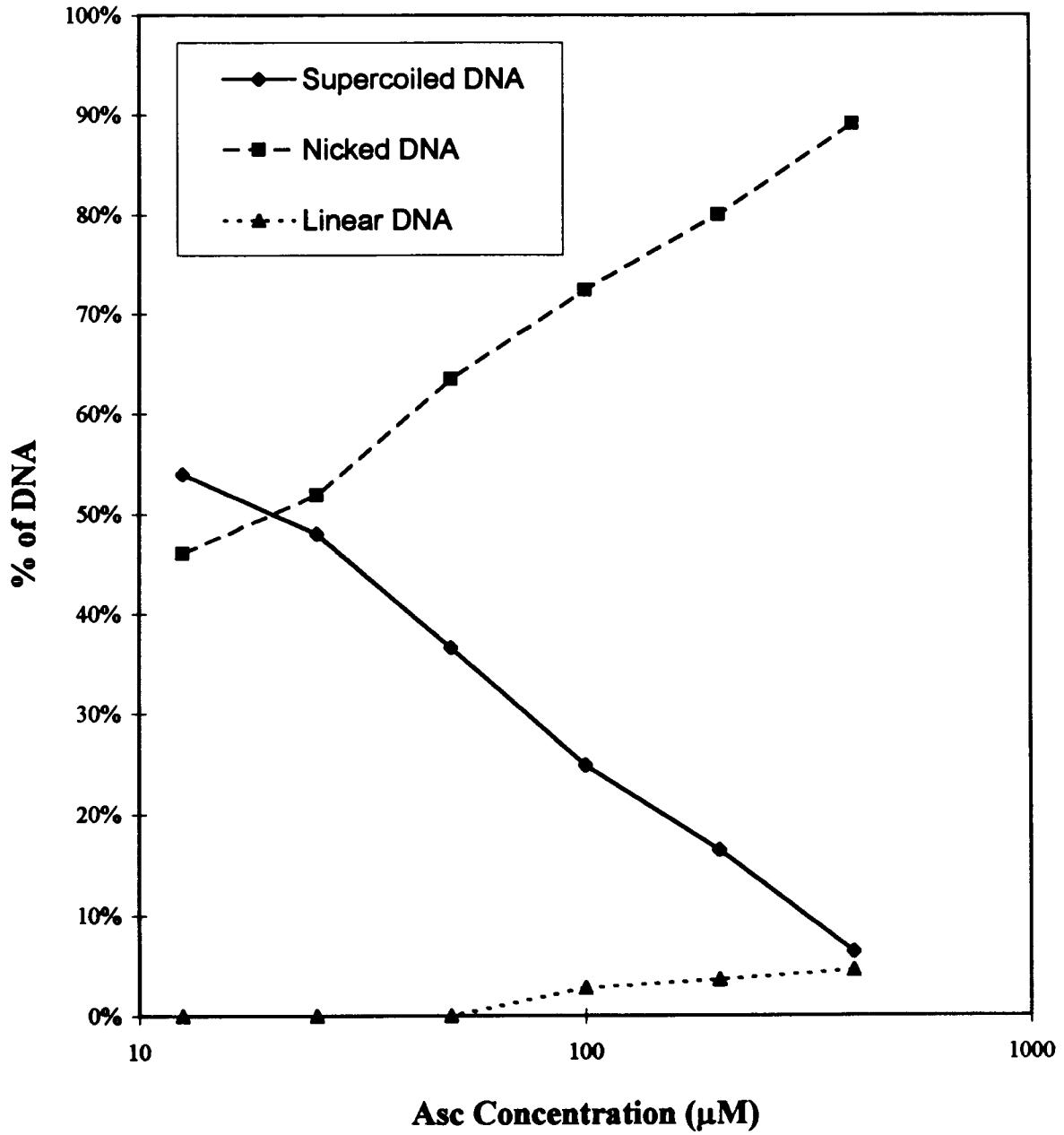


Fig. 2
The Effect of Fe(III) on DNA Damage

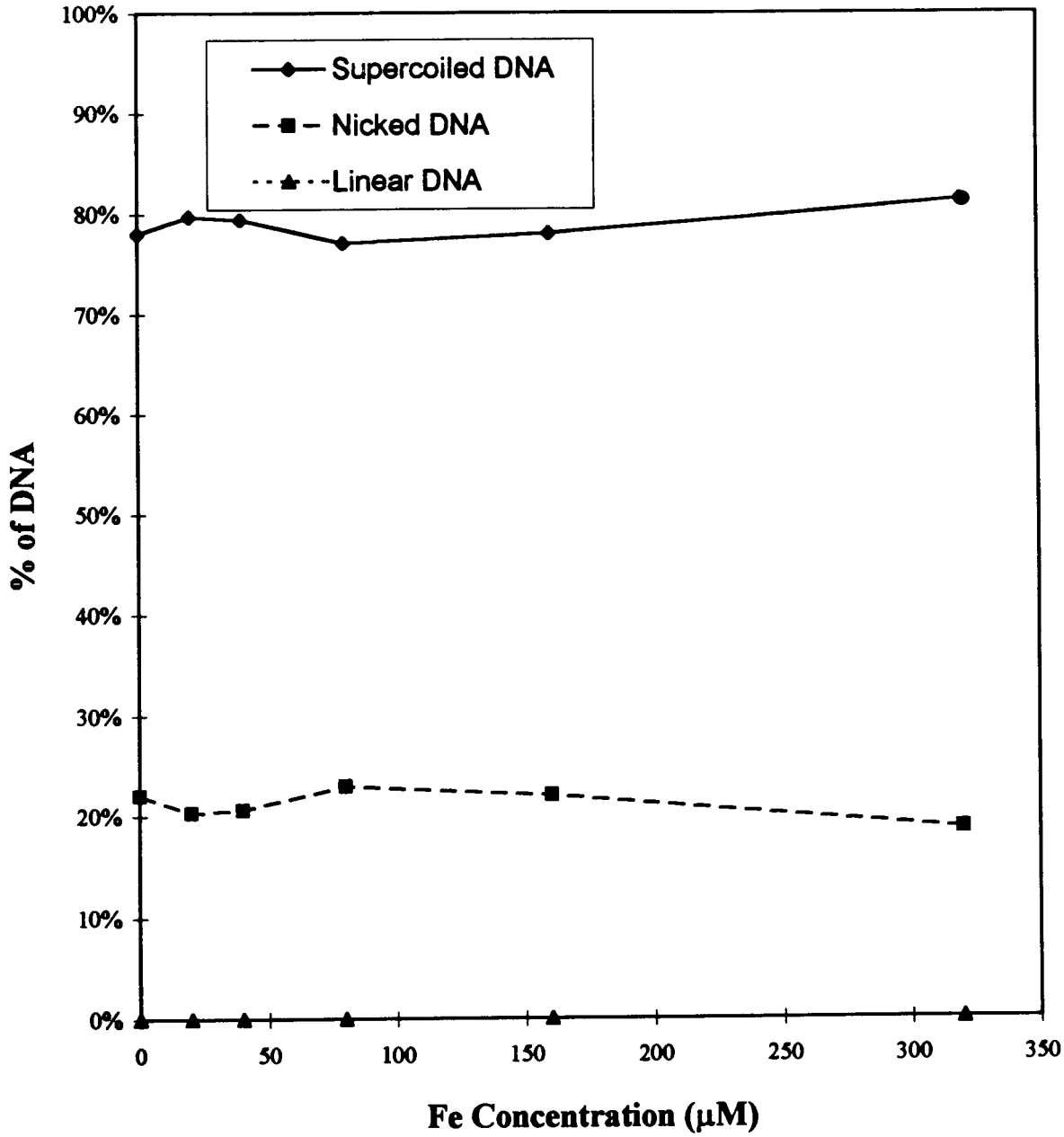


Fig. 3 (a)
The Effect of Fe(III) on DNA Damage Induced by Ascorbate

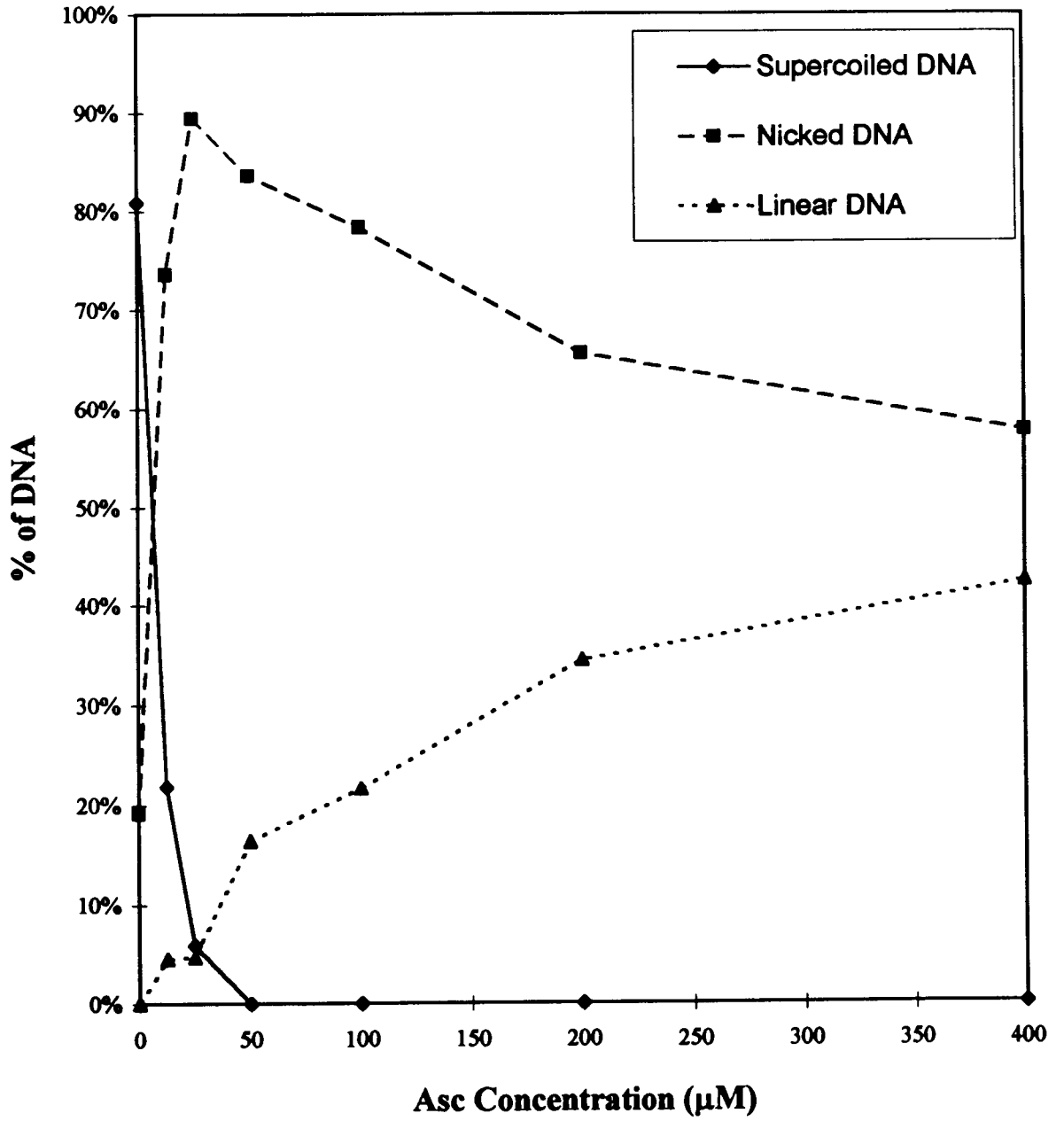


Fig. 3 (b)
The Effect of Fe(III) on DNA Damage Induced by Ascorbate

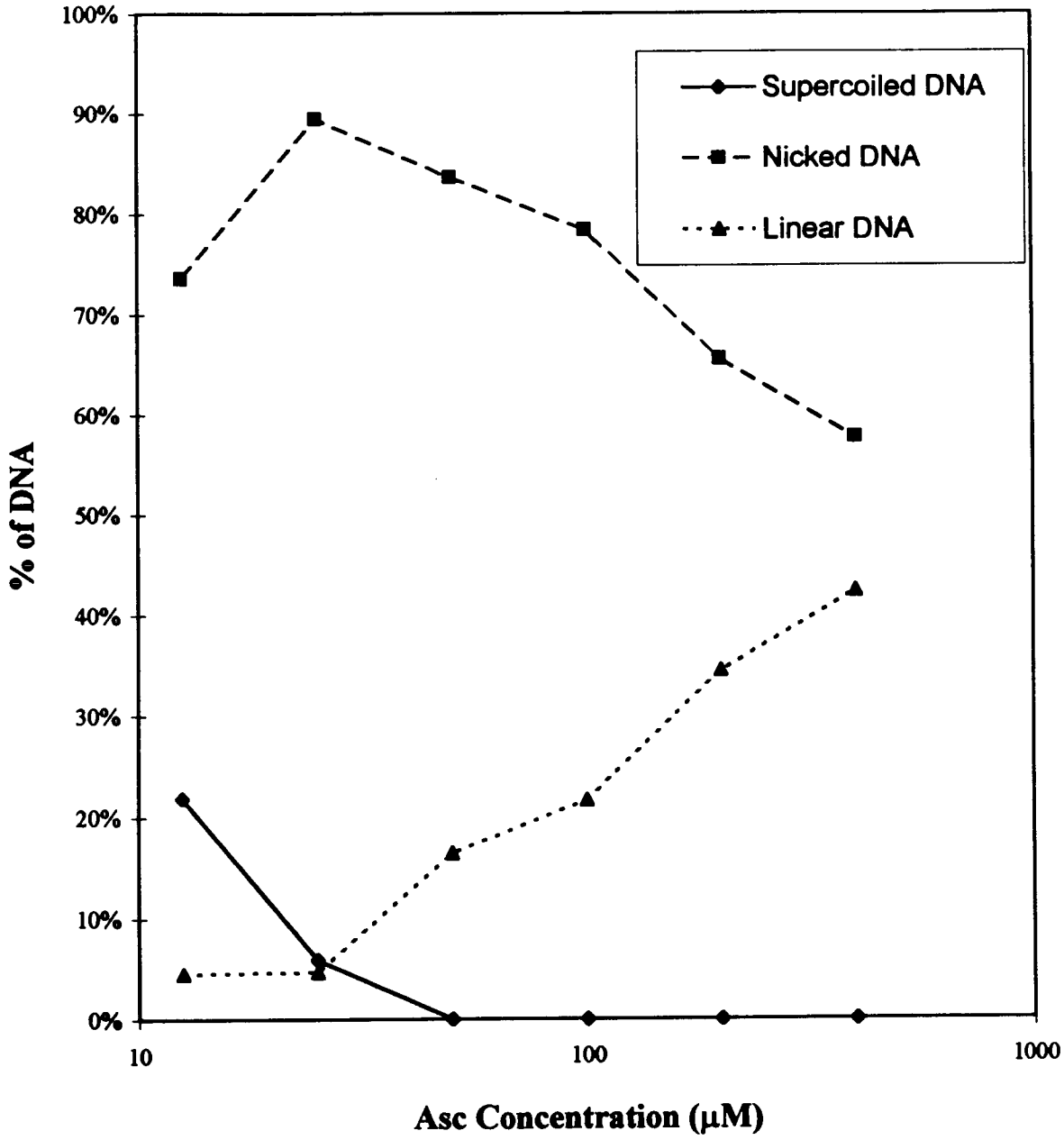


Fig. 4 (a)
The Effect of Graded Concentrations of Fe(III) on DNA Damage Induced by Ascorbate

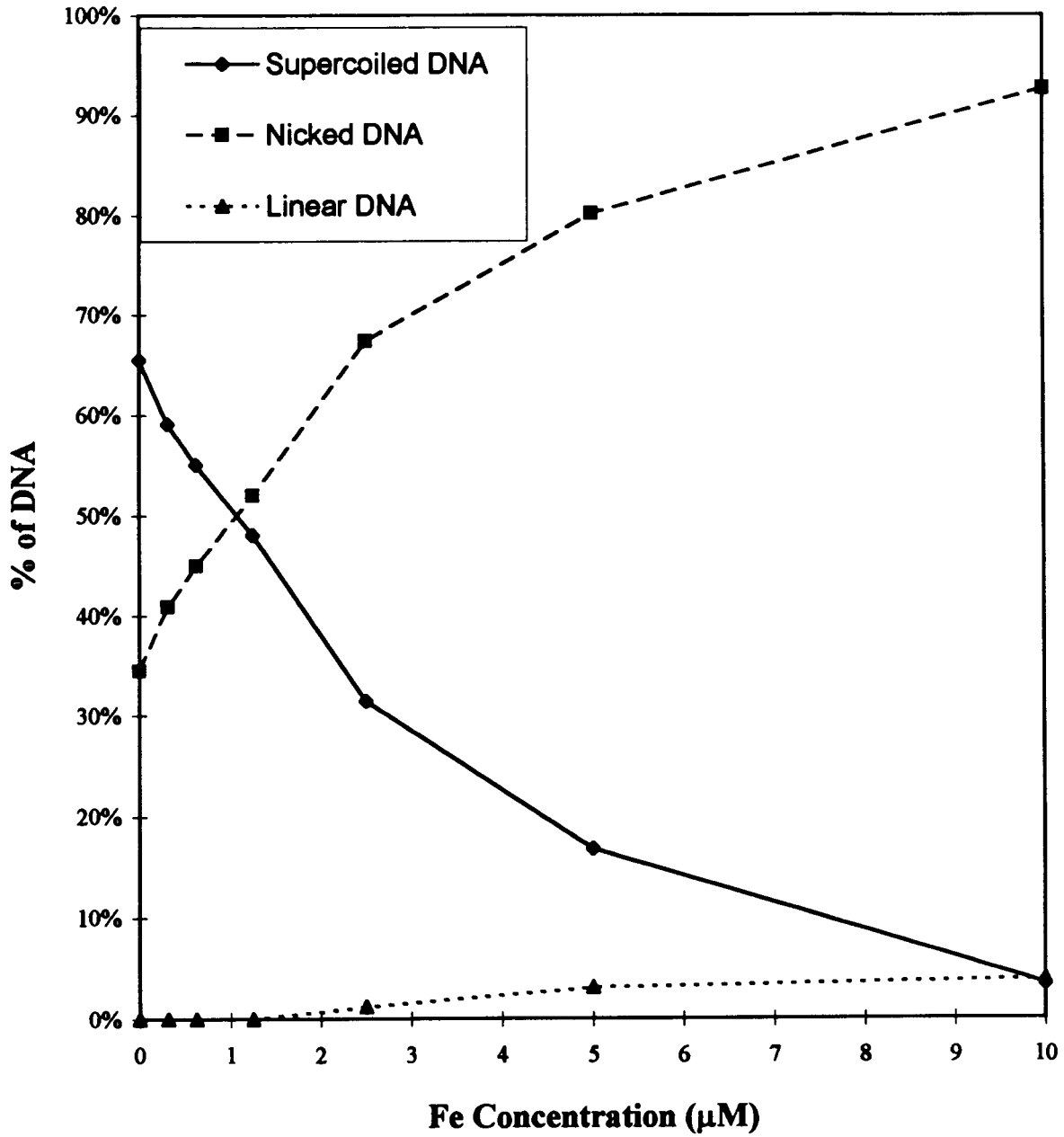


Fig. 4 (b)
The Effect of Graded Concentrations of Fe(III) on DNA Damage Induced by Ascorbate

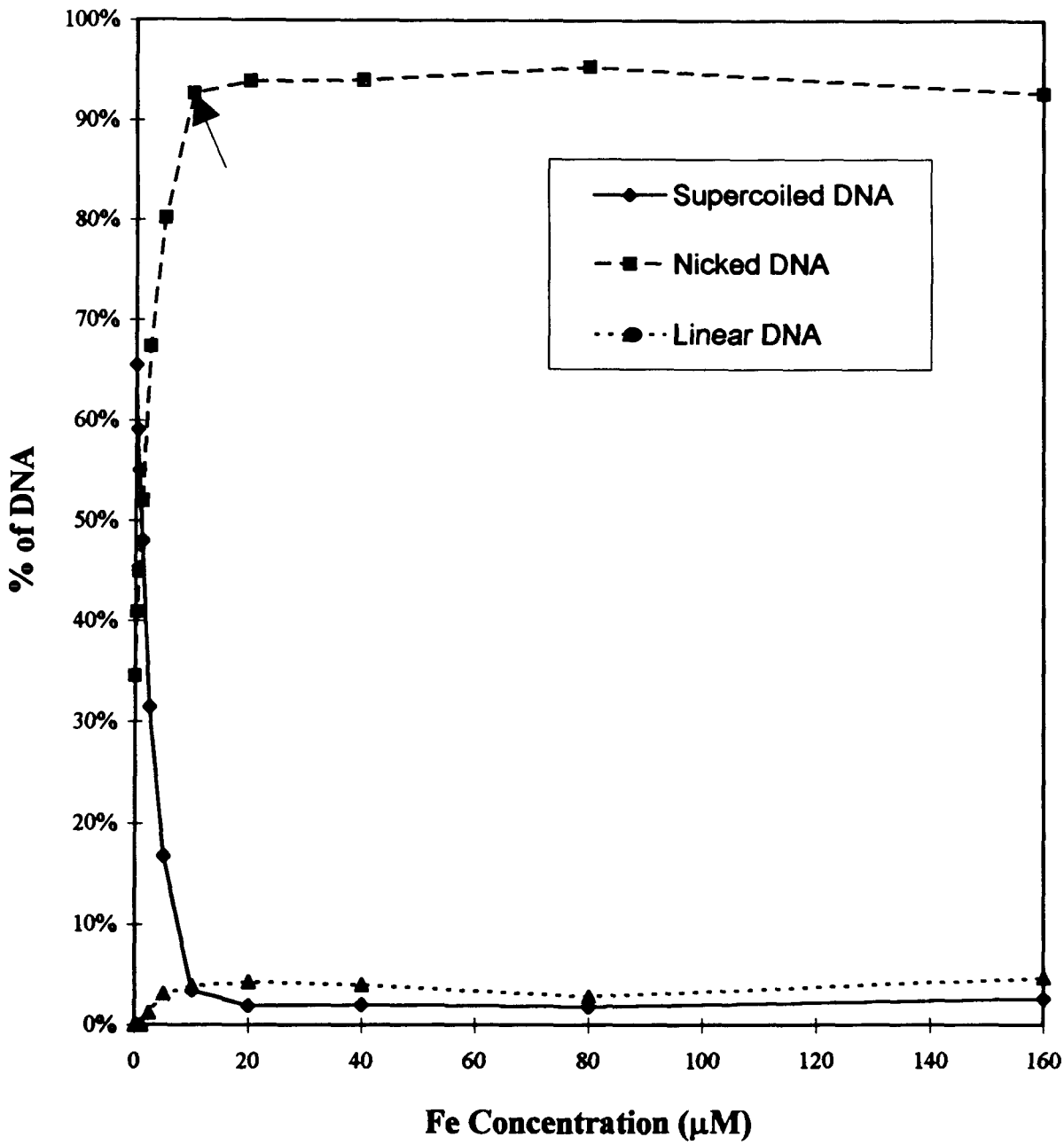


Fig. 5
DNA Damage Induced by High Concentrations of Ascorbate

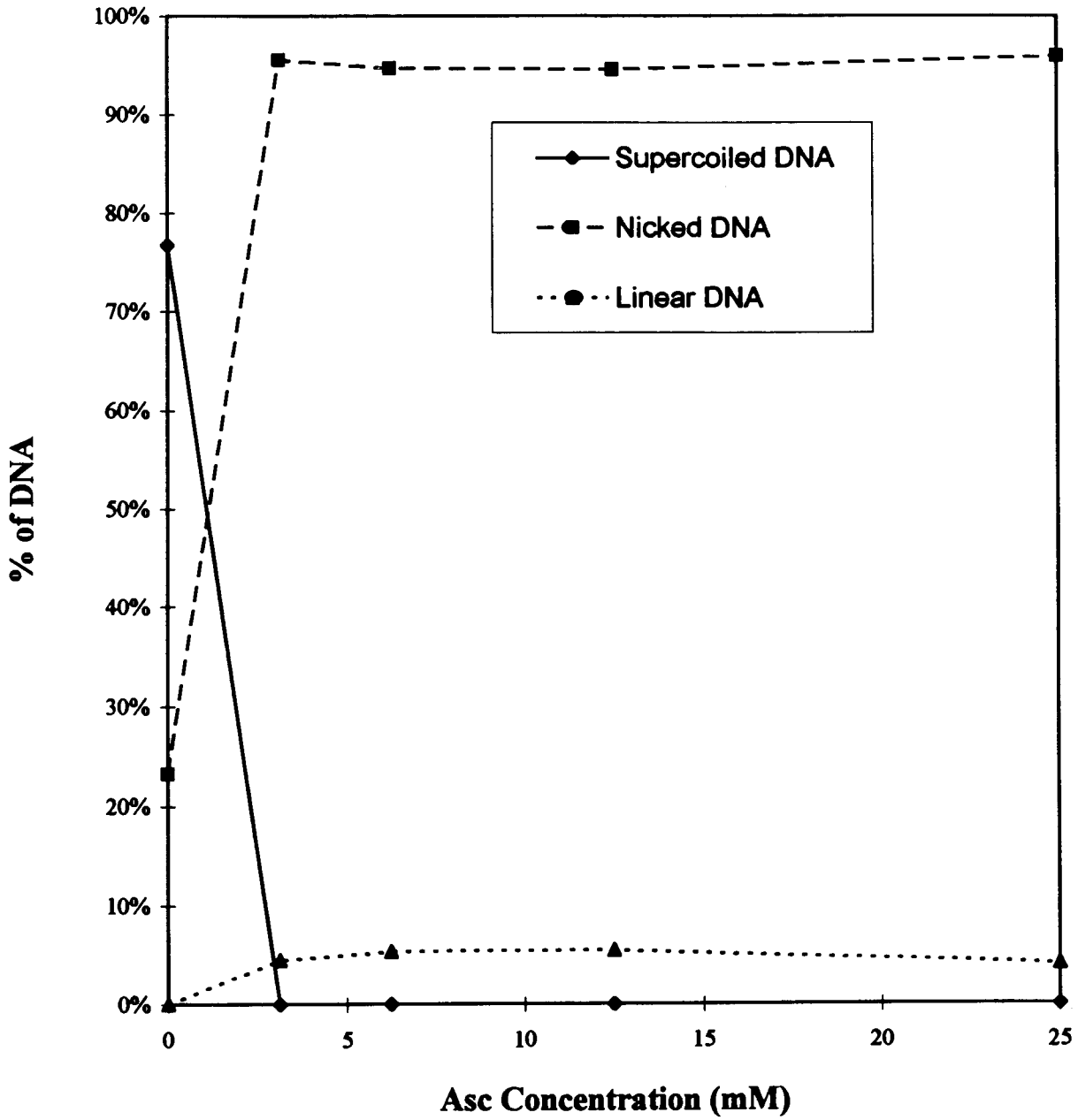


Fig. 6 (a)
The Effect of Fe(III) on DNA Damage Induced by High Concentrations of Ascorbate

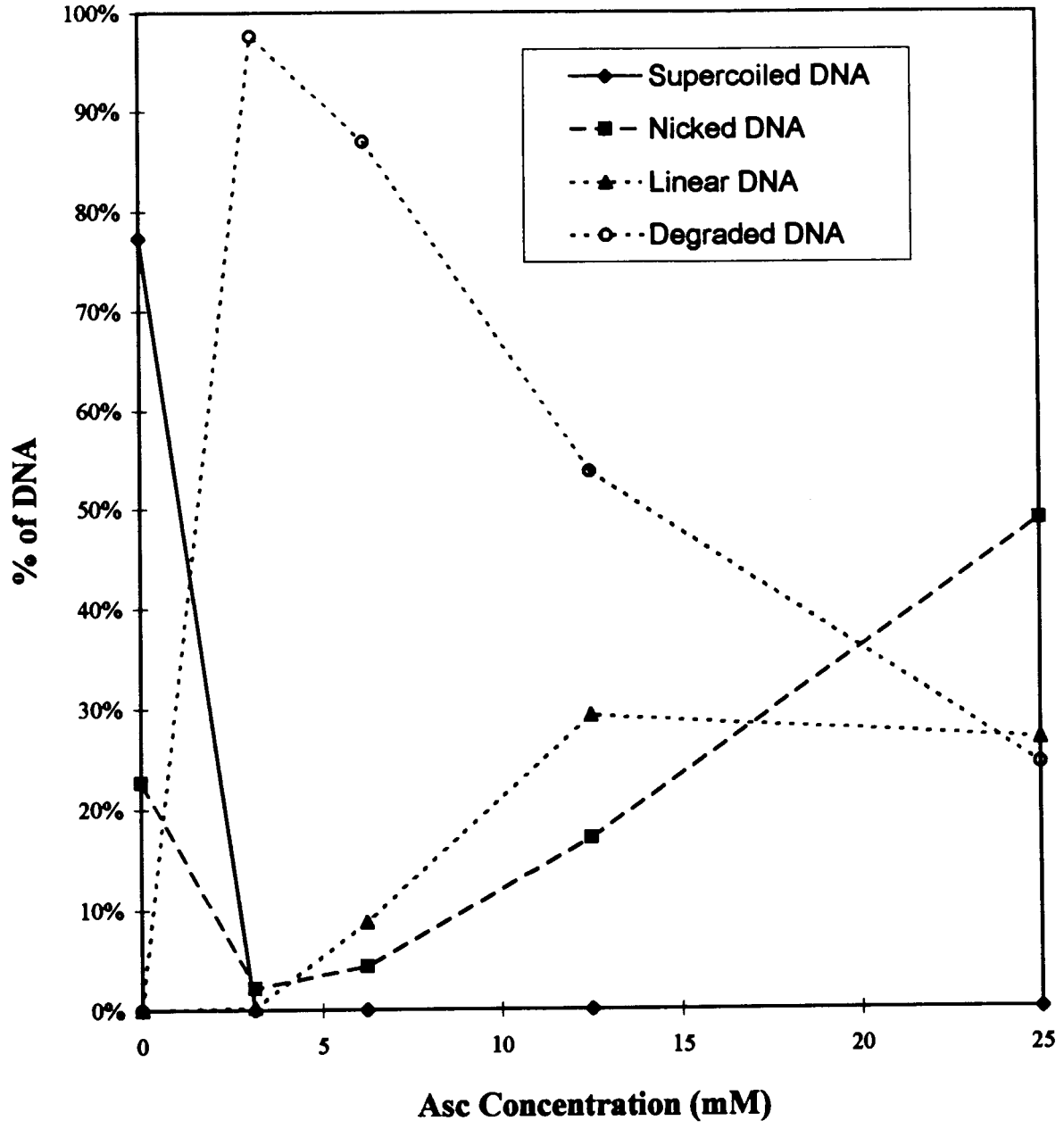


Fig. 6 (b)
The Effect of Fe(III) on DNA Damage Induced by High Concentrations of Ascorbate

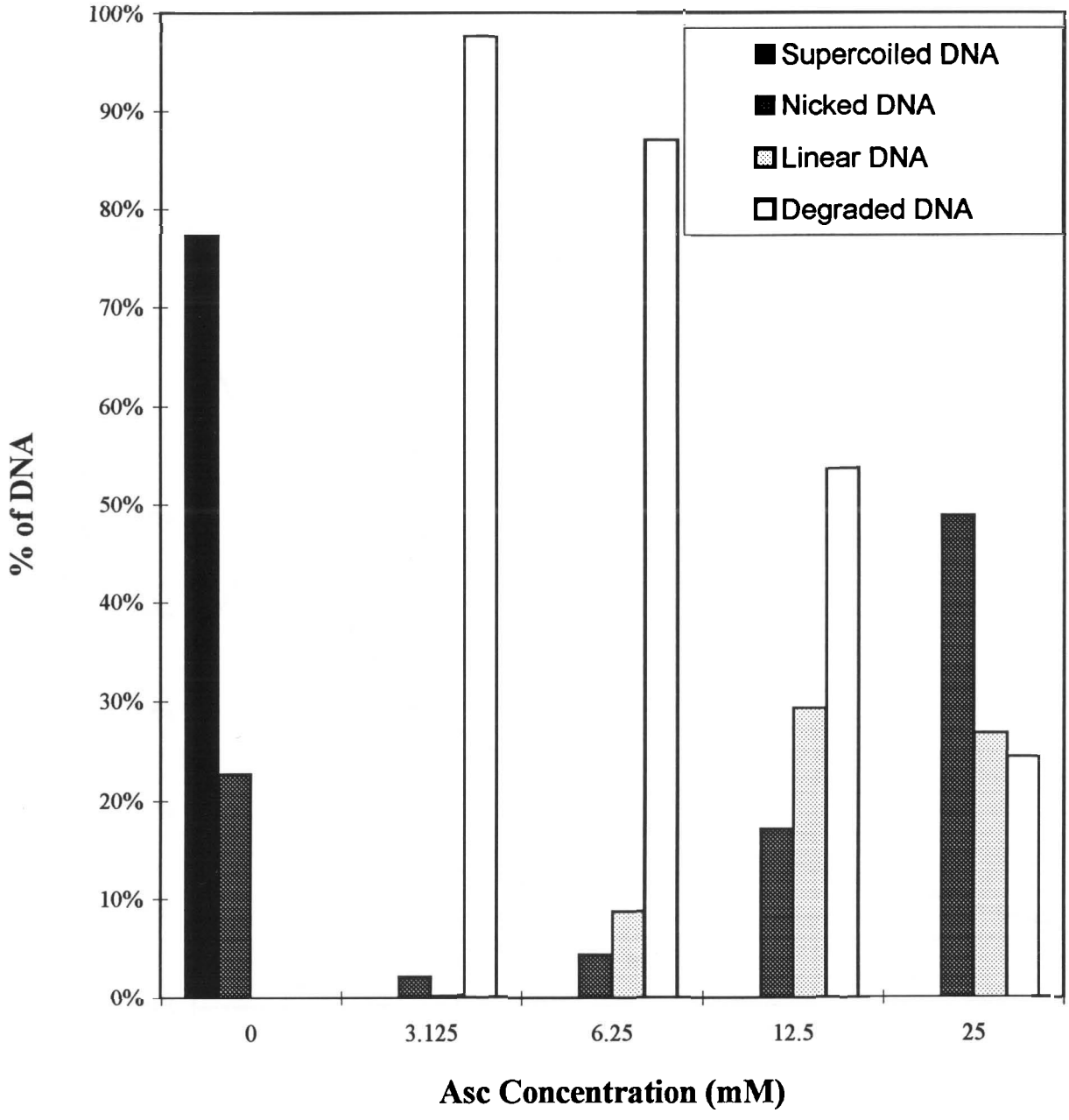


Fig. 7 (a)

The Effect of Desferrioxamine on DNA Damage Induced by Ascorbate and Fe(III)

(Asc Alone)

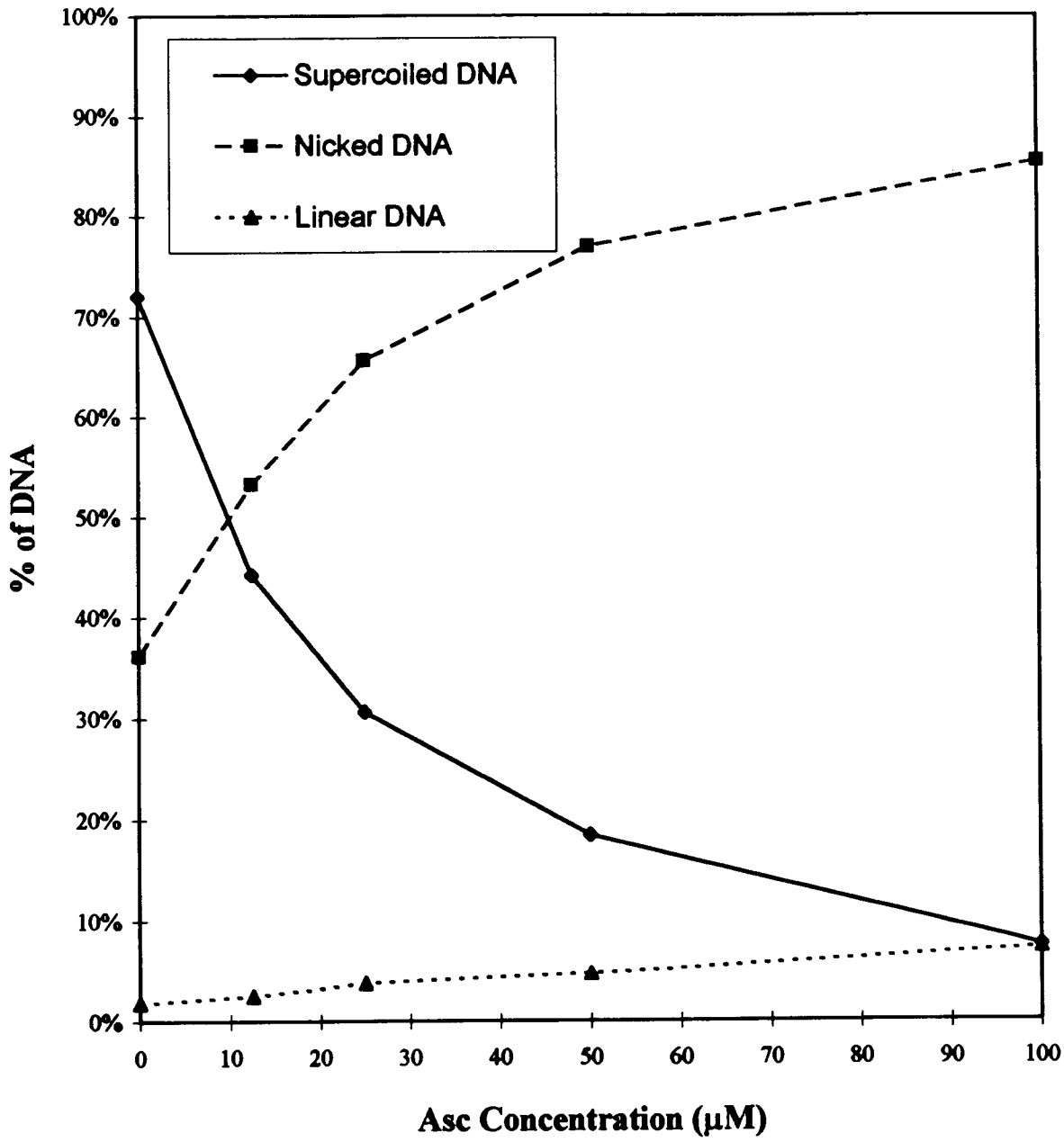


Fig. 7 (b)

The Effect of Desferrioxamine on DNA Damage Induced by Ascorbate and Fe(III)

(Asc + 80 μM Fe)

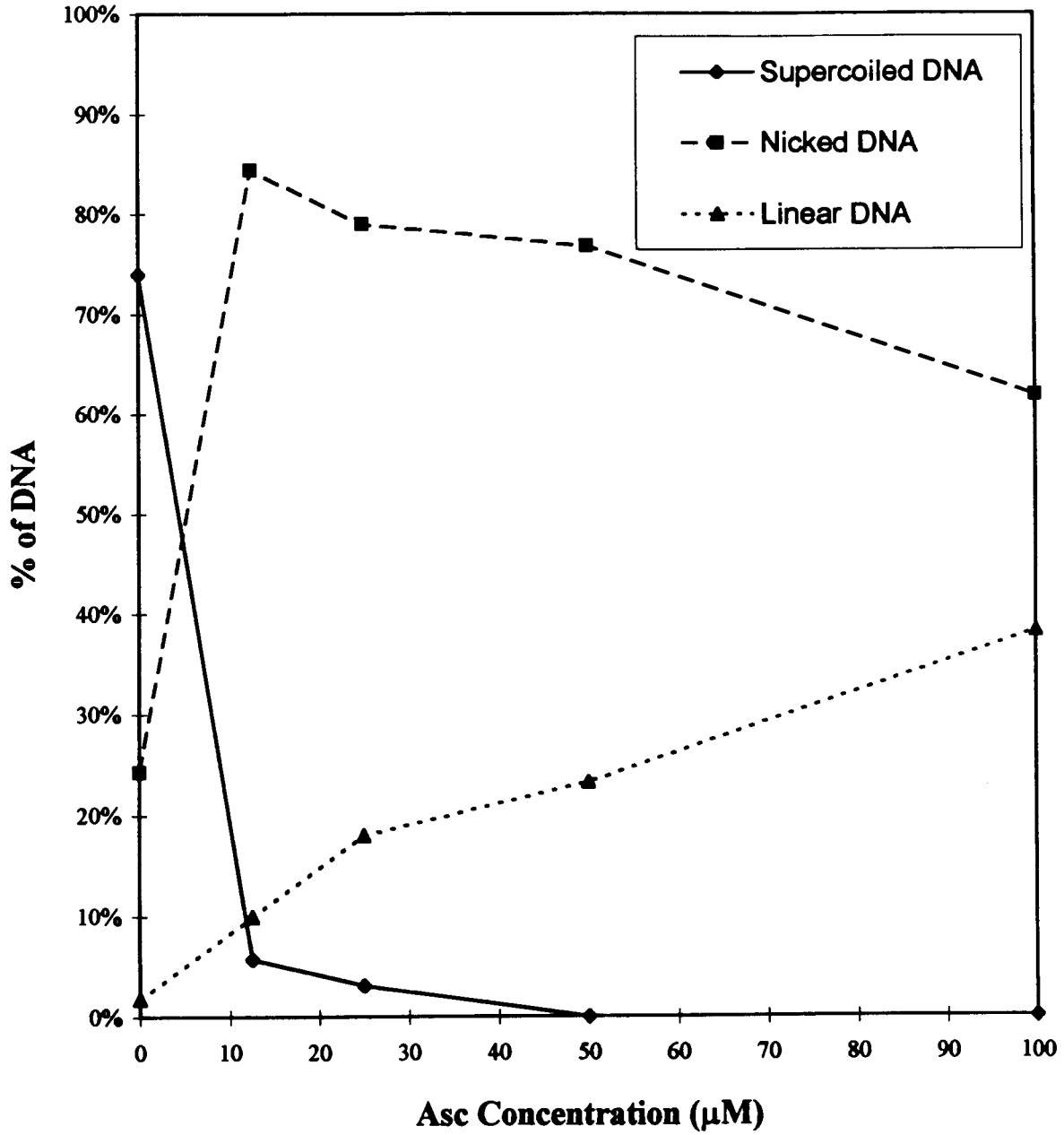


Fig. 7 (c)
The Effect of Desferrioxamine on DNA Damage Induced by Ascorbate and Fe(III)

(Asc + 100 μM Desf.)

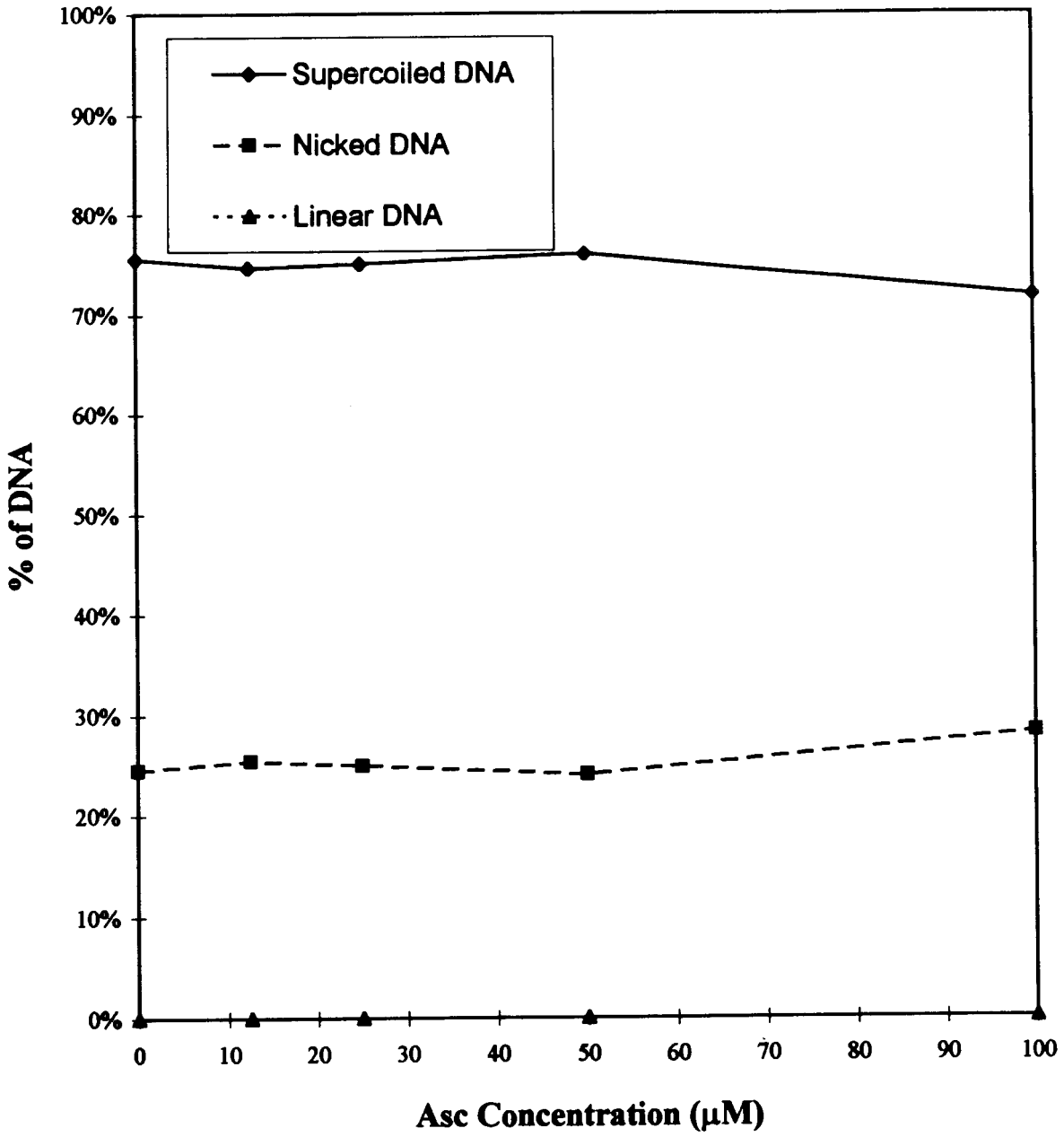


Fig. 7 (d)

The Effect of Desferrioxamine on DNA Damage Induced by Ascorbate and Fe(III)

(Asc + 80 μM Fe + 100 μM Desf.)

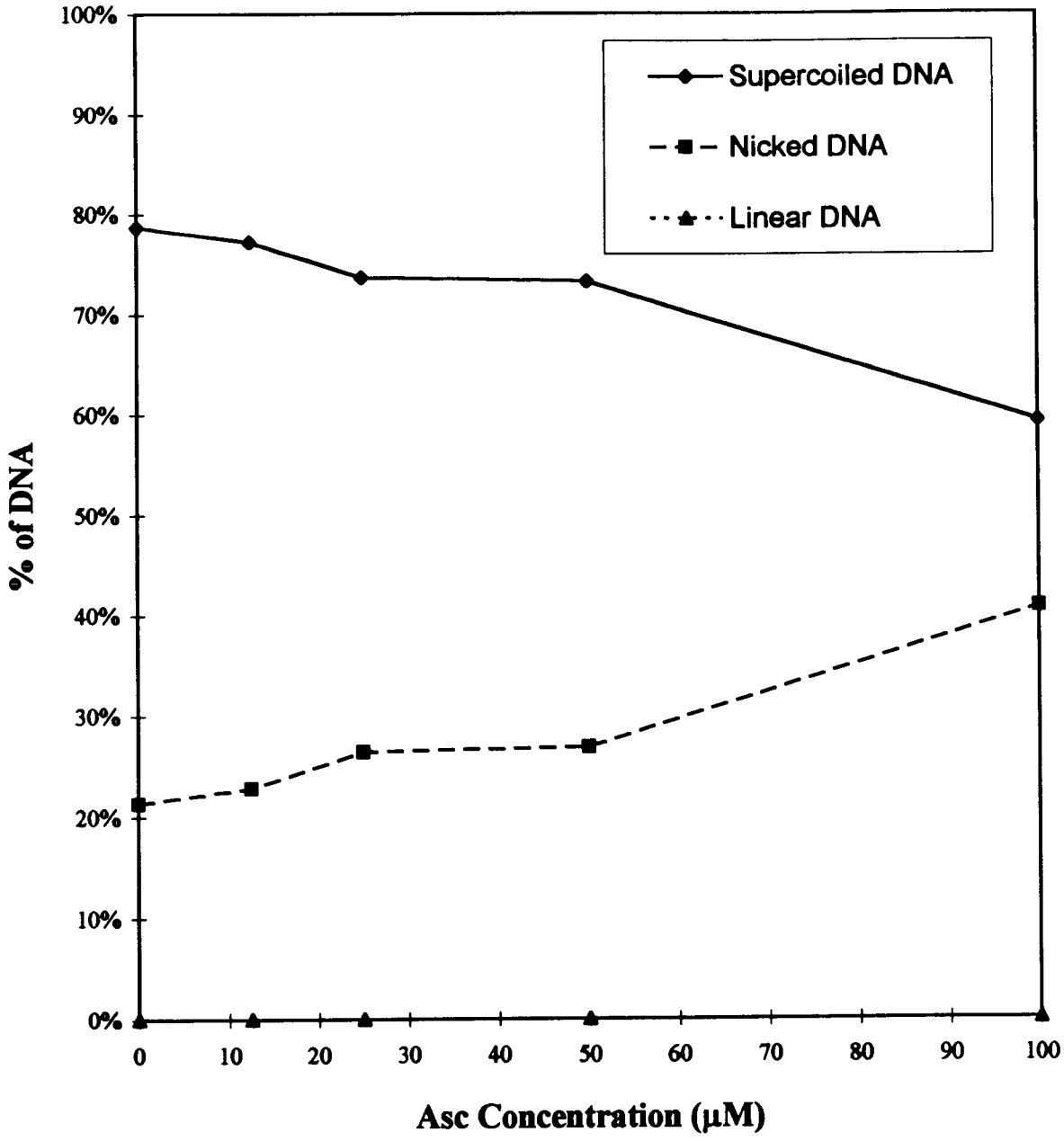


Fig. 8

The Effect of Concentrations of Desferrioxamine on DNA Damage Induced by Ascorbate and Fe(III)

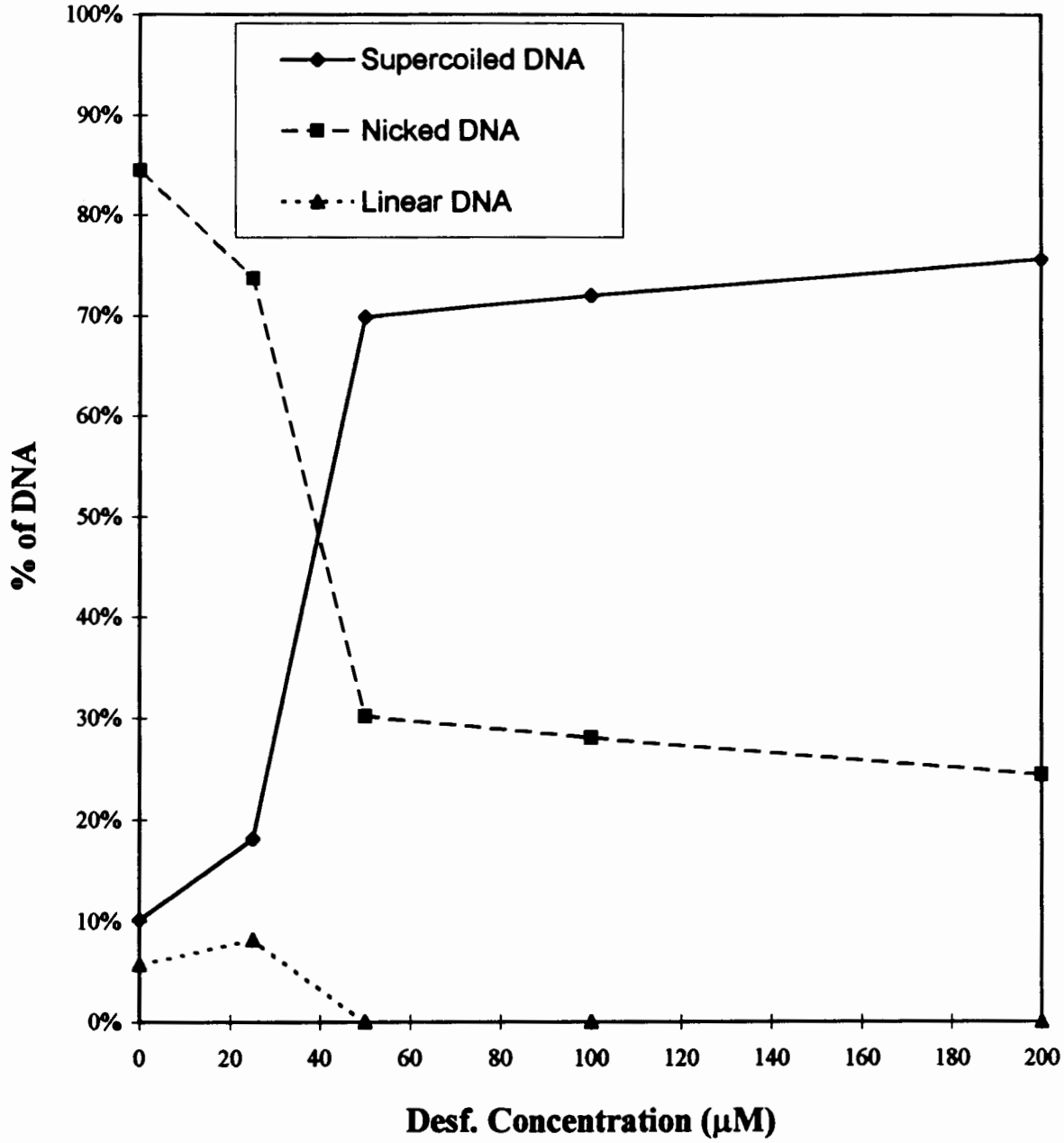


Fig. 9
The Effect of EDTA on DNA Damage

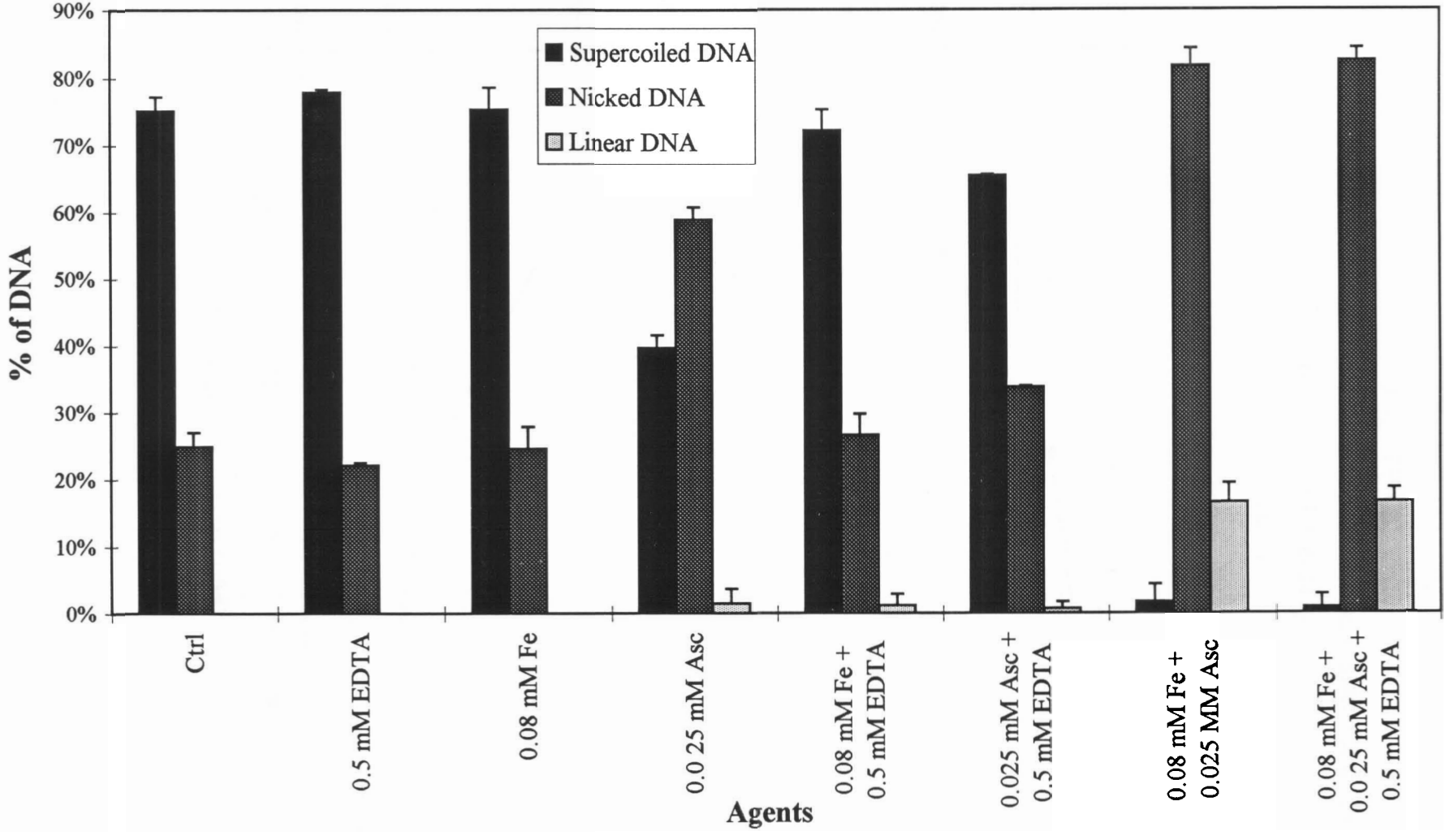


Fig. 10 (a)
The Effect of EDTA on DNA Damage Induced by Ascorbate and Fe(III)
(Ascorbate Alone)

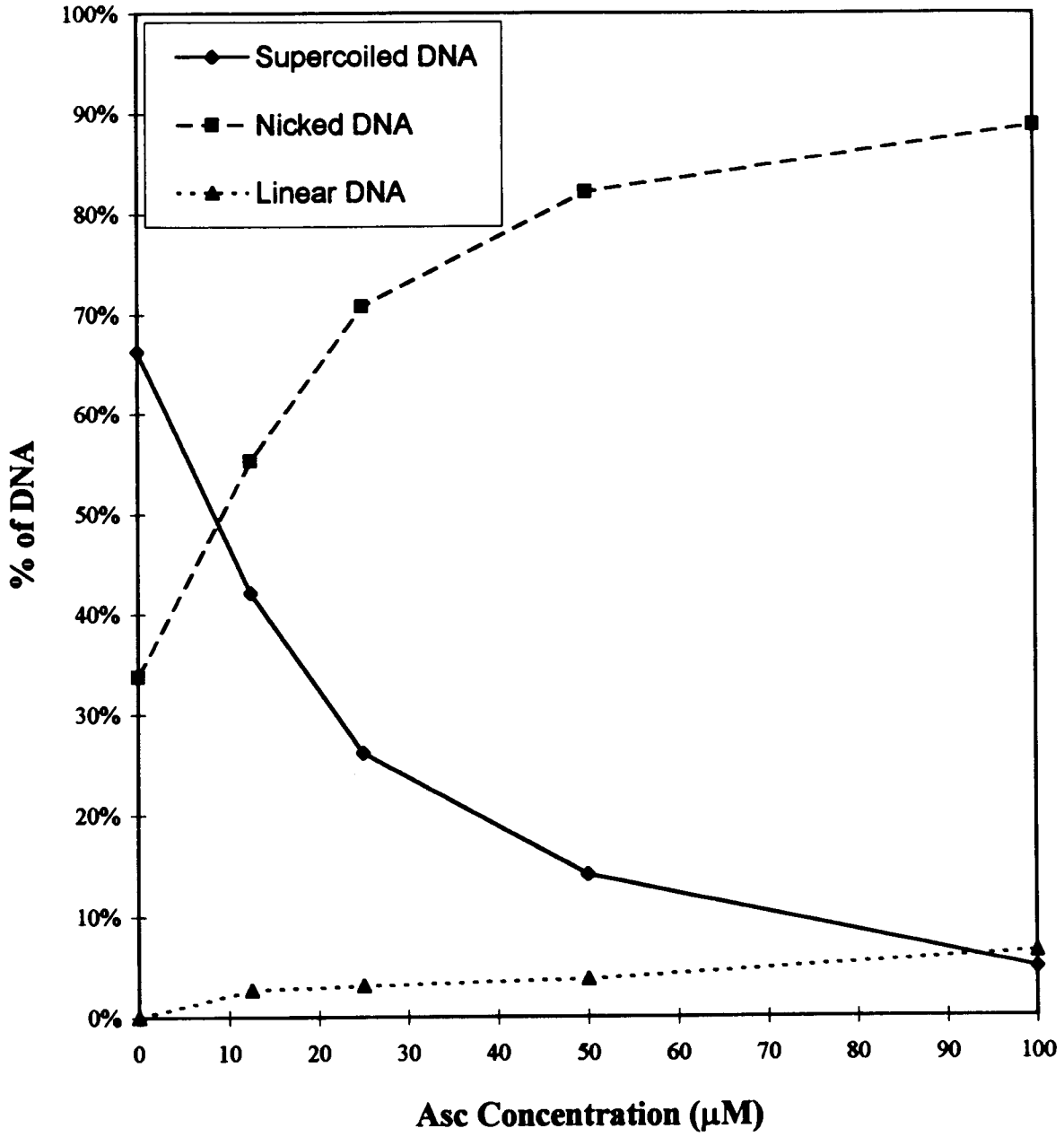


Fig. 10 (b)
The Effect of EDTA on DNA Damage Induced by Ascorbate and Fe(III)

(Asc + 80 μM Fe)

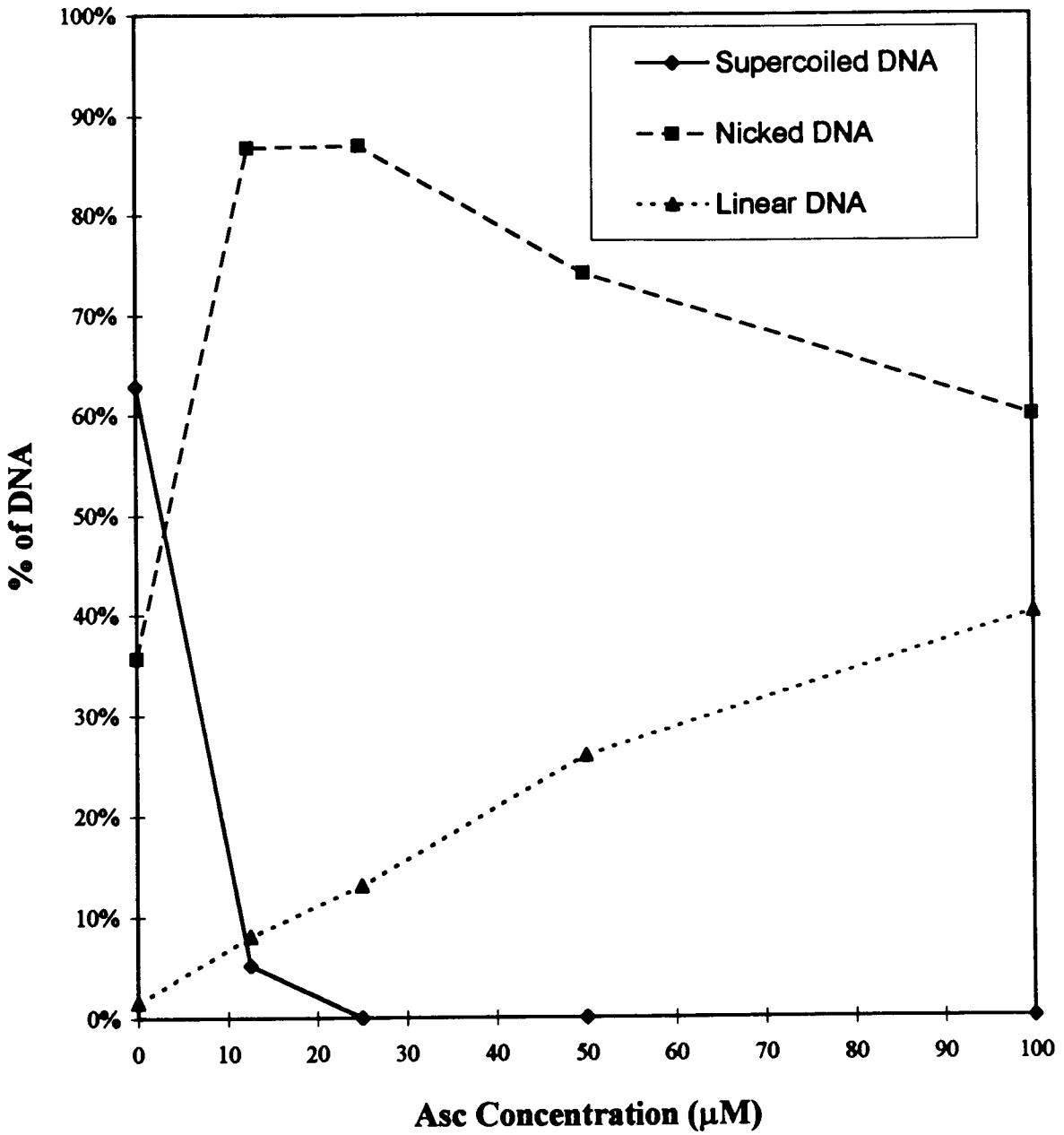


Fig. 10 (c)
The Effect of EDTA on DNA Damage Induced by Ascorbate and Fe(III)

(Asc + 500 μM EDTA)

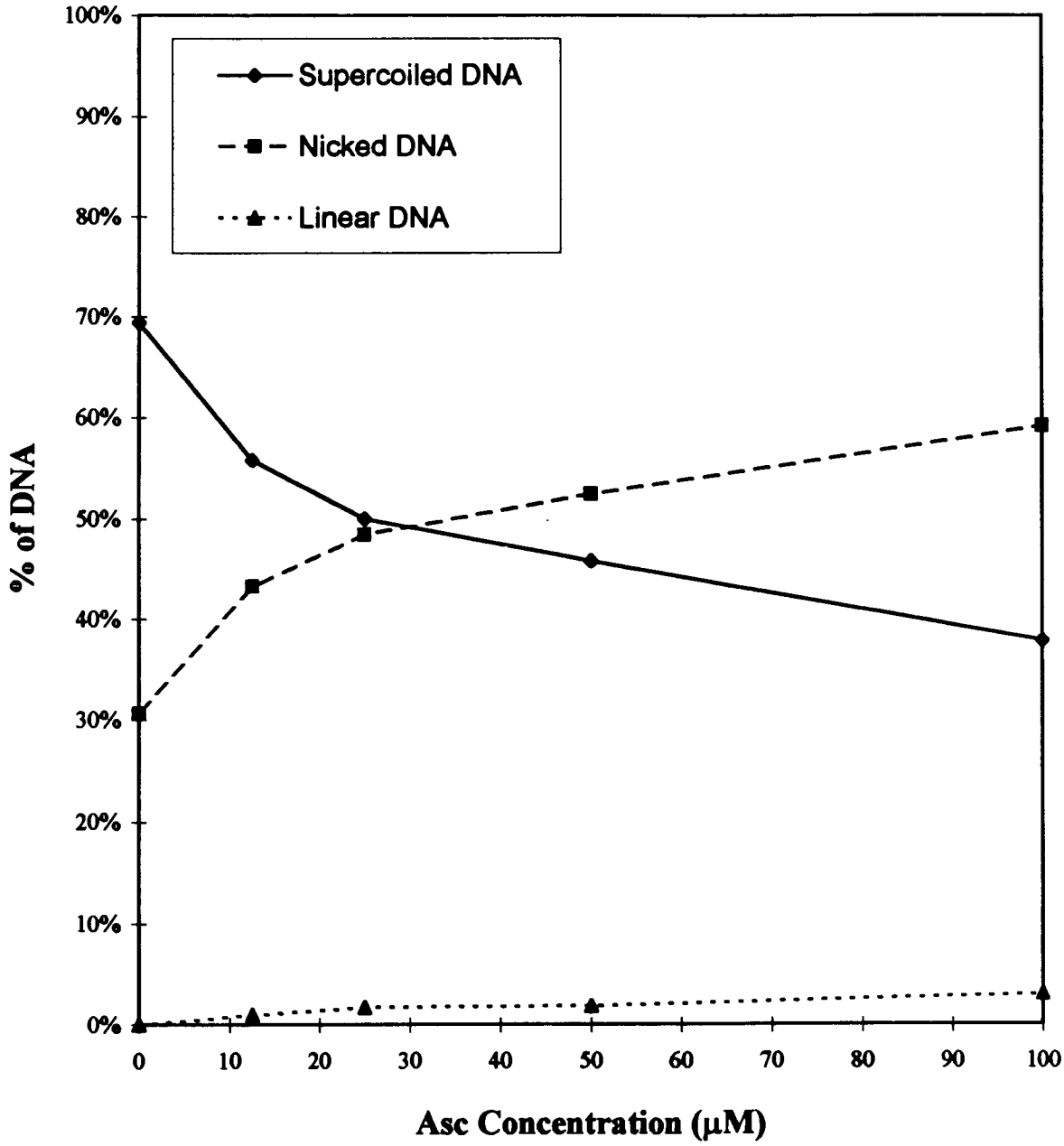


Fig. 10 (d)
The Effect of EDTA on DNA Damage Induced by Ascorbate and Fe(III)
(Asc + 80 μM Fe + 500 μM EDTA)

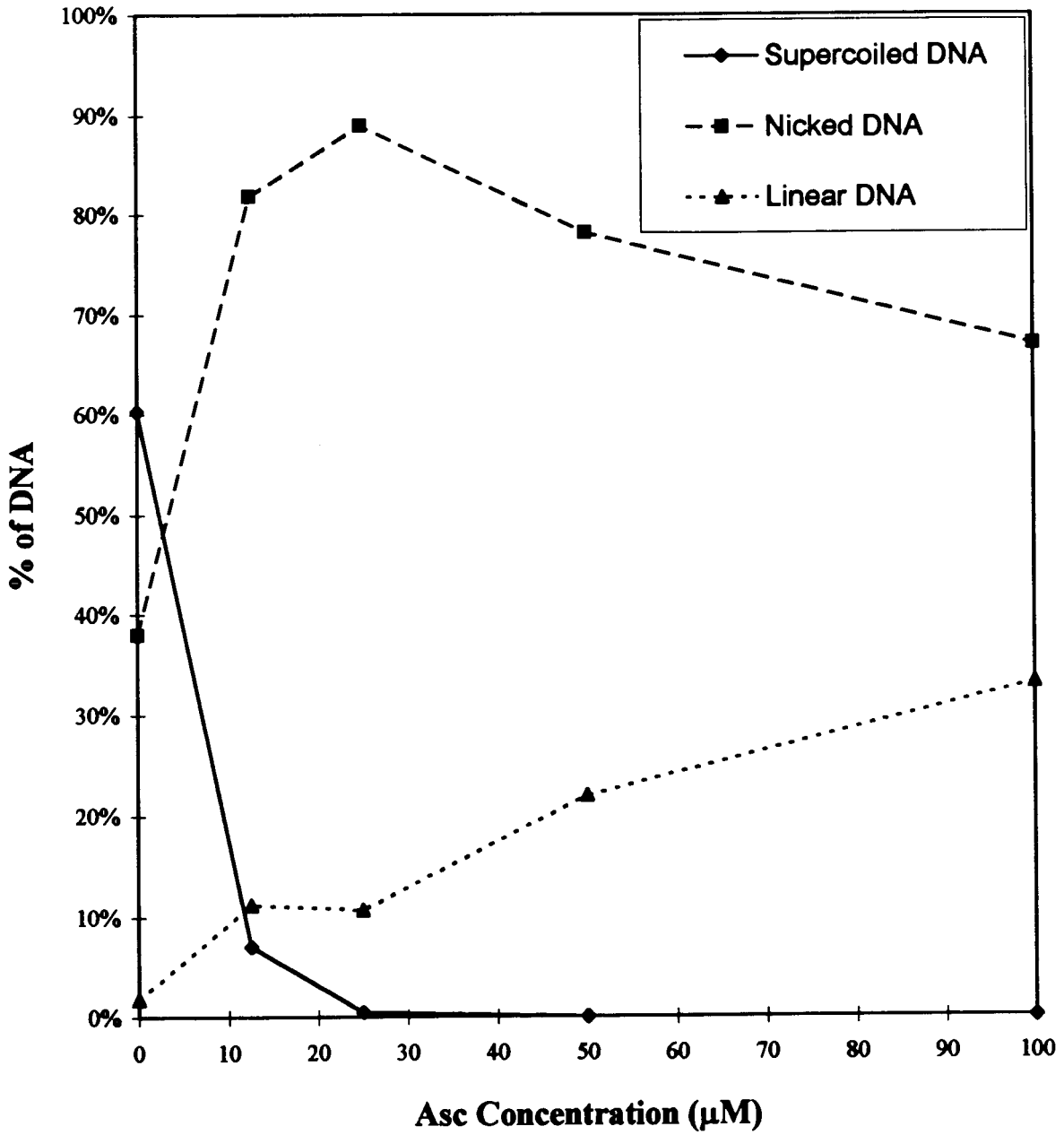


Fig. 11
The Effect of ADP on DNA Damage

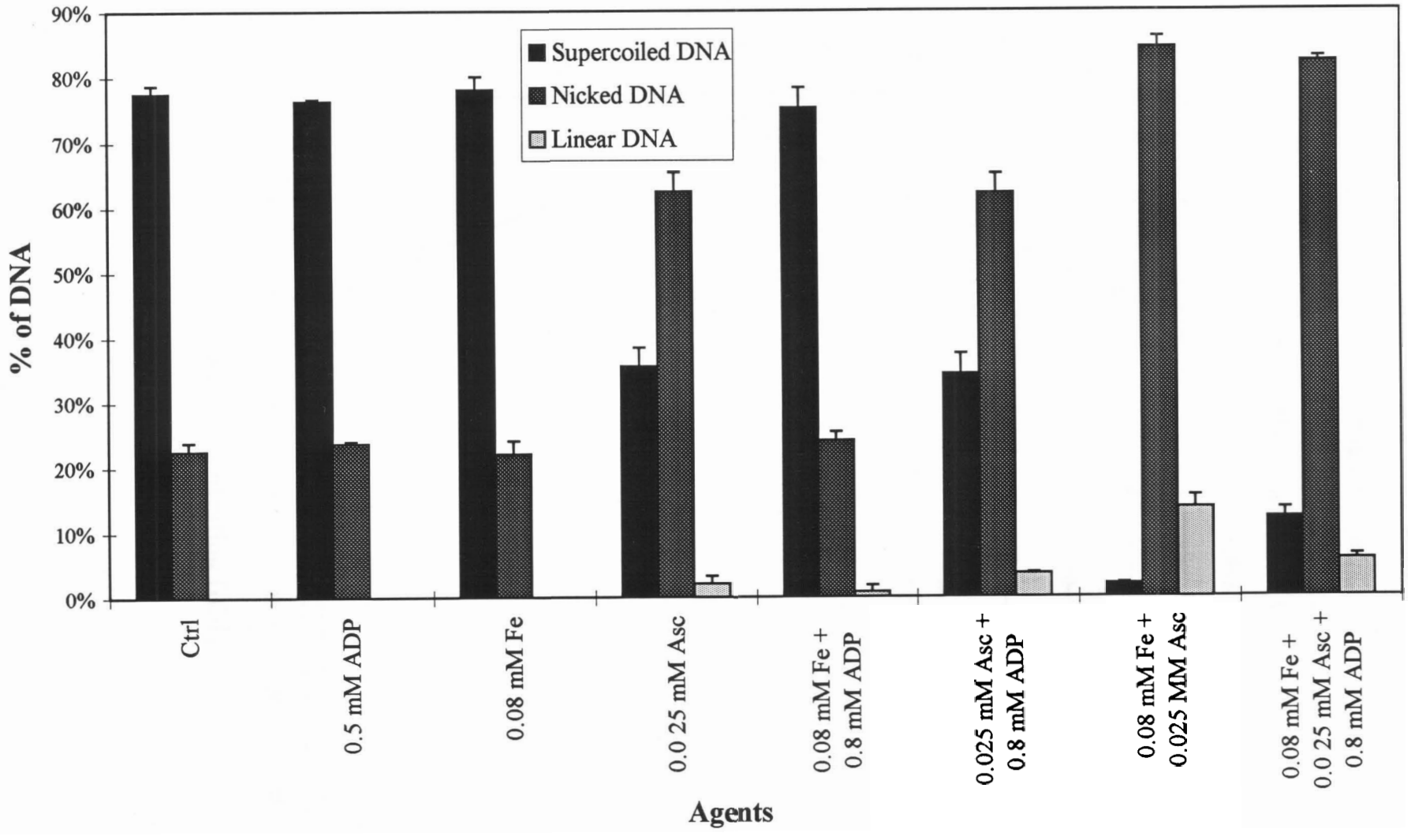


Fig. 12 (a)
The Effect of ADP on DNA Damage Induced by Ascorbate and Fe(III)
(Ascorbate Alone)

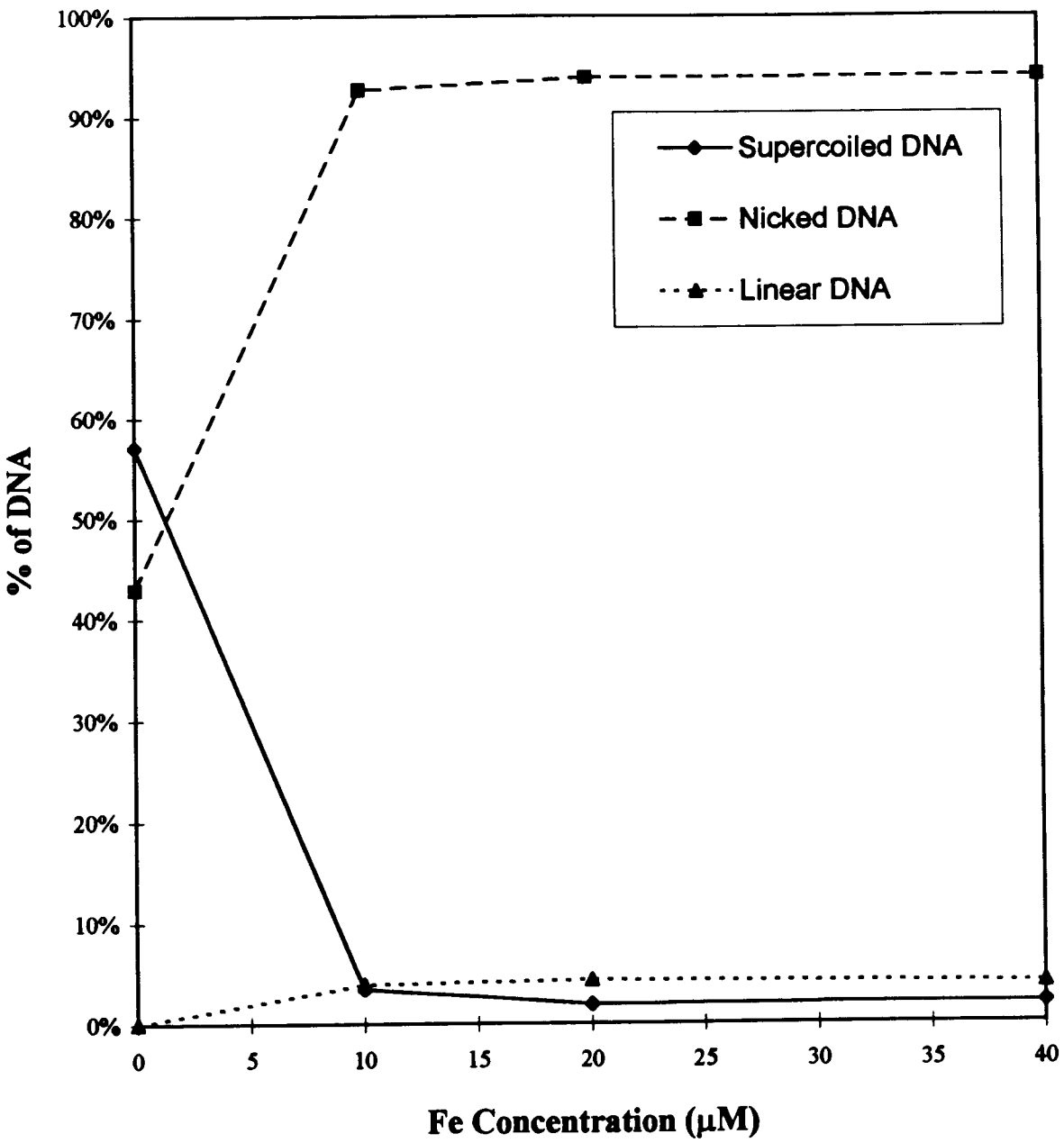


Fig. 12 (b)
The Effect of ADP on DNA Damage Induced by Ascorbate and Fe(III)

(Asc + 800 μM ADP)

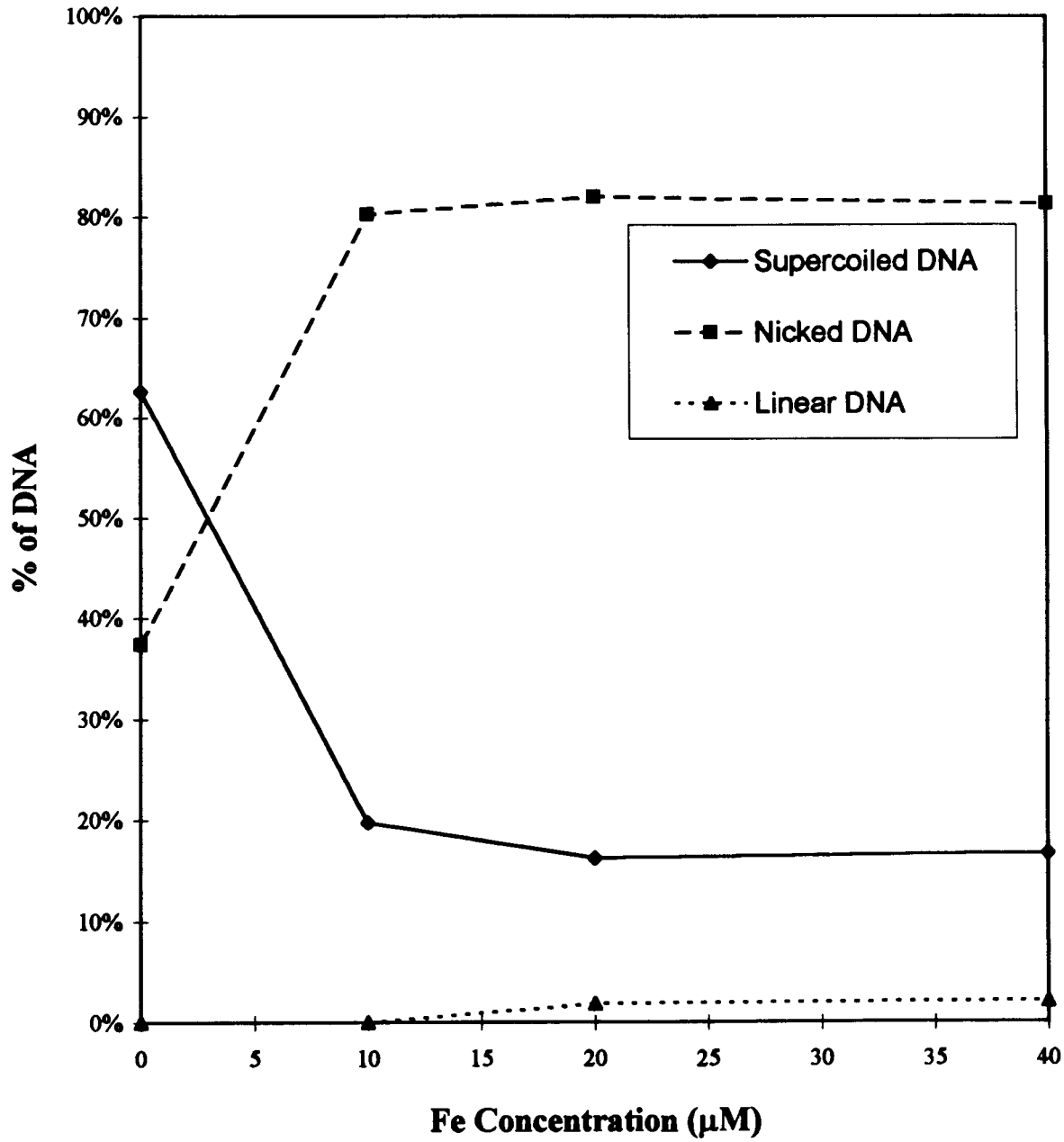


Fig. 13 (a)
The Effect of Active Catalase on DNA Damage Induced by Ascorbate and Fe(III)

(Ascorbate Alone)

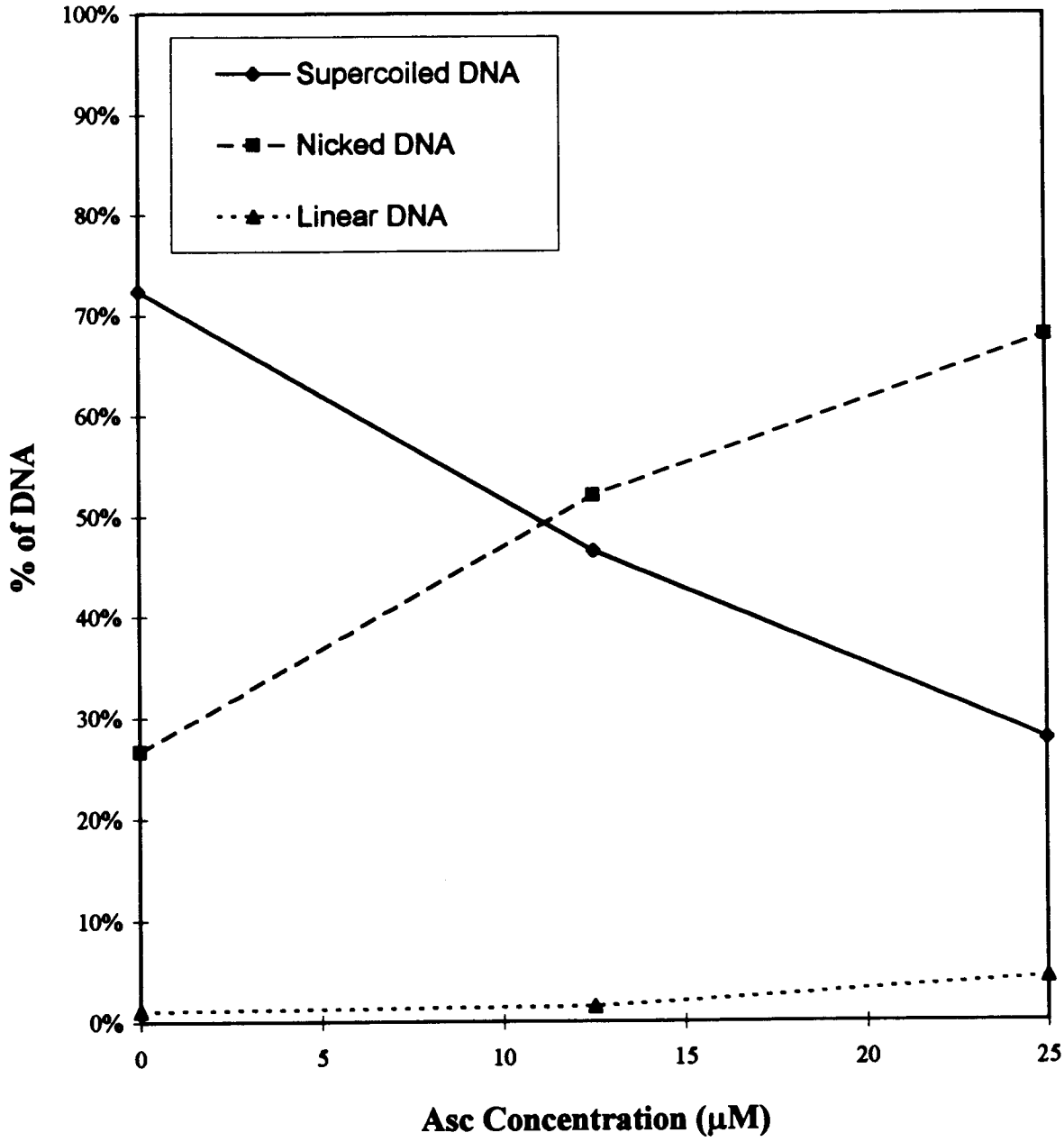


Fig. 13 (b)
The Effect of Active Catalase on DNA Damage Induced by Ascorbate and Fe(III)

(Asc + 80 μM Fe)

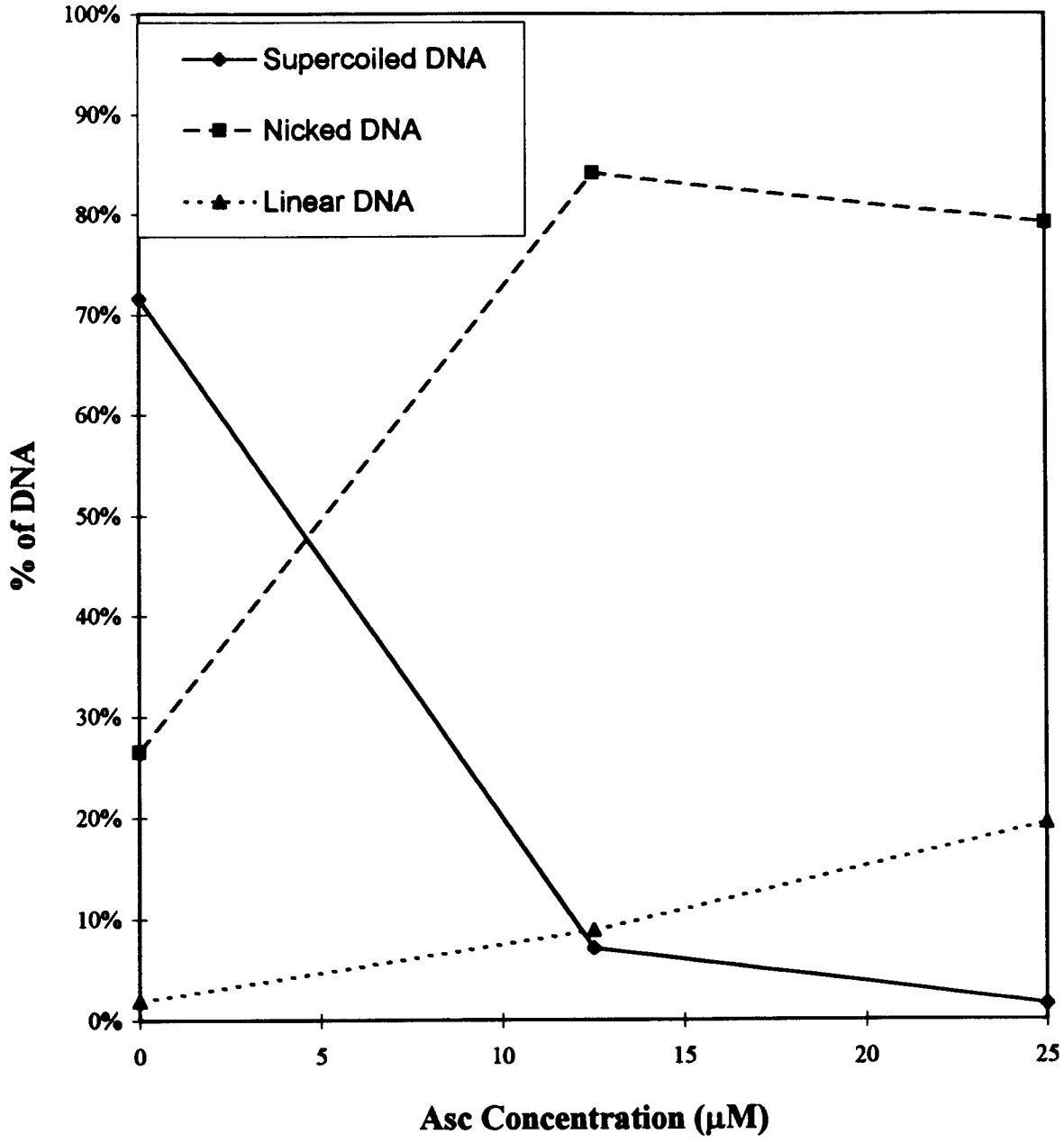


Fig. 13 (c)
The Effect of Active Catalase on DNA Damage Induced by Ascorbate and Fe(III)

(Asc + 10 U/ml Cat)

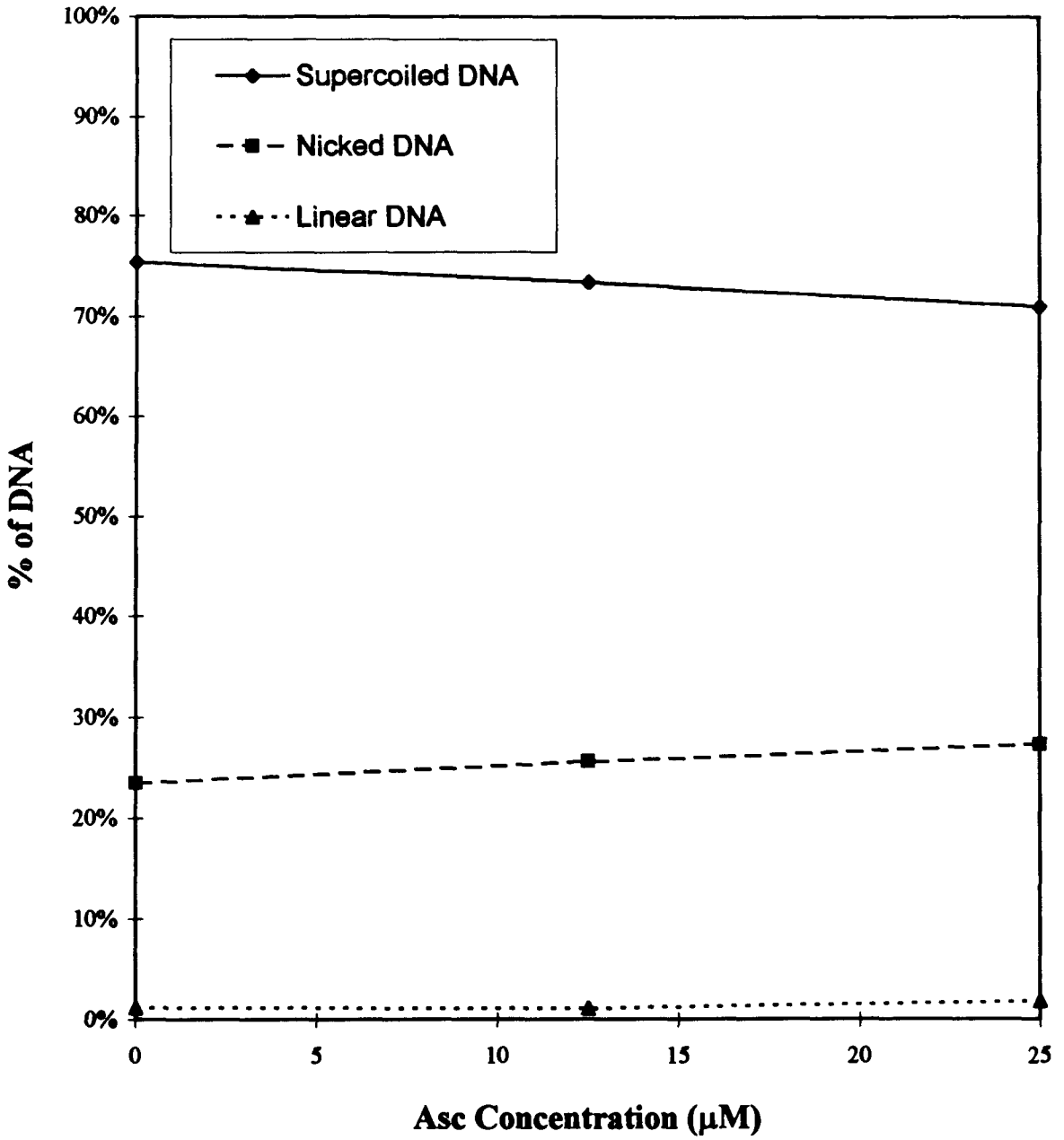


Fig. 13 (d)

The Effect of Active Catalase on DNA Damage Induced by Ascorbate and Fe(III)

(Asc + 80 μM Fe + 10 U/ml Cat)

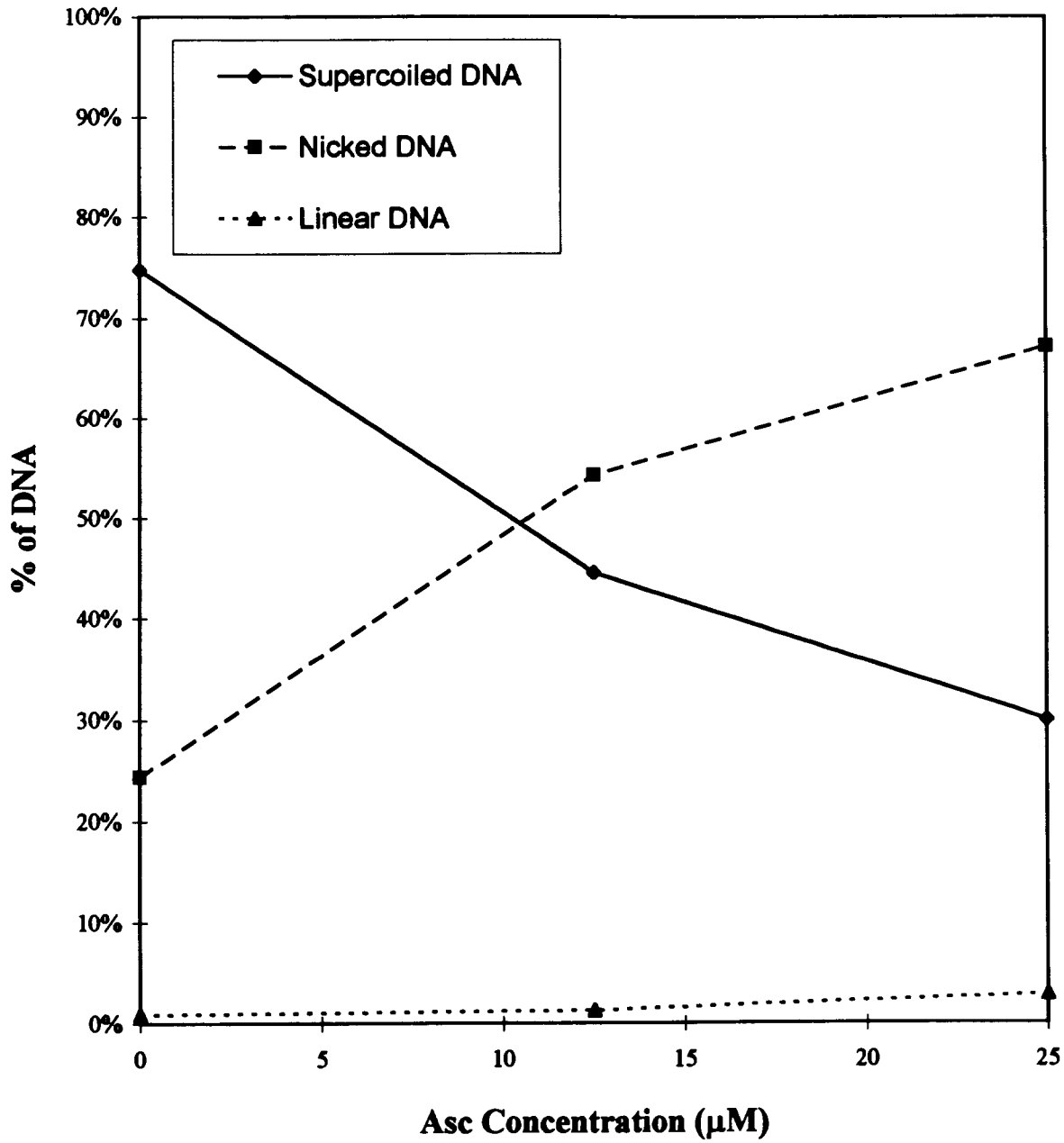


Fig. 14 (a)
The Effect of Denatured Catalase on DNA Damage Induced by Ascorbate and Fe(III)

(Ascorbate Alone)

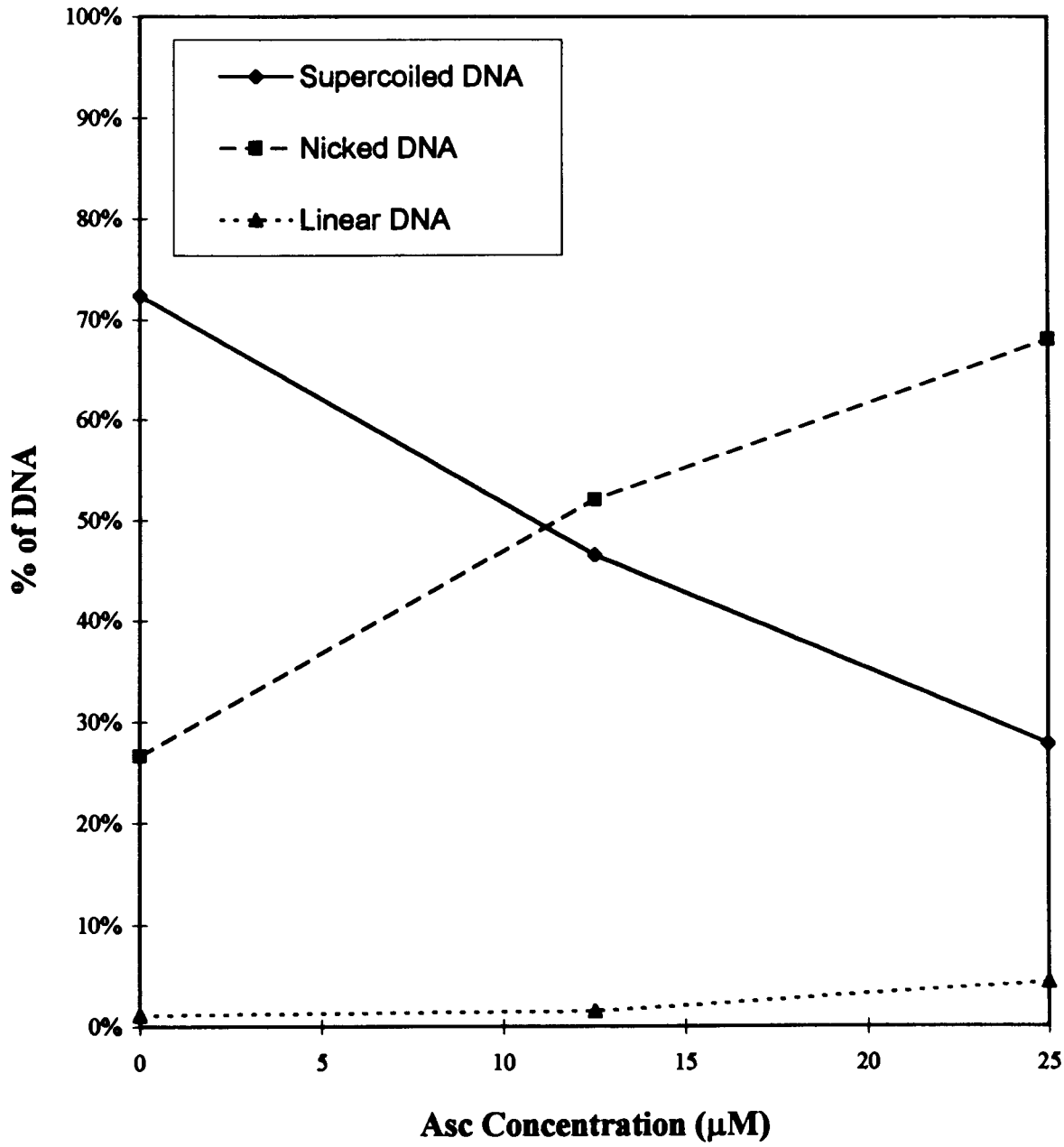


Fig. 14 (b)
The Effect of Denatured Catalase on DNA Damage Induced by Ascorbate and Fe(III)

(Asc + 80 μM Fe)

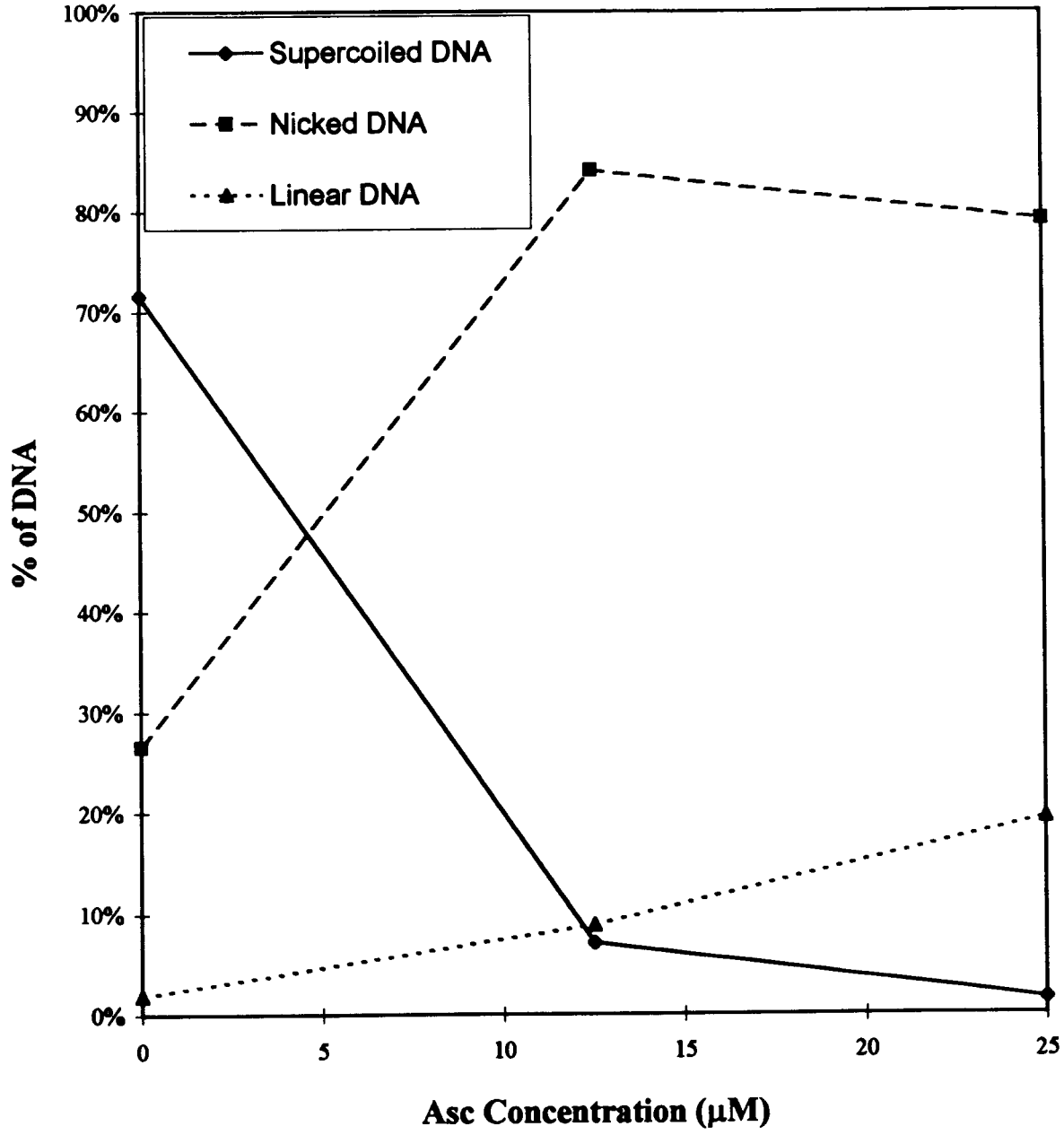


Fig. 14 (c)
The Effect of Dentatured Catalase on DNA Damage Induced by Ascorbate and Fe(III)

(Asc + 10 U/ml Cat)

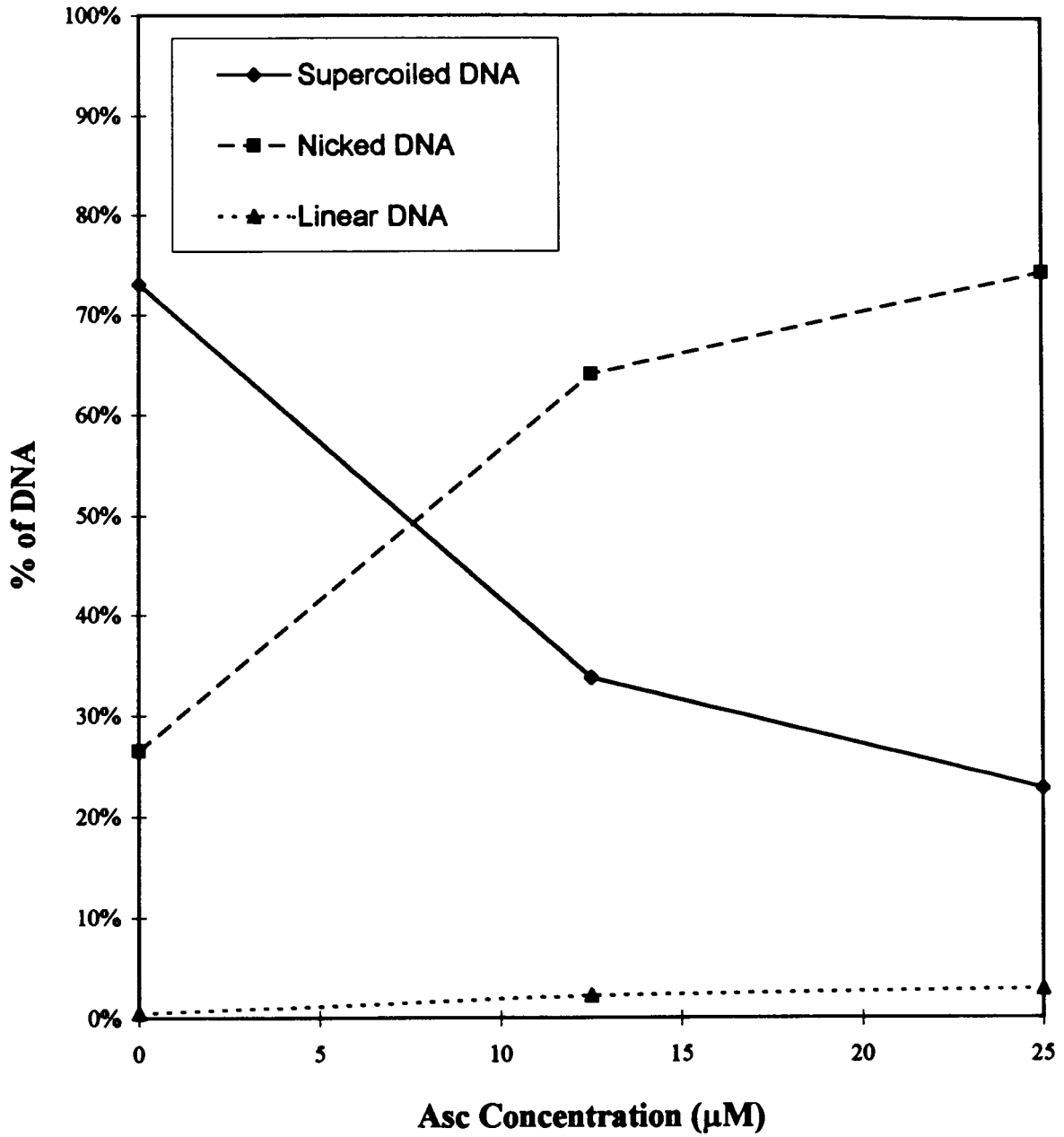


Fig. 14 (d)
The Effect of Denatured Catalase on DNA Damage Induced by Ascorbate and Fe(III)

(Asc + 80 μM Fe + 10 U/ml Cat)

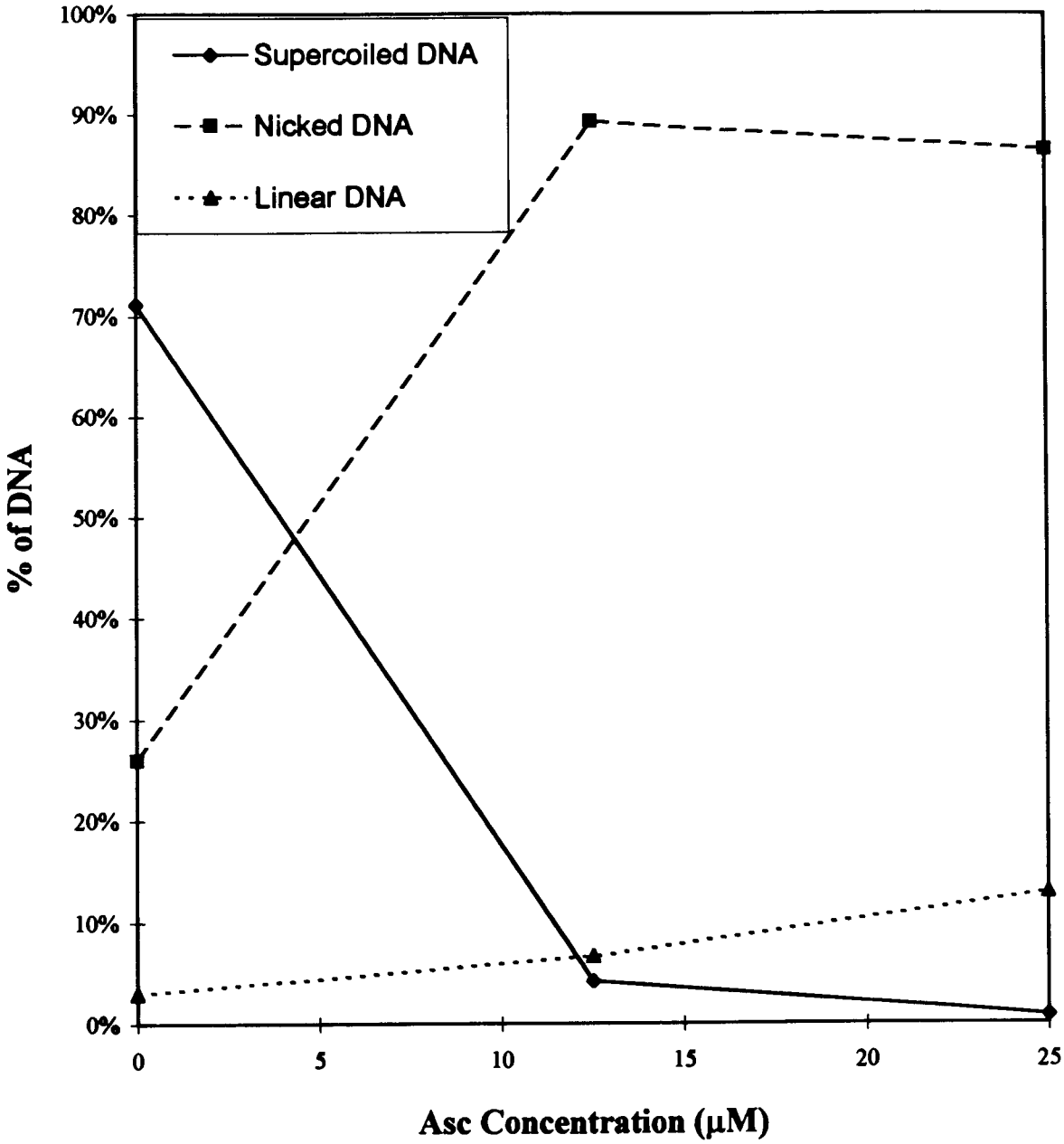


Fig. 15 (a)
The Effect of Active SOD on DNA Damage Induced by Ascorbate and Fe(III)

(Ascorbate Alone)

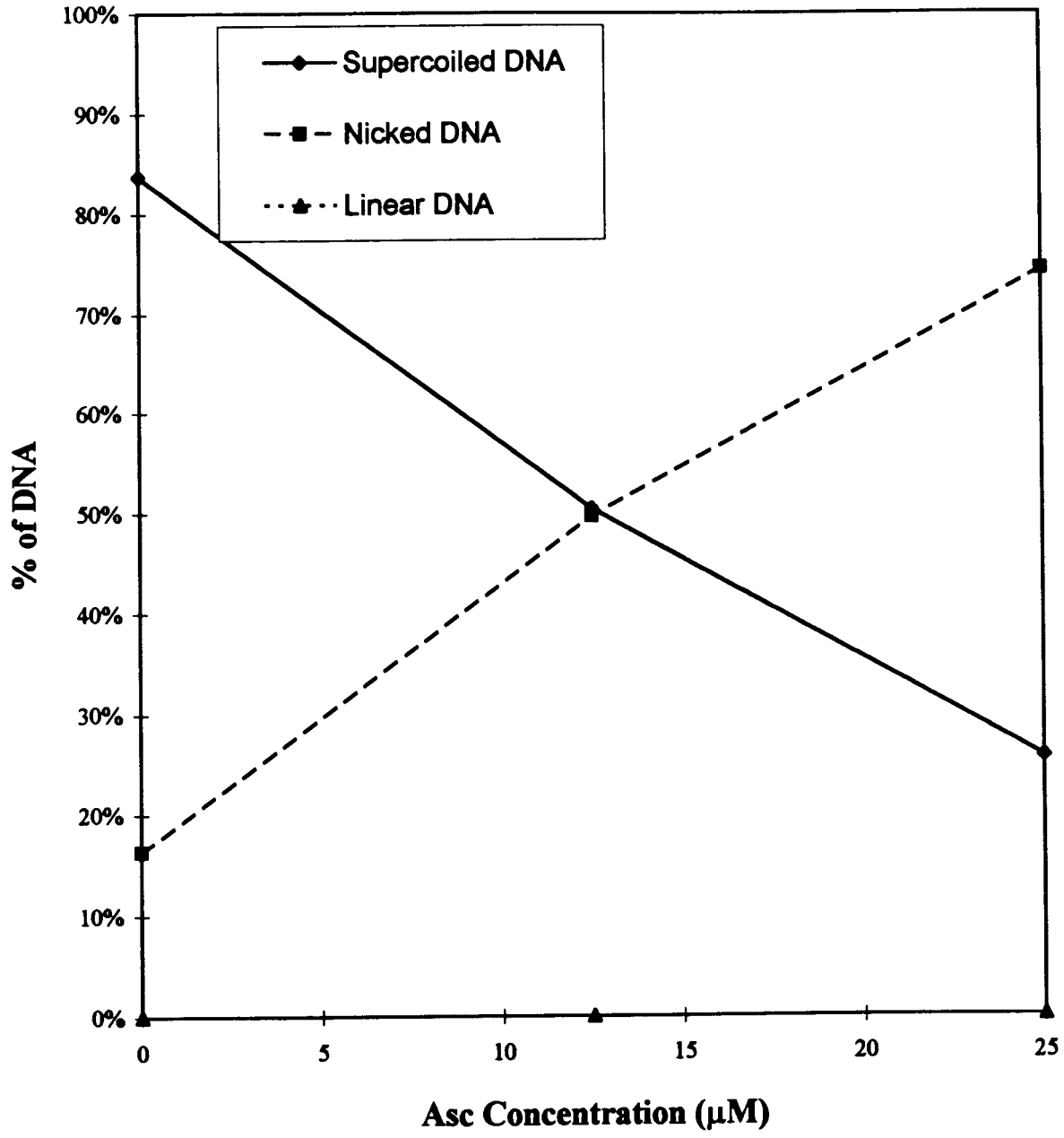


Fig. 15 (b)
The Effect of Active SOD on DNA Damage Induced by Ascorbate and Fe(III)

(Asc + 80 μM Fe)

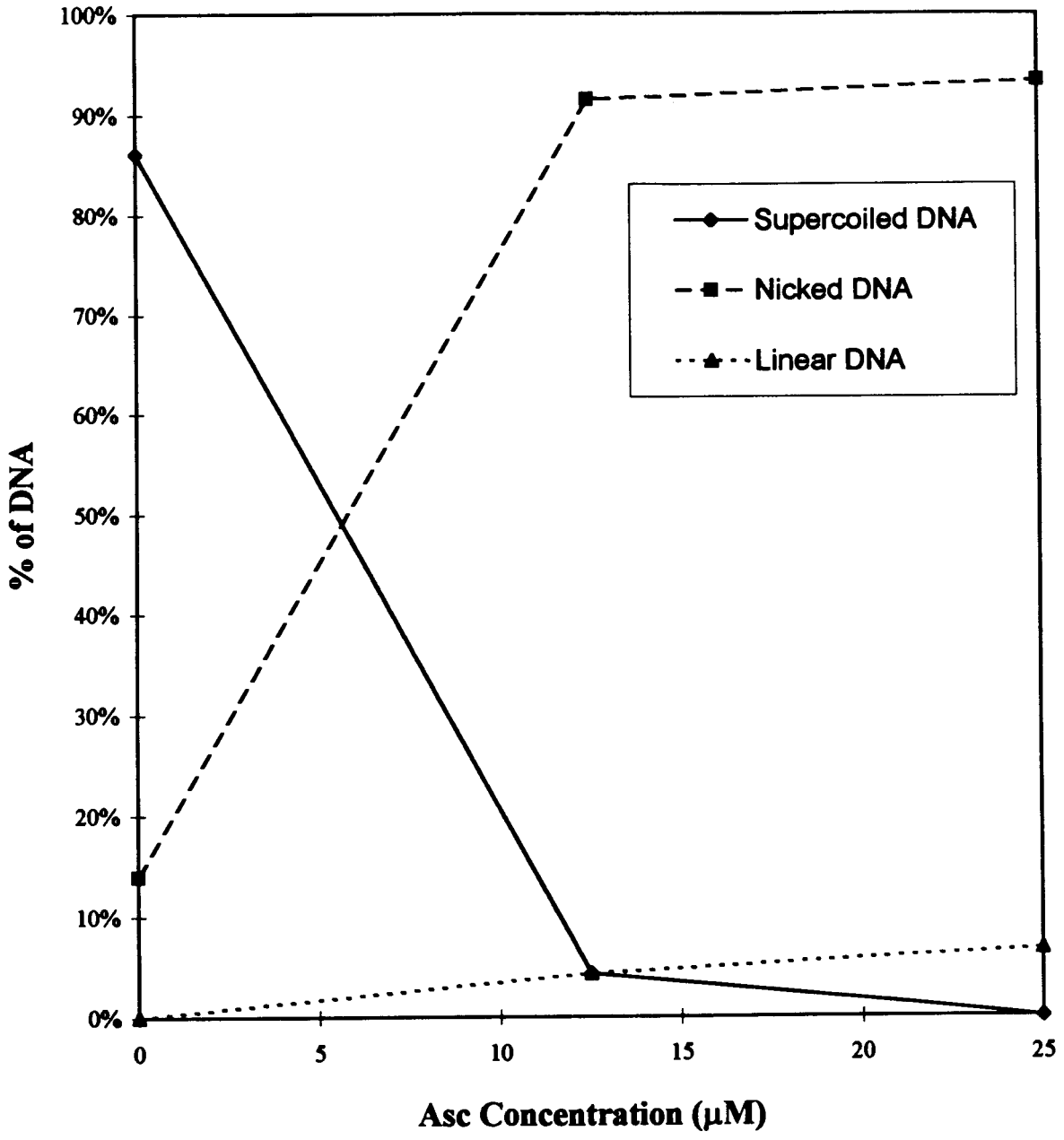


Fig. 15 (c)
The Effect of Actvie SOD on DNA Damage Induced by Ascorbate and Fe(III)
(Asc + 20 U/ml SOD)

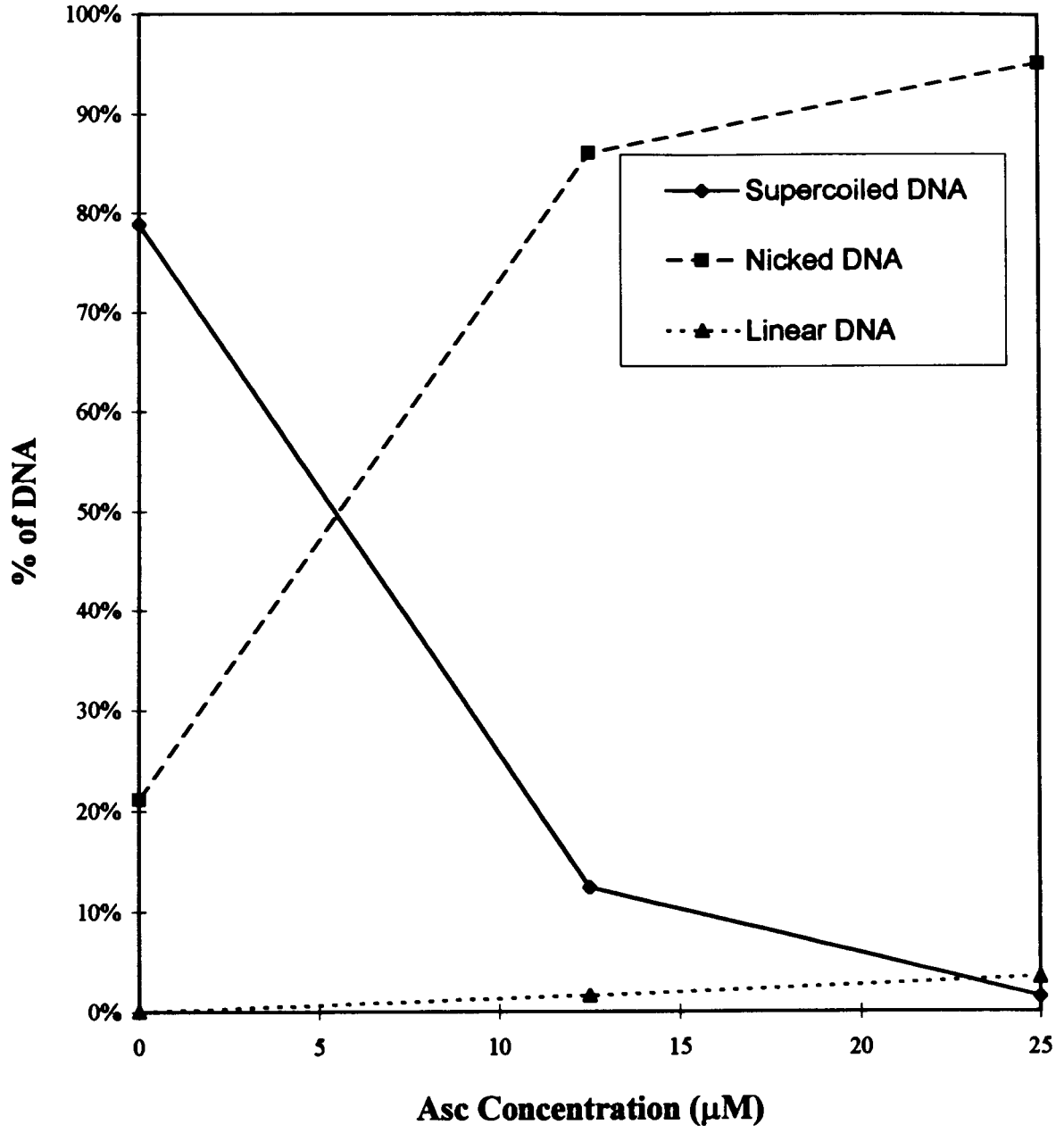


Fig. 15 (d)

The Effect of Active SOD on DNA Damage Induced by Ascorbate and Fe(III)

(Asc + 80 μM Fe + 20 U/ml SOD)

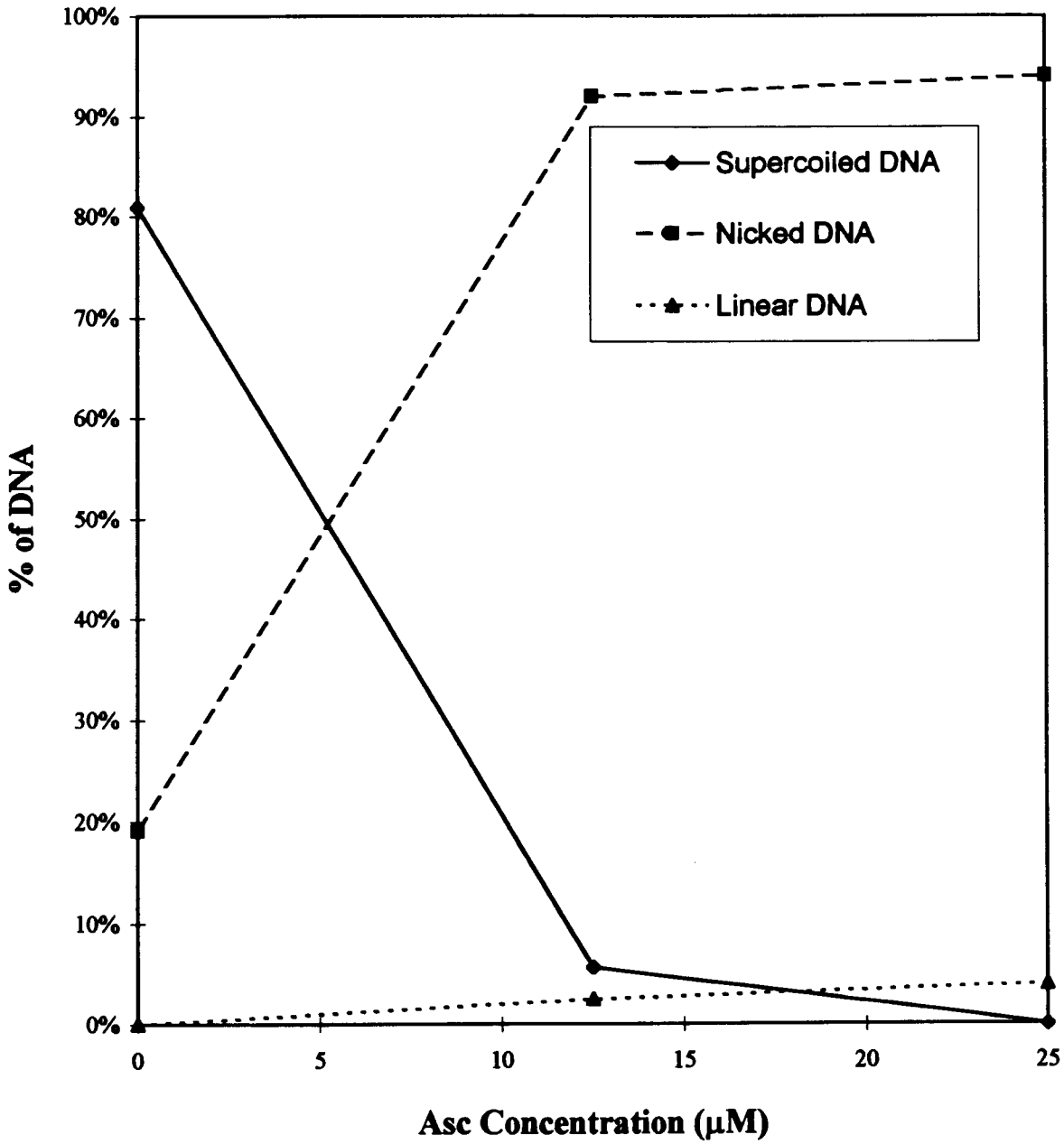


Fig. 16 (a)
The Effect of Inactivated SOD on DNA Damage Induced by Ascorbate and Fe(III)

(Ascorbate Alone)

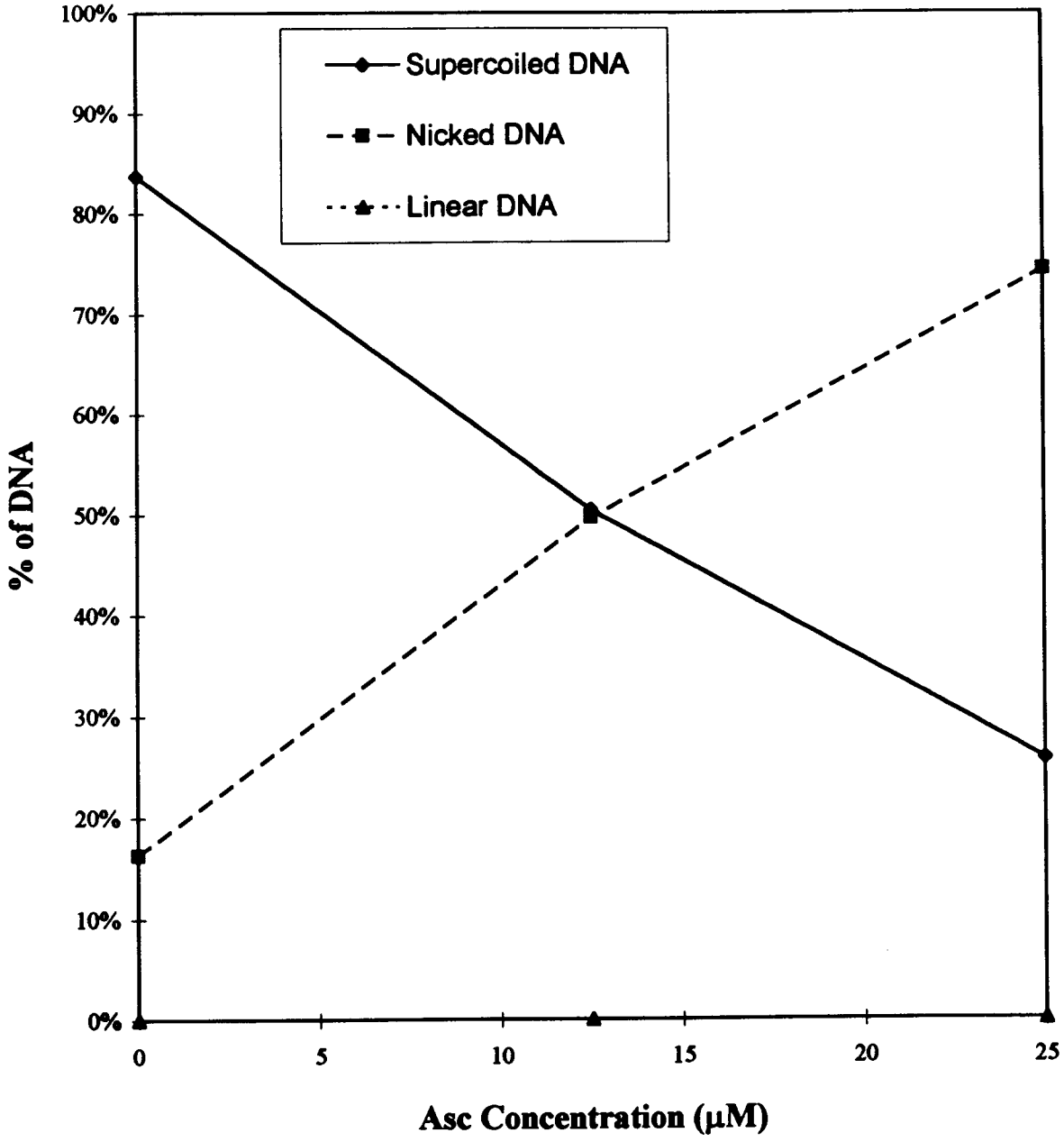


Fig. 16 (b)
The Effect of Inactivated SOD on DNA Damage Induced by Ascorbate and Fe(III)

(Asc + 80 μM Fe)

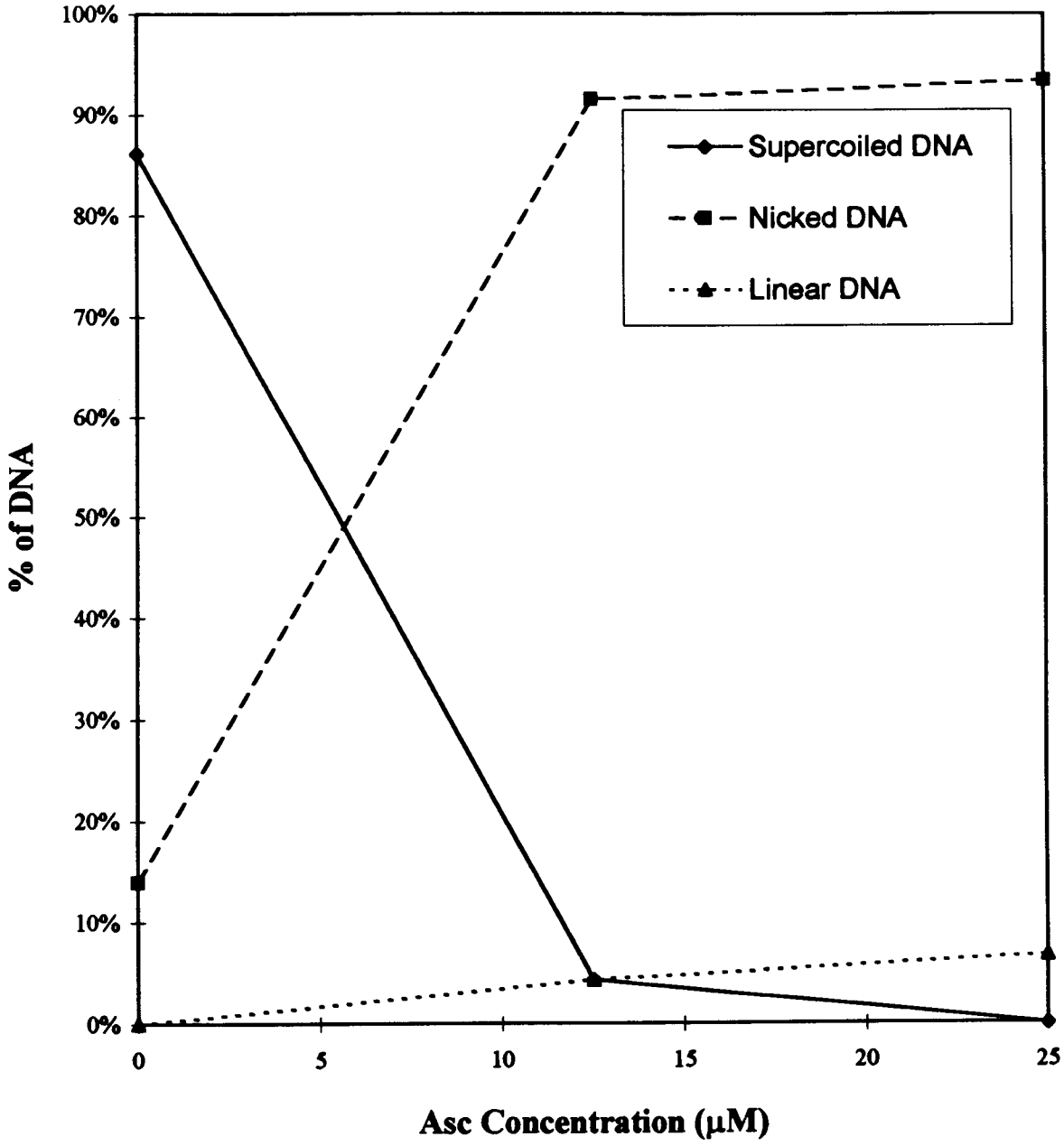


Fig. 16 (c)
The Effect of Inactivated SOD on DNA Damage Induced by Ascorbate and Fe(III)
(Asc + 20 U/ml SOD)

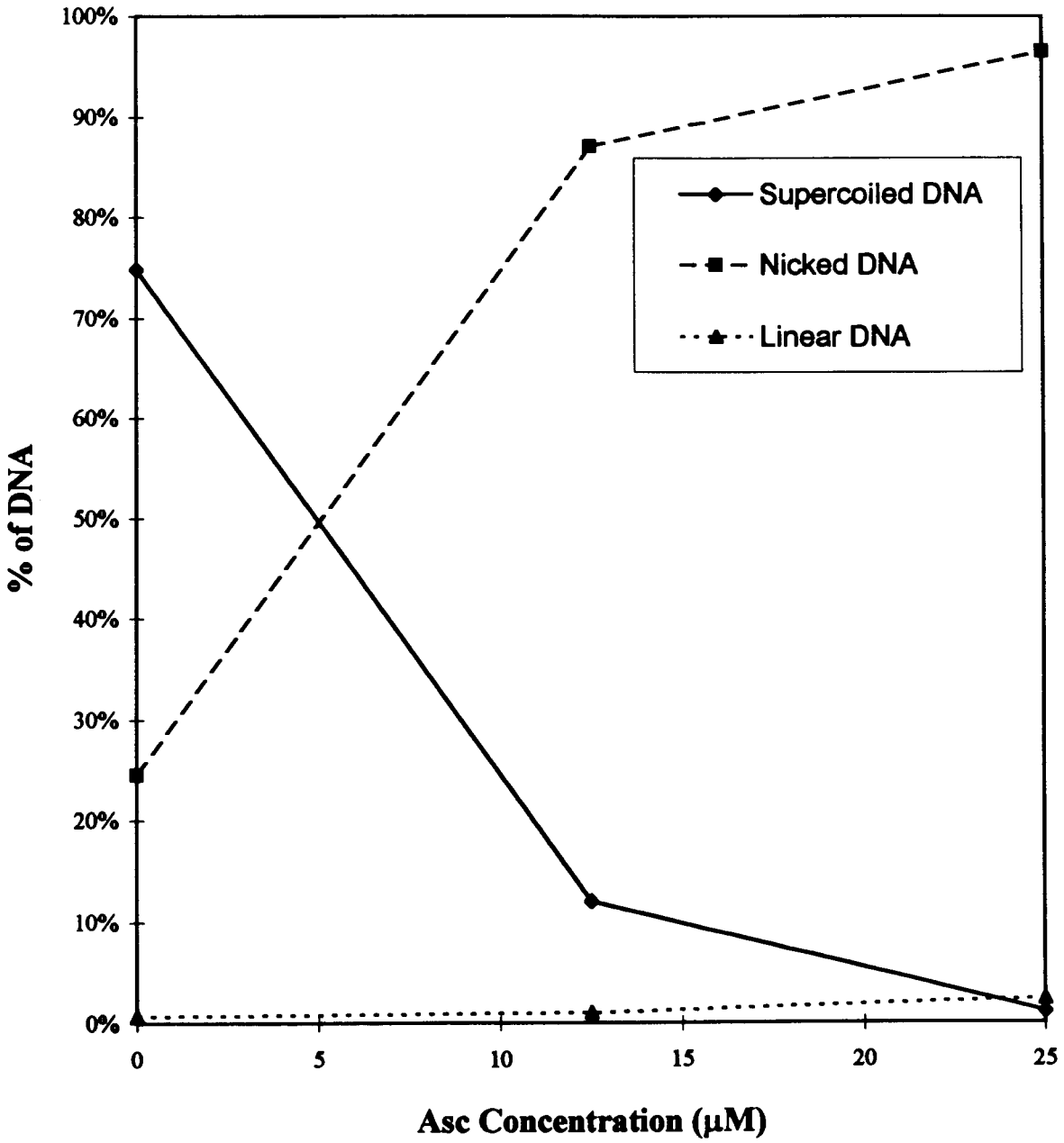


Fig. 16 (d)
The Effect of Inactivated SOD on DNA Damage Induced by Ascorbate and Fe(III)

(Asc 80 μM Fe + 20 U/ml SOD)

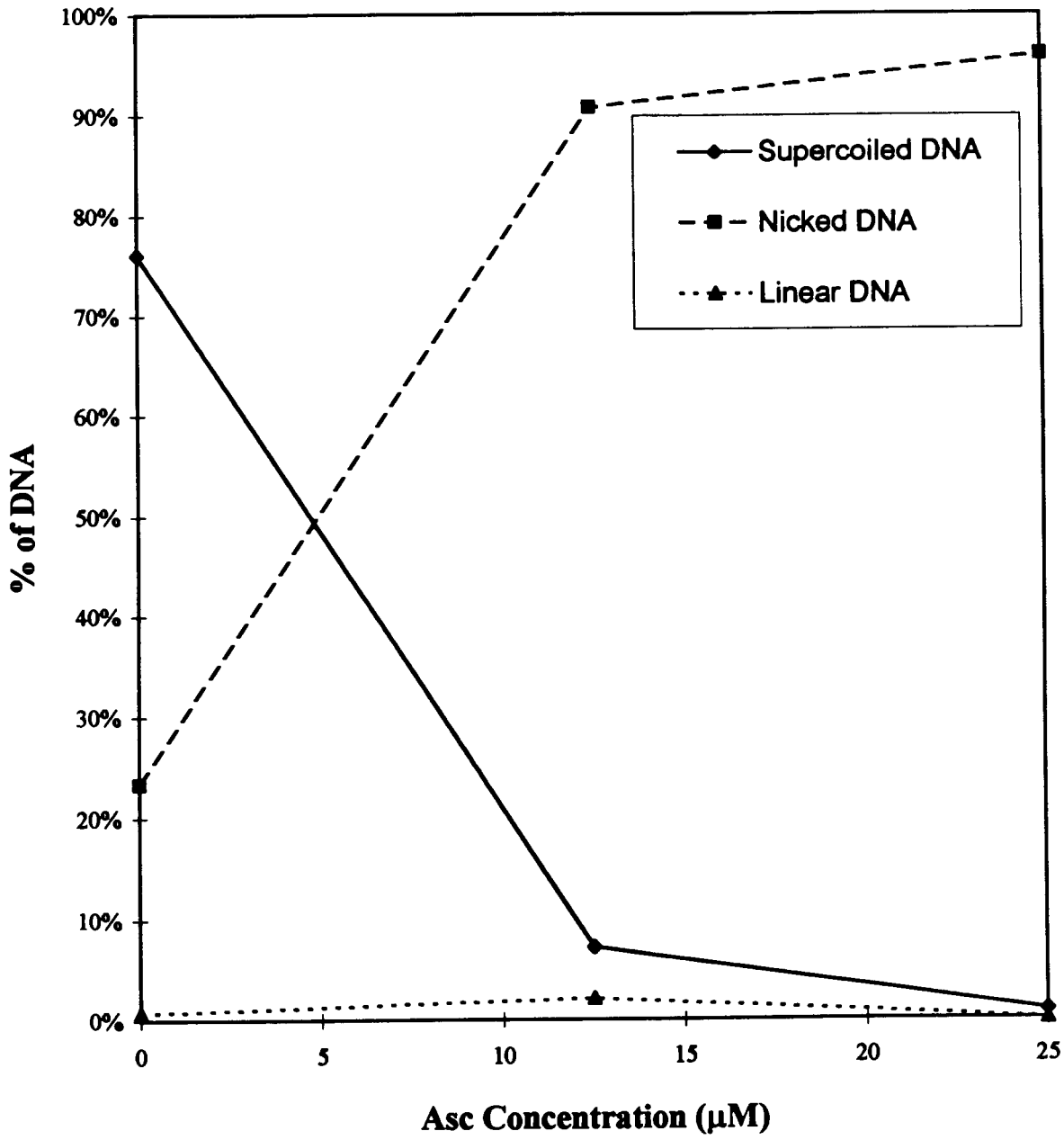


Fig. 17
The Effect of Formate on DNA Damage Induced by Ascorbate and Fe(III)

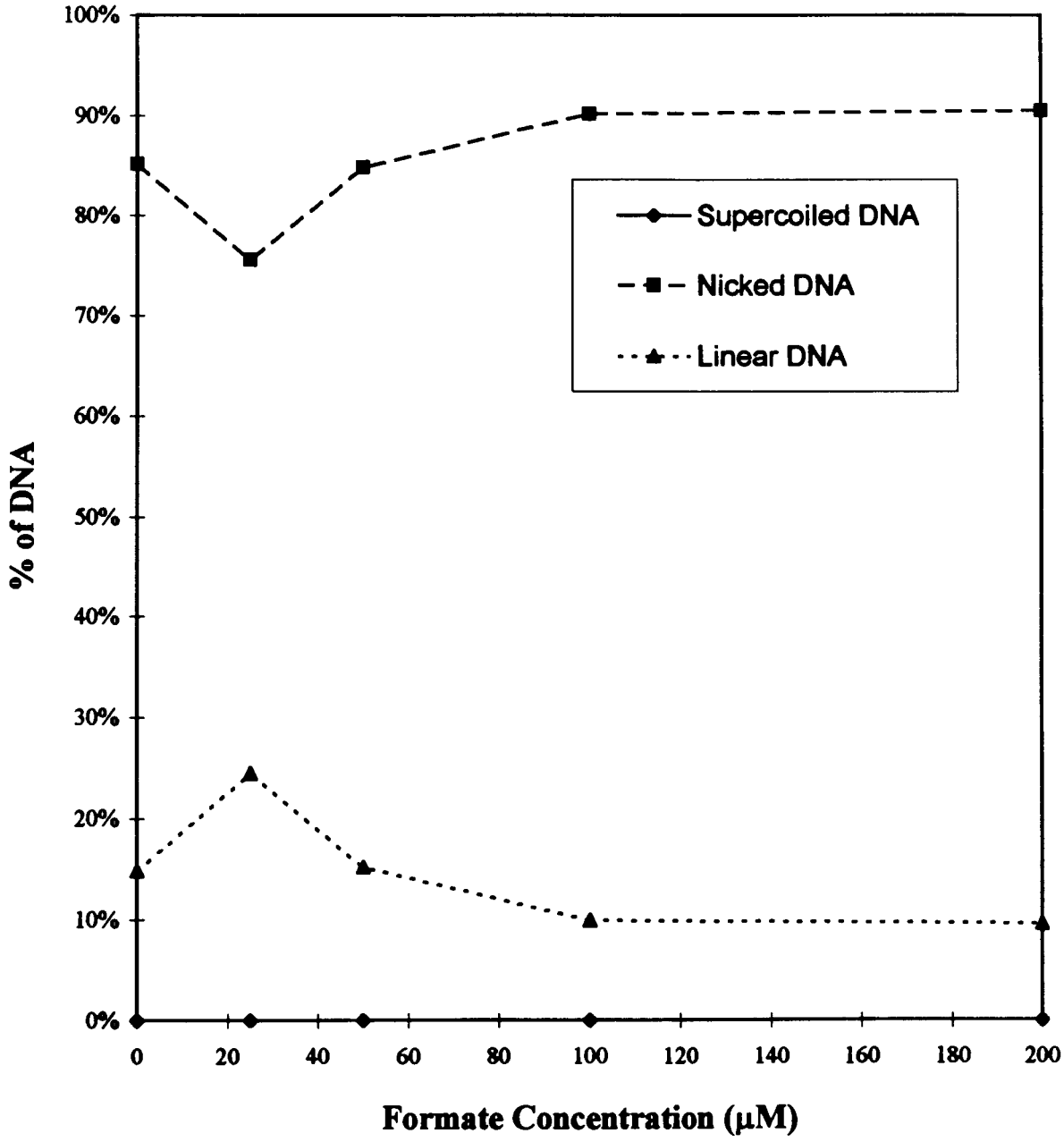


Fig. 18
The Effect of Mannitol on DNA Damage Induced by Ascorbate and Fe(III)

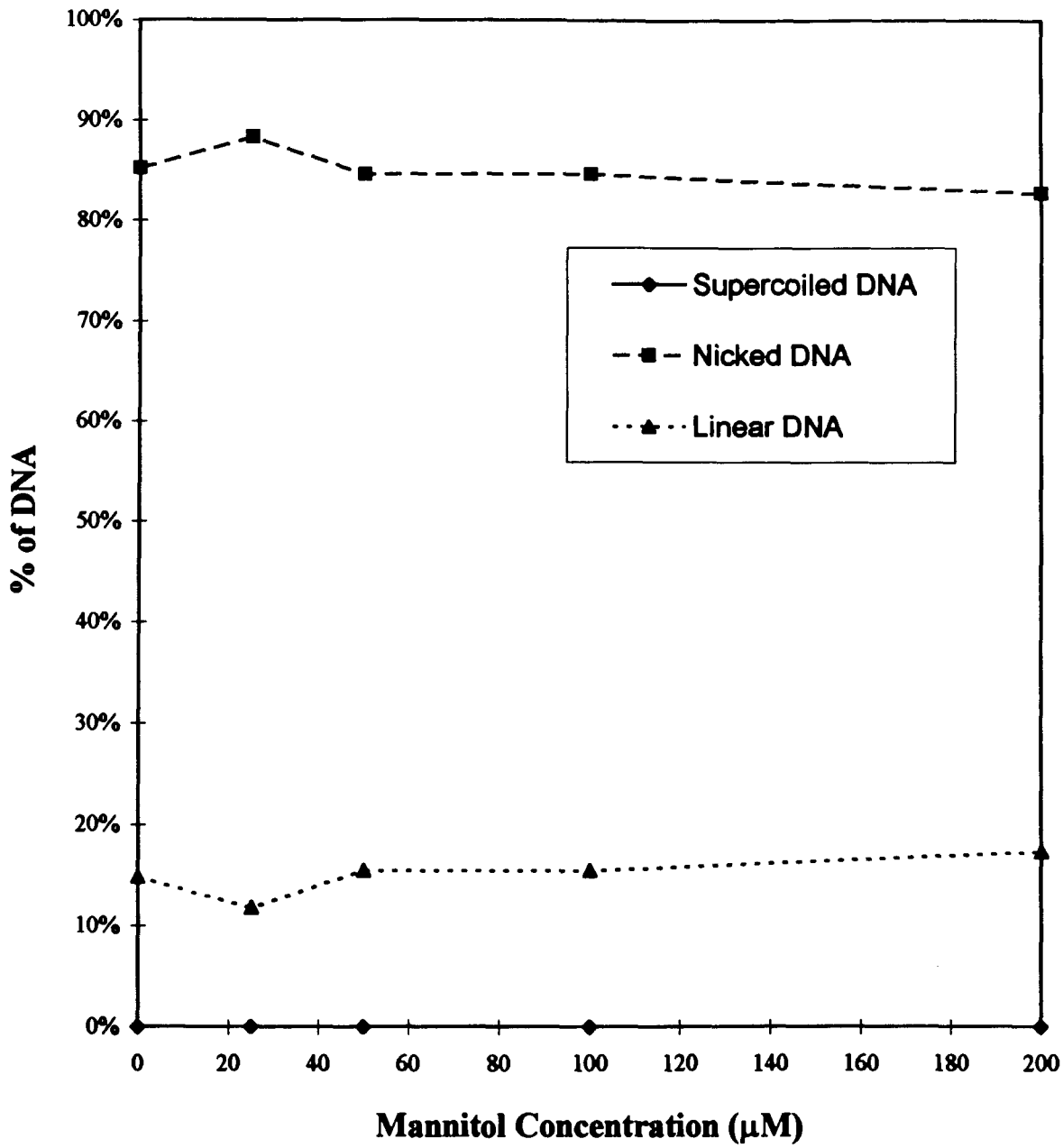
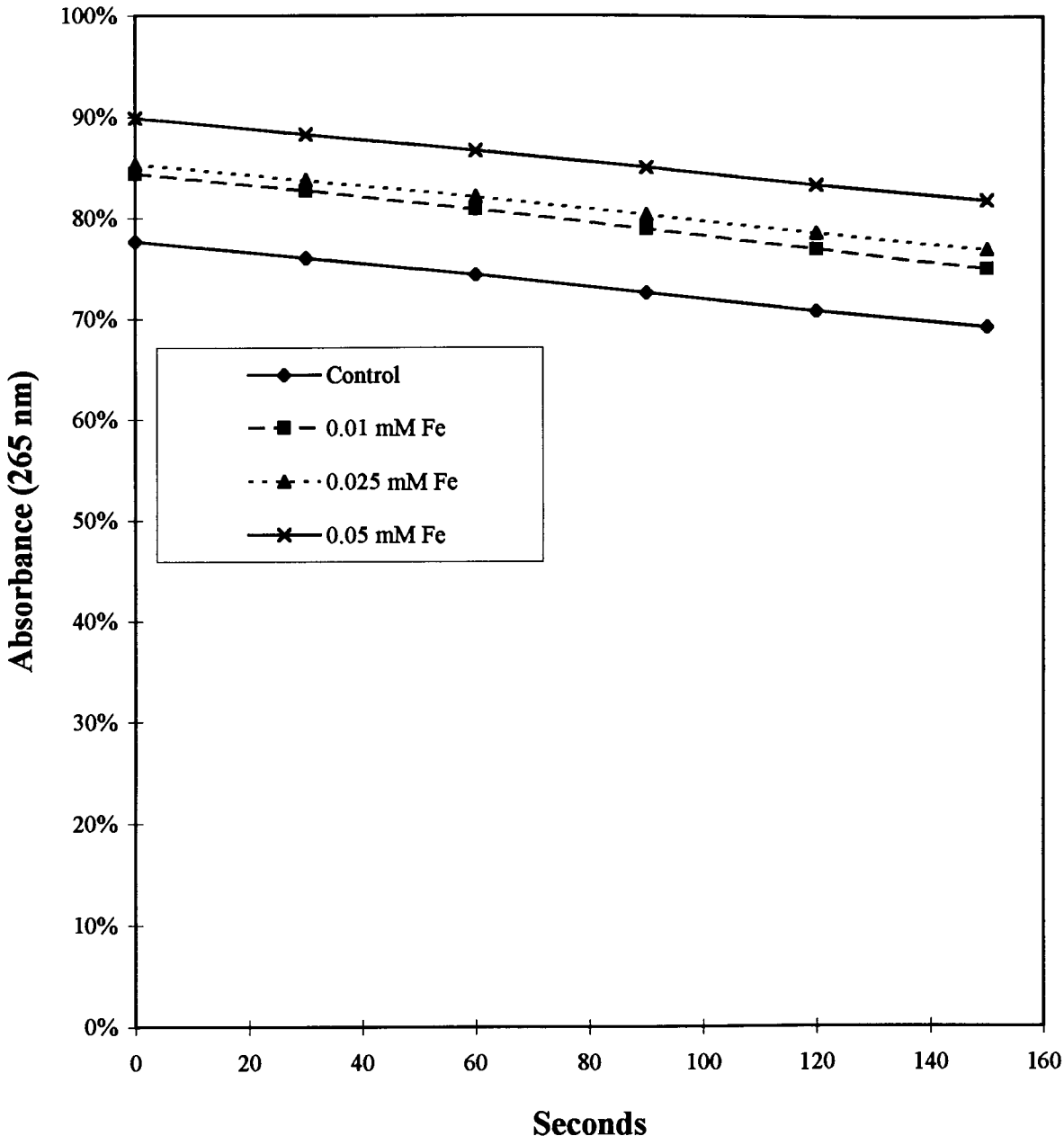


Fig. 19
The Effect of Fe(III) on Oxidation of Ascorbate



General discussion and perspective

Using PM2 DNA as a target test system, we examined the impact of iron on the effects of ascorbate. Based on the current data we summarize our observations and conclusions as follows:

Ascorbate itself does not cause any DNA damage

Ascorbate alone in free solution did not cause any damage to supercoiled DNA molecules. The DNA damage induced by ascorbate "alone" is probably due to the interaction of ascorbate with trace metals in buffer solution. This follows from the observation that desferrioxamine (100 μM) almost completely inhibited this damage. However, this does not mean ascorbate alone has no adverse effect to other important biological molecules. Several studies report that ascorbate alone inactivates catalase.[1][2][3] Thus, a logical next step is to investigate the role of ascorbate alone in a wider range of target systems.

In the presence of Fe(III), ascorbate acts as a pro-oxidant

The impact of iron on the effects of ascorbate is dramatic. In the presence of iron, ascorbate acts mainly as a pro-oxidant, increasing free radical production. Ascorbate presumably acts by recycling Fenton reactive metals, accelerating damage through "site-specific" Fenton-type mechanisms. The implication of this result for iron-overloaded individuals with even traces of "decompartmentalized" iron is that increasing dietary intake of ascorbate may induce severe oxidant stress. Decompartmentalized iron can remove the kinetic barrier between oxygen and ascorbate, and catalyzing the oxidation of ascorbate, and producing free radicals. The findings in the present study and others[4][5][6] that Fe(III) does

not increase the oxidation of ascorbate at neutral pH suggests that the impact of iron on ascorbate-induced tissue damage is at the site and in the action of radicals rather than their generation.

Ascorbate at high concentrations causes less DNA damage

The present study shows that, both in the absence and presence of iron, ascorbate at millimolar levels causes less DNA damage when its concentration exceeds a certain threshold. The suggestion that patients with iron over-loaded diseases should take amounts of ascorbate large enough to enjoy its anti-oxidant effects cannot be supported by this observation. The concentrations of ascorbate we used in the test tube is not transferable to the situation in tissues. On the other hand, the observation that ascorbate causes DNA damage in the presence of iron does not means that patients with iron over-loaded diseases should avoid all foods containing vitamin C. Lack of this essential nutrient in their bodies will lead to another fatal disease —— scurvy.

Desferrioxamine protects against damage by ascorbate and Fe(III)

Although desferrioxamine inhibits DNA damage induced by ascorbate and iron, there have been reports of desferrioxamine toxicity. Thus, the common recommendation that patients with hemochromatosis be injected with desferrioxamine is controversial. Ascorbate reportedly accelerates urinary iron excretion induced by desferrioxamine,[7],[8] this ability suggests that ascorbate might be used together with desferrioxamine in treating hemochromatosis. Current knowledge suggests caution in following this suggestion.

Catalase, but not superoxide dismutase inhibits DNA damage by ascorbate and Fe(III)

Consistent with failure of superoxide dismutase to slow oxidation of ascorbate, superoxide dismutase fails to protect DNA against damage by iron/ascorbate in the present study. Resistance to scavengers might result from DNA molecules combining with ascorbate, iron, and oxygen, forming a complex prior to the damaging reactions with hydrogen peroxide. The entire electron transfer process might occur within a solvent cage surrounding this complex. The inhibition by catalase of DNA damage induced by ascorbate and iron suggests that hydrogen peroxide produced outside the solvent shell plays a key role.

Since several anti-oxidants are present at the same time *in vivo*, the pathway of free radical reactions and mechanisms will not follow the same steps as *in vitro*. Furthermore the DNA used in this study is bacteriophage DNA. The difference between bacteriophage DNA and human chromosomal DNA introduces many uncertainties in extrapolating the result to humans. To improve our understanding of the mechanism of free radical damage in our body, the impact of combinations of anti-oxidants on damage induced by iron/ascorbate deserves a high priority.

Conclusions

In conclusion then, this study has answered some of the questions posed at the start. For example, we have confirmed in part that ascorbate can cause damage to DNA and this damage is diminished by raising ascorbate concentration, by catalase, or by the presence of desferrioxamine. More importantly, the current data raise several new questions and open new avenues for future study. These relate to the extent to which the effects of iron on ascorbate-induced DNA damage are paralleled in other targets, and in systems more closely related to biological damage. We have seen how disferrioxamine, catalase, superoxide dismutase, EDTA, ADP, formate, and mannitol affect the interaction of ascorbate and iron on DNA individually. A logical next step is to examine combined effects of these anti-oxidants. What are the optimal levels of ascorbate *in vivo* for anti-oxidant activity and how close are the levels of ascorbate to optimal conditions in normal individuals and in those with hemochromatosis? In order to solve these uncertainties, we need to extend this study to cellular and tissue levels. For example, it will be important to investigate the impact of iron on anti-oxidant and pro-oxidant actions of ascorbate in erythrocytes and liver-biopsy samples from patients with hemochromatosis, and to establish an iron over-loaded animal model to study these issues.

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