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MECHANISM OF TOXICITY OF ANTHRACYCLINE COMPOUNDS: HYDROXYL RADICAL FORMATION

The Ohio State University

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MECHANISM OF TOXICITY OF ANTHRACYCLINE COMPOUNDS:
HYDROXYL RADICAL PRODUCTION
DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate School
of The Ohio State University

By
Abraham J. Tobia, B.S., M.S.

The Ohio State University
1984

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This dissertation is dedicated to my parents, George and Linda, and my brothers and sisters, Fred, Mike, Mary, Martha and Georgette, who have given me their endless love and support. I will be eternally thankful.

My only wish is that my sister, Linda, could have lived to see this dissertation. She would have been very proud of me.

I would also like to give a very special dedication to Jane Carolyn Allen who in the recent year has brought joy to my life that I have never encountered previously. She has given me total and undivided support in writing this dissertation. Her continual support will enable me to reach new heights as a scientist and as an individual. If I may be poetic, "She is like the sun which gives life and eternal beauty to the cosmos."
ACKNOWLEDGEMENTS

Whatever achievements I may obtain in the past or future, both within and without science, many individuals have gratiously given me their support, wisdom, encouragement and guidance, to these individuals I will be eternally indebted.

First, I would like to specifically acknowledge the fundamental role which my adviser, Dr. Daniel Couri has had in my development as a scientist and as an individual. His dedication to science is a trait which I will strive to attain. He has shown me that being a scientist and caring individual are equal. He will continue to serve as a source of endless determined inspiration in my development as a scientist.

My deepest appreciation is also given to Dr. Arthur Sagone for his endless advice and invaluable guidance in so many areas.

I would like to thank the faculty and staff in the Department of Pharmacology and the Division of Hematology/Oncology for providing me with the basic knowledge and talents to enable me to complete my graduate studies and that I will use in future training.

I would also like to thank Dr. Michael Milks for being a
fellow graduate student and colleague, for his invaluable assistance and friendship.

I would be totally remiss if I did not acknowledge the assistance and gratifying friendship of my fellow colleagues, Daniel Mullet, Bart Beverly, Carl Miller, Abraham Ali, Shaker Mousa, Paul Kirk, Steve Wilt and Mike Alexander. I would like to thank the following individuals: Suzy, Madeleine, Beth, Mark, Rose, Gia, Hans, Judy, Bev, Doug, John, Clara, Keith and Lucy for their friendship and support throughout my graduate years.

To the many other individuals whom I did not specifically mention, for they will forever be remembered by me, for their contribution.

A very special acknowledgement goes to Gretchen Lowry for her patience in typing this dissertation and being a friend whom I could always rely on in needed times.

In closing, I would like to thank everybody so very, very much.
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The effects of heart microsomal fractions (9,000 and 100,000 g supernate and microsomal fraction) on decarboxylation of benzoate.

Structures of adriamycin and mitomycin C
LIST OF ABBREVIATIONS

a  Active
ADRIA  Adriamycin
CAT  Catalase
\textsuperscript{14}C  Radiolabelled Carbon
C  Degree Centigrade
CO\textsubscript{2}  Carbon Dioxide
CON  Control
Conc.  Concentration
CYTOXAN  Cyclophosphamide
DMSO  Dimethylsulfoxide
DDC  Diethyldithiocarbamate
DHAQ  Mitozantrion
e\textsuperscript{-}  Electron
Fe  Iron
Fu  5-Fluorouracil
g  Gram
\textsuperscript{g}  Centrifugation Force
GSH  Glutathione
GSSG  Reduced Glutathione
H\textsubscript{2}O\textsubscript{2}  Hydrogen Peroxide
HR  Hour
1  Inactive

xx
<table>
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<th>Full Form</th>
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<td>I.P.</td>
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<tr>
<td>I.V.</td>
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</tr>
<tr>
<td>kg</td>
<td>Kilogram</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>mCi</td>
<td>Millicurie</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
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<tr>
<td>ml</td>
<td>Milliliter</td>
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<tr>
<td>mM</td>
<td>Millimole</td>
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<td>mMole</td>
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<tr>
<td>MAN</td>
<td>Mannitol</td>
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<tr>
<td>min.</td>
<td>Minute</td>
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<td>MITO. or M.C.</td>
<td>Mitomycin C</td>
</tr>
<tr>
<td>N</td>
<td>Normal</td>
</tr>
<tr>
<td>NADPH</td>
<td>B-Nicotinamide Adenine Dinucleotide Phosphate</td>
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<tr>
<td>nM</td>
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<tr>
<td>O₂</td>
<td>Oxygen</td>
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<tr>
<td>O₂⁻</td>
<td>Superoxide Radical</td>
</tr>
<tr>
<td>OH⁻</td>
<td>Hydroxide</td>
</tr>
<tr>
<td>OH·</td>
<td>Hydroxyl Radical</td>
</tr>
<tr>
<td>%</td>
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<td>S-9</td>
<td>9,000 g Supernate</td>
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<td>Standard Error</td>
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<td>Abbreviation</td>
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<td>S.E.M.</td>
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<td>Microcurie</td>
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<tr>
<td>ug</td>
<td>Microgram</td>
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<td>Micromole</td>
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INTRODUCTION

The cellular damage produced by ionizing radiation is known to be enhanced by molecular oxygen. This increased damage appears to be related to the oxidant injury induced by several reactive oxygen species generated in oxygenated aqueous solutions by radiation. Examples of these molecules include superoxide (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), and hydroxyl radicals (OH·).

There is now evidence that oxidant injury may also be important in the cellular damage produced by some chemotherapeutic drugs. For example, adriamycin and mitomycin C, are agents known to be effective in the treatment of patients with neoplasia may generate free radicals in some tissues similar to those generated by ionizing radiation. This capacity of adriamycin and mitomycin C to generate free radicals may be directly related to their antitumor effects and the well described cardiac toxicity of adriamycin. In this regard, activation of mitomycin C, adriamycin and other benzanthraquinone drugs to form free radicals has been demonstrated in microsomal systems. The reactions require NADPH as a cofactor and oxygen as a terminal acceptor. Theoretically, activation of these drugs in vivo also occurs by this
mechanism. In microsomal systems, NADPH is a required cofactor for these reactions. This raises the question whether or not the hexose monophosphate shunt pathway (HMPS) provides the energy for the activation of these drugs in the intact cell since this pathway is the major source of cellular NADPH. Thus, the HMPS pathway which may normally protect the cell against oxidant damage may provide the cellular energy for the activation of drugs to drug free radicals with several reactive oxygen species being generated from molecular oxygen. Both the drug radical and the reactive oxygen species could then cause damage to the cell.

Mechanism of Glutathione: Protection Against Reactive Oxygen Species

The cellular resistance to oxidant damage in the intact cell is highly complex, but appears to be dependent on enzyme systems which are capable of the rapid degradation of these oxidants. These include superoxide dismutase, catalase, and glutathione peroxidase. The latter enzyme is linked to the HMPS activity of the cell by glutathione reductase. Several types of tissues may be particularly sensitive to oxidant injury because they have lower concentrations of these enzymes. These include cancer cells, cardiac tissue, myeloblasts, and
lymphoid cells. Thus, the mechanism for the selective oxidant injury of adriamycin and mitomycin to some tissues could be related to the capacity of the cell to activate these drugs via its microsomal system combined with a sensitivity of the tissue to oxidant injury.

As previously stated, adriamycin and mitomycin C have been reported to produce free radicals in a NADPH microsomal system according to Bachur, et. al.; 1977 and 1978. Myers, et. al. (1977), and Doroshow, et. al. (1980) have investigated the possibility that the cardiac toxicity of adriamycin and mitomycin C toxicity may be related to the generation of free radicals by these drugs. These investigators found that mice receiving adriamycin have a significant fall in the concentration of myocardial reduced glutathione. Further, 100% of the mice receiving adriamycin and diethylamine, an agent which depletes soluble sulfhydryls, died.

In contrast, mice receiving cysteamine, a sulfhydryl donor, were protected against death as a result of adriamycin. The observations of these investigators indicate that the cardiac toxicity produced by adriamycin is associated with a depletion of soluble sulfhydryls. Although this biochemical event may not necessarily prove that this fall in glutathione occurring in the myocardium is the result of the generation of "free radicals" in vivo, it is strongly suggestive. In any event, the depletion of glutathione,
whatever the mechanism should increase the sensitivity of the myocardium to oxidant injury. This abnormality may be directly related to the lipid peroxidation (Myers, et. al., 1976) and altered cyclic nucleotide concentrations (Levey, et. al., 1979) reported in cardiac tissue following administration of adriamycin. Whether this effect of adriamycin occurs in other tissues and its relationship to the chemotherapeutic value of the drug still remains unclear. For this reason the effect of adriamycin on the reduced thiol content of other cells caused by reactive oxygen species is of importance. Several other chemotherapeutic drugs in addition to adriamycin have been shown to have the capacity to oxidize NADPH in microsomal systems. These drugs have been studied and published by Bachur, 1978.

Energy Production Mechanism

It has been postulated by Bachur et. al., 1977 and 1978, that microsomal activation of benzenanthraquinones and N-heterocyclic quinones to drug free radicals in vivo may be related to the cytotoxic capacity of these drugs. Further, NADPH appears to be the major cofactor in the activation of these drugs by microsomal systems in vitro. This raises the question of whether or not the
hexose monophosphate shunt (HMPS) pathway provides the energy for the activation of these drugs in the intact cell. Recently, Kimpel and Sagone (in press) have shown that mitomycin C increased HMPS activity under aerobic conditions, while having no effect on KREB cycle activity. Under hypoxic conditions HMPS or KREB cycle activity was not observed in mitomycin C treated or untreated liver cell preparations. This evidence suggests the requirement for oxygen for mitomycin C stimulation of the HMPS. Further, their data showed oxygen dependence of endogenous and mitomycin C stimulated shunt activity, and their results provided evidence for mitomycin C activation of HMPS by a pathway which was independent of reactive oxygen species and involved microsomal fraction and NADPH (provided by the HMP shunt). In addition, since oxygen appears to act as a terminal acceptor for these reactions, activation of these drugs in vivo may be associated with the generation of reactive oxygen species such as $\cdot O_2^-$, $H_2O_2$, or $OH^·$. These compounds, as well as the drug free radical, could also cause oxidant damage to the cell. Whether these drugs are actually activated to drug free radicals in all tissue, as predicted by the microsomal system, is uncertain. It seems reasonable to conclude that such activation would be highly dependent on the concentrations of microsomes, NADPH, and probably the enzymes of the hexose monophosphate shunt.
pathway. However, even if the concentrations of these components and of the microsomal system are known for a particular tissue, it still does not seem possible to predict with certainty the activity of this system in the intact cell since there may be significant compartmentalization. Therefore, the study of the mechanism of action of these drugs in the presence of microsomal fraction seems important in fully understanding their cytotoxic capacities. Suggestive evidence indicate that oxygen radicals may also be related to the cellular damage produced by bleomycin as described by Oberley, et. al., 1979. Superoxide has been shown to increase the number of DNA breaks occurring during incubation with this drug.

Several enzymes are known to be important in the protection of mammalian cells and bacteria against oxidant injury as postulated by Fridovich (1975). Fridovich (1975) states these enzymes include superoxide dismutase, catalase, and glutathione peroxidase. The degradation of hydrogen peroxide by glutathione peroxidase is dependent on the pentose phosphate pathway. Glutathione peroxidase is known to require reduced glutathione as a cofactor. GSH which is oxidized during the degradation of $H_2O_2$ is regenerated by glutathione reductase, an enzyme dependent on NADPH as a cofactor. The NADPH required for the reduction of GSSG is provided by the hexose monophosphate shunt pathway of the
cell. This pathway may also provide energy for the reduction of other potential antioxidants such as ascorbic acid.

In addition to the requirement for NADPH in the regeneration of GSH, there is experimental evidence as stated by Jacobs, et. al., 1966 and Sanger, 1965 that under certain circumstances NADPH may be able to react directly with oxidants. Fridovich (1975) stated the relative importance of these enzymes in the protection against oxidant injury is still controversial. Cells which are deficient in these enzymes are known to be sensitive to oxidant injury. A classic example is RBC's deficient in glucose 6-phosphate dehydrogenase.
There are several ways in which the generation of reactive oxygen species by a chemotherapeutic drug might cause additional damage to the cell. First, the requirement for GSH and NADPH in the degradation of $\text{H}_2\text{O}_2$, or other oxygen radicals, would not allow these substances to be available for the repair of macromolecules. For example, Bachur, et. al., (1977 and 1978) has postulated that the drug free radical may induce a free radical in macromolecule such as DNA or RNA. This could result in irreversible damage to the DNA or alternately allow binding of the drug to the DNA molecule. Potentially, NADPH or GSH might prevent such damage or binding. Second, the oxidant injury might be associated with a depletion of cellular GSH or NADPH resulting in a loss of one of the major protective mechanisms of the cell against further oxidant injury. Fridovich (1975) stated this condition might occur if the rate of oxidation of GSH or NADPH was more rapid than the ability of the cell to generate these cofactors and might ultimately lead to irreversible damage to the cell because of the inability to degrade the reactive oxygen species which are normally generated in biological systems. Further, depletion of cellular GSH or the generation of substantial concentrations of GSSG might have additional implications in regard to cellular function. Recent studies by Oliver, et. al. (1976) in granulocytes suggest a possible
role of GSH in microtubular function and a role of GSH in cell division has been postulated for some time. In addition Nugteren (1973) showed GSH may be required in prostaglandin synthesis. Further, GSSG is known to be a potent inhibitor of protein synthesis in enzyme systems as shown by Frischer and Ahmod (1977). Thus, the oxidation of cellular GSH might directly impair cellular function by several mechanisms.

There is also experimental evidence that other chemotherapeutic drugs can impair the HMPS pathway by mechanisms which do not involve the production of reactive oxygen species. Frischer and Ahmod (1977) has demonstrated that carmustine is a potent inhibitor of glutathione reductase. Sagone, et. al. (1979) showed cells with inhibition of this enzyme have impaired capacity to regenerate reduced glutathione.

Nitrogen mustard has been known to be an effective chemotherapeutic agent for some time. This alkylating agent has recently been shown by Roth, et. al. (1975) to react with reduced glutathione in red cells. This results in an impaired capacity of RBCs to degrade \( \text{H}_2\text{O}_2 \) via the glutathione peroxidase system. This raises the question whether other cells might have a similar depletion in GSH after incubation with this drug \textit{in vitro} and whether a similar depletion of tissues GSH occurs after administration of nitrogen mustard \textit{in vivo} and thus allowing formation of hydroxyl radicals from \( \text{H}_2\text{O}_2 \).

If impaired hexose monophosphate shunt activity occurs in
several tissues in vivo after administration of chemotherapeutic drugs, there should be some period of time required for recovery. This may be quite variable from one tissue to another. During this period, the tissue would have an enhanced sensitivity to further oxidant injury.

Hydroxyl Radical Formation: Decarboxylation of Benzoate as an Indicator of Formation of OH•

To answer the questions raised, our laboratory recently developed an assay for the detection of hydroxyl radicals in a biological system. Preliminary experiments (Sagone, et al., 1983) have confirmed the observation of Frabrinkant, Matthew and Sangster, which indicates that benzoate is decarboxylated upon interaction with the hydroxyl radicals generated during radiolysis. Sagone, et al., (1980) reports that (carboxyl-14C) benzoate is decarboxylated by the xanthine-xanthine oxidase system as a consequence of hydroxyl production. The generation of hydroxyl radicals by this enzyme system requires the interaction of superoxide (O2•⁻) and hydrogen peroxide (H2O2) (Haber-Weiss reaction). This assay was used to detect hydroxyl radical production by stimulated polymorphonuclear cells and arachidonic acid stimulated platelets (Sagone, et al., 1980a, 1980b, 1981, 1983, and Singh, et al., 1981). These studies suggest that this assay is specific for the detection of hydroxyl
radicals. Recently, benzoate was used as a probe for detection of the oxygen radicals generated by a NADPH dependent, microsomal electron transfer system. Initial studies indicate that $^{14}\text{C}O_2$ production from (carboxyl-$^{14}\text{C}$) benzoate serves as a simple, sensitive, and specific probe for the production of oxidative radicals generated during microsomal electron transport.

It has been suggested by several investigators (Berlin and Haseltine 1981; Dorshow, 1980; Hand and Sato, 1976; Komiyama, et al., 1982; Pan and Bachur, 1979) that the anthracycline and related anticancer quinone drugs may generate hydroxyl radicals in the presence of rat liver microsomal fraction (i.e. NADPH cytochrome P-450 reductase enzymes). Further, it has been postulated that the mechanism for the production of hydroxyl radicals in this system is similar to that observed for the xanthine-xanthine oxidase enzyme system (i.e. involves Haber-Weiss type reaction). Egan, et. al., (1979) has demonstrated several characteristics of the reaction, however, the mechanism of the production of hydroxyl radicals is still under investigation. During the reaction (Mimnaugh, et. al., 1981, 1982a) the anthracycline drugs appear to be metabolized to a semiquinone free radical intermediate. Bachur (1977 and 1978) showed under aerobic conditions, these drugs stimulate oxygen consumption and enhance the rate of lipid peroxidation in the system.

In this study, Adriamycin and analogs and Mitomycin C are
examined in relationship to the enhancement of the
decarboxylation of (carboxyl-\textsuperscript{14}C) benzoate by the liver
microsomal system. The capacity of these drugs to decarboxylate
benzoate will be correlated with oxygen consumption in a similar
system. In addition, the effect of three other drugs:
cyclophosphamide, methotrexate, and 5-fluorouracil, will be
studied for their capacity to decarboxylate benzoate. These
agents were selected for the following reason; a) they appear to
be inactive in oxygen consumption experiments b) do not possess
the quinone type structure, c) their spectrum of anticancer
activity is similar to Adriamycin and mitomycin C, d) 5-fluorouracil
and methotrexate's modes of action differ from Adriamycin and
mitomycin in that they do not alkylate. DNA, however,
cyclophosphamide's mode of action is similar to Adriamycin and
mitomycin in alkylating DNA, and e) cyclophosphamide and
5-fluorouracil are metabolized in the liver as are Adriamycin
and mitomycin, whereas, methotrexate is not metabolized by liver
and is excreted by the renal system unchanged. Finally, using the
decarboxylation of benzoate as a method for detection of hydroxyl
radical (OH\textsuperscript{-}), the mechanism by which reactive oxygen species are
produced by the microsomal system can be characterized.
Mechanisms of Reactive Oxygen Species

1. Oxygen to Superoxide Anion (\(O_2^-\)) and Hydrogen Peroxide (\(H_2O_2\))

Molecular oxygen (\(O_2\)) in its molecular state has two unpaired electrons in its outermost orbital (Badney and Karnovsky, 1980). These electrons are in their lowest energy state and are located in separate orbitals with electron spin in the same direction. Each of these orbitals can be hybridized to possess an additional electron. When an electron is added to one of these orbitals, a superoxide radical (\(O_2^-\)) is produced. Also, oxygen can be reduced by both enzymatic and nonenzymatic processes to the superoxide radicals. Superoxide radicals are postulated to be formed in vivo in cells through activation of some iron-sulfur enzymes and flavoproteins (i.e. xanthine-xanthine oxidase). Superoxide radical can be dismutated to hydrogen peroxide (\(H_2O_2\)) by superoxide dismutase (SOD) and according to the following reaction:

\[
2O_2^- + 2H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2 \quad (\text{Equation 1})
\]

Superoxide radicals can also lose an electron and be oxidized back to oxygen. In equation 1, superoxide is acting both as
a reducing and oxidizing agent. In addition, superoxide radicals are weak bases with a pKa of 4.8.

Fridovich (1975) has postulated that superoxide dismutase is a metalloenzyme (copper-zinc) which promotes the dismutation of superoxide radicals. Superoxide dismutase is primarily found in cells that utilize oxygen. The rate constant of interaction of SOD with $O_2^-$ is approximately $2 \times 10^9 M^{-1} \text{Sec}^{-1}$ (McCord and Fridovich, 1969). Superoxide dismutase is found in two forms, one in the extramitochondrial cytosol and another in the mitochondria. The mitochondrial superoxide dismutase of eukaryotes is similar to superoxide dismutase of many bacteria with respect to its characteristic content of Mn$^{+2}$ and many homologies in amino acid and sequence. The cytosol form of superoxide dismutase, on the other hand, has quite a different structure and contains Cu$^{+2}$ and Zn$^{+2}$. These enzymes are present in high concentrations and are extraordinarily active suggesting that superoxide radicals are being continuously produced during the enzymatic reduction of oxygen by various enzymes and enzyme systems.

From equation 1, hydrogen peroxide is formed by dismutation and flavin-linked oxidases (i.e. xanthine-xanthine oxidase, aldehyde, and dihydroorotate oxidase) systems. The pKa of H$_2$O$_2$ is 10.6. It was stated previously that $O_2^-$ is quickly dismutated to H$_2$O$_2$, due to the fact that H$_2$O$_2$ is less reactive than superoxide radical.
addition, there are enzymes in animal tissues capable of metabolizing hydrogen peroxyde to less reactive products by the following reaction:

\[ 2\text{H}_2\text{O}_2 \xrightarrow{\text{catalase}} 2\text{H}_2\text{O} + \text{O}_2 \]  \ (Equation 2)

The enzyme responsible for this reaction is catalase which is a heme containing enzyme that is found in the microbodies of animal cells, also called peroxisomes.

2. Hydroxyl Radical (OH\(^{-}\)) Production

Haber and Weiss suggested in 1934 that hydroxyl radical could be formed from the reduction of \(\text{H}_2\text{O}_2\) by \(\text{O}_2^{-}\) by the following reaction:

\[ \text{O}_2^{-} + \text{H}_2\text{O}_2 \rightarrow \text{OH}^{•} + \text{OH}^{-} + \text{O}_2 \]  \ (Equation 3)

However, a number of investigators, Hallwell (1978); McCord and Day (1978); and Badney and Karnovsky (1980) suggested that the Haber-Weiss reaction (equation 3) could not produce physiological amounts of hydroxyl radicals due to the fact that the rate constant for the reaction of \(\text{O}_2^{-}\) with \(\text{H}_2\text{O}_2\) \((10^{-4} \text{ to } 3.4 \text{ M}^{-1} \text{ sec}^{-1})\) is significantly below that of the competing reaction, the
spontaneous dismutation of superoxide radical. To settle this dilemma, McCord and Day (1978) proposed a modified Haber-Weiss reaction in which a metal serves as the redox catalyst. This is similar to the Fenton type reaction using iron as the reduction-oxidation catalyst. In the presence of iron, they calculated that the degree of efficiency of the modified Haber-Weiss reaction is sufficient to have significant biological impact.

Fridovich (1975) was the first to show that hydroxyl radical could be generated in significant amounts from a reaction between $O_2^-$ and $H_2O_2$ in an aqueous enzymatic system (xanthine-xanthine oxidase). Originally the method for demonstrating the production of hydroxyl radical involved the ability of this radical to react with methional and subsequently form ethylene.

Evidence that hydroxyl radical were generated from the interaction of $H_2O_2$ with $O_2^-$ or $Fe^{+2}$ are provided by the following points, a) this reaction was inhibited by either superoxide dismutase or catalase, b) scavengers of hydroxyl radicals (benzoate, mannitol, ethanol) effectively inhibited the reaction and c) the reaction was stimulated by the addition of $H_2O_2$ (Beaucham and Fridovich 1970).

Recently, the use of methional to detect $OH^-$ has fallen out of favor because the reaction is far more complex than originally thought (Bors, et. al. (1982); and Pryor, et. al., ...)
1978). Bors found that the yields of products are highly dependent upon assay conditions and methional, like many aldehydes, can undergo spontaneous oxidation to form hydroperoxides. These compounds are known to form alkoxy radicals. Alkoxy radicals could then react with methional to release ethylene, thus indicating the non-specificity of the reaction (Pryor, et. al., 1978).

Pharmacology of Adriamycin

The anthracycline antibiotics and their derivatives are produced by the fungus streptomyces peucetius var. caesius. Daunorubicin was isolated independently by Di Marco and by Dubost and colleagues in 1963. Doxorubicin was identified by Arcamone and co-workers in 1969. Though their structures differ slightly, Daunorubicin has been used primarily in acute leukemias, whereas, doxorubicin has activity against a wide variety of human neoplasms and solid tumors. The clinical value of both agents is limited by an unusual cardiomyopathy; its occurrence is related to the total dose of the drug and is often irreversible (Chabner, et. al., 1975; Di Marco, 1975; and Goldberg, et. al., 1977).
1. Chemistry

The anthracycline antibiotics have a tetracycline ring structure with an unusual sugar, daunosamine attached by glycoside linkage. Cytotoxic agents of this class all have hydroquinone moieties on adjacent rings that permit them to function as electron-accepting and electron-donating agents. The chemical structure of Adriamycin is shown in Appendix 1.

2. Mechanism of Action

X-ray differentiation and other studies suggest that these agents may intercalate between adjacent base pairs of DNA and that the sugar, daunosamine, plays an essential role in binding. Pigram, et al. (1972) showed that the DNA helix is untwisted to permit intercalation; this change produces a longer, thinner molecule and inhibits the template activity of the nucleic acid. Oberly and Buettner (1979) postulated a biochemical action of potential importance resulting from the quinone-hydroquinone structure of adriamycin, which permits this molecule to accept electrons to form semiquinones. They showed addition of adriamycin to hepatic microsomal fractions significantly augments the oxidation of NADPH and the transfer of electrons to molecular oxygen, resulting in the formation of superoxide anion radicals (O$_2$•⁻).
Bachur, et. al. (1977 and 1978) suggested that the enzyme responsible for this effect is NADPH-cytochrome P-450 reductase. The production of free radicals is also shown by the marked accumulation of malonyldialdehyde in cardiac tissue after treatment of mice with adriamycin. This substance is known to be a product of free radical attack on unsaturated fatty acids. Further supportive evidence for a role of free radicals in anthracycline-induced cardiotoxicity is the protective effect afforded by free radical scavengers such as ALPHA-tocopherol and co-enzyme Q. Myers, et. al. (1977) stated that these manipulations do not alter the antitumor effect of adriamycin. Tritton, et. al. (1978) suggested that adriamycin has a disruptive effect on cellular membrane as a possible mechanism of adriamycin toxicity. This process may be related to radical production.

3. Absorption, Rate, and Excretion

Adriamycin is usually administered intravenously, and cleared from the plasma rapidly. The disappearance curve for adriamycin is triphasic, with an initial $t_{1/2}$ of 12 minutes, an intermediate $t_{1/2}$ of 3.3 hours, and a terminal phase value of 30 hours. Adriamycin is rapidly taken-up by the heart, kidneys, liver, spleen, and does not cross the blood-brain barrier. Adriamycin is mostly excreted
unchanged, and has multiple metabolites. Adriamycin is predominantly metabolized in the liver and excreted in the bile. Hepatic clearance is roughly 60% of hepatic blood flow. Adriamycin should not be administered at the usual dosage should a patient present with impaired hepatic function (Chabner, et. al., 1975; Takanashi and Bachur, 1975a, 1975b; Benjamin, et. al., 1977; Riggs, et. al., 1977; and Ahmed, et. al., 1978).

4. Dosage

The current recommended dose of adriamycin is 60 to 75 mg/sqM, administration in a single rapid intravenous infusion and repeated after 21 days. The drug may also be given 0.5 to 1 mg/kg daily for 2 to 6 days or in doses of 20 to 30 mg/sqM daily for 3 days or once weekly. Care should be given while administering this agent, due to tissue necrosis developing if not given intravenously.

5. Therapeutic Uses

According to Blum and Carter (1974), Gottlieb and Hill (1977) and Di Marco (1975) adriamycin is effective in acute leukemias and malignant lymphomas. When used in combination with other antineoplastic drugs (such as cytoxan,
vincristine, bleomycin, and prednisone), adriamycin is successful in the treatment of non-Hodgkin's lymphomas. In combination with cisplatin, it has considerable activity against carcinomas of the ovary. In addition, when given with other agents it has activity against breast and small (oat) cell carcinomas of the lung. It is also effective as a single agent in the treatment of a wide range of sarcomas (including osteogenic, Ewing's and soft tissue), metastatic adenocarcinoma of the breast, carcinoma of the bladder, bronchogenic carcinoma and neuroblastoma. Adriamycin is the most single effective agent in the treatment of metastatic thyroid carcinoma. It has been shown to have activity against carcinoma of the endometrium, testes, prostate, cervix, head and neck, and plasma-cell myeloma.

6. Toxicity

Blum and Carter (1974); Chabner, et. al. (1975); Minow, et. al. (1977); and Bristow, et. al. (1978) have characterized adriamycin toxicity. They indicated myelosuppression is usually the major dose-limiting complication, with leukopenia usually reaching a nadir during the second week of therapy. Recovery is seen by the fourth week; thrombocytopenia and anemia follow a similar pattern but are less pronounced than the myelosuppression. Stomatitis, gastrointestinal disturbances, and alopecia are common but reversible with cessation of
drug. Cardiomyopathy is a unique characteristic of the anthracycline drugs. Two types of cardiomyopathies may occur: (1) an acute form is characterized by abnormal EEG changes, including ST-T wave alterations and arrhythmias. This is a brief problem. Studies have shown that these changes are reversible if the drug is removed and are only reversible in the initial treatments, (2) chronic, cumulative dose related toxicity is manifested by congestive heart failure that is unresponsive to digitalis treatment. The mortality rate is in an excess of 50%. Total dosage of adriamycin as low as 250 mg/sqM can cause myocardial toxicity, as demonstrated subendocardial biopsies. Non specific alterations including a decrease in the number of myocardial fibrils, mitochondrial changes, and cellular degeneration are observed by electron microscopy. Therefore, the total dosage suggested to avoid permanent cardiotoxicity is 500 mg/sqM over a protracted period of time. Also, there is no reliable indication or test for the cardiotoxicity of this agent.

Pharmacology of Mitomycin C

1. Source and Chemistry

Mitomycin C was isolated from streptomyces caespitiosus by Wakaki and Associates in 1958. Mitomycin contains a
urethane and a quinone group in its structure, including an aziridine ring, which is essential for its antineoplastic activity according to Kersten (1975); Crooke and Bradner (1976). Structurally mitomycin C is shown in Appendix 1.

2. Mechanism of Action

After intracellular enzymatic reduction of the quinone and loss of the methoxy group, mitomycin becomes a bifunctional or trifunctional alkylating agent. It inhibits DNA synthesis and cross-links DNA to an extent proportional to its content of guanine and cytosine. Its action is most prominent during the late phase of G₂ and early phase of S of cell cycle. Crooke and Bradner (1976) show that mitomycin C is teratogenic and carcinogenic in rats and its immunosuppressive properties are relatively weak.

3. Absorption, Fate, and Excretion

Mitomycin C is administered intravenously, and disappears rapidly from blood, distributing throughout the body tissue and is not detected in the blood. Mitomycin does not cross the blood brain barrier. It is metabolized primarily in the liver and less than 10% of the active drug is excreted unchanged in the urine or the bile.
4. Dosage

The current recommended dosage of mitomycin is 2 mg/sqM or 50 ug/kg daily for 5 days. This course is repeated after a 2 day interval. Should myelosuppressive toxicity occur, the treatment should be discontinued. Treatment can proceed following disappearance of myelosuppressive toxicity.

5. Therapeutic Uses

Mitomycin can be used to treat gastric adenocarcinoma, carcinomas of the cervix, colon, rectum, pancreas, breast, bladder, head and neck, lung and in melanoma. It has shown activity against lymphomas and leukemia.

6. Toxicity

The toxicity of mitomycin C is myelosuppression, characterized by marked leukopenia and thrombocytopenia. These toxicities may be delayed and additive. Other symptoms are nausea, vomiting, diarrhea, stomatitis, dermatitis, fever, and malaise. Moore, et. al. (1978); Crook and Bradner (1976) and Orwoll, et. al. (1978) have shown that mitomycin produces interstitial pneumonia and glomerular damage resulting
in renal failure in a small percentage of the patient on mitomycin.
STATEMENT OF THE PROBLEM

Activation of adriamycin and other benzanthraquinones to drug free radicals has been demonstrated in microsomal systems. The reactions require NADPH as a cofactor and oxygen as a terminal acceptor. Theoretically, activation of these drugs in vivo also occurs by this mechanism. In microsomal systems, NADPH is the best cofactor for these reactions. This raises the question of whether or not activation of adriamycin and other benzanthraquinones occurs in the intact cell. In addition, whether the hexose monophosphate shunt pathway provides the energy for the activation of these drugs in the intact cell since this pathway is the major source of cellular NADPH. Thus, the HMPS pathway which may normally protect the cell against oxidant damage may provide the cellular energy for the activation of drugs to drug free radicals with several reactive oxygen species being generated from molecular oxygen. Both the drug radical and the reactive oxygen species could then cause damage to the cell.

In this study, we will determine the capacity of chemotherapeutic agents to generate reactive oxygen species, in particular formation of hydroxyl radicals, using decarboxylation of benzoate as the indicator of hydroxyl radical production in the presence of rat liver microsomal
fraction and NADPH. This will enable us to determine the capacity of these agents to be activated to drug free radical in the microsomal system and whether it correlates with the capacity to generate reactive oxygen species. In addition, the type of reactive oxygen species generated will be ascertained and the mechanism by which they are generated.
MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (150-250 g, or 45 days old) were obtained from Harlan Sprague-Dawley (Indianapolis, IN) and were acclimated to the animal housing facilities for three to seven days prior to treatment. The Ohio State University medical school is an American Association for Accreditation of Laboratory Animal Care (AAALAC) - approved facility and provides climate controlled conditions for a twelve hour light cycle (6 a.m.-6 p.m.), room temperature of 22-23°C and a relative humidity of 45%. All animals received Purina Lab Chow and tap water ad libitum during the time housed in these facilities.

Materials

(Carboxyl-C\textsuperscript{14}) benzoic acid (specific activity 25 mCi/m mole or 48 mCi/m mole), \textsuperscript{14}C-formate (sodium salt, freeze dried preparation, specific activity 59 mCi/m mole) and \textsuperscript{14}C-1-glucose (specific activity 3.94 mCi/m mole) was obtained from Amersham Corporation (Arlington, Heights, IL). Sodium benzoate, dimethylsulfoxide (DMSO), catalase, (Thymol Free, 14,000 units/mg), superoxide dismutase (2500
units/mg protein, type 1), B-nicotinamide adenine dinucleotide phosphate (NADPH), (chemically reduced form, type 1), methotrexate, mitomycin C, and diethyldithiocarbamate were obtained from Sigma Chemical Company (St. Louis, MO). Sodium azide and potassium phosphate (mono and dibasic) were obtained from Fisher Scientific Co. (Fairlawn, NJ). Mannitol was obtained from Abbott Laboratory (Chicago, IL). Cyclophosphamide was obtained from Mead Johnson and Company (Evansville, IN). SKF-525 A was a gift from Smith, Kline and French (Philadelphia, PA). 5-Fluorouracil was obtained from Adria Laboratories (Columbus, OH). Adriamycin (pure chemical form) and other anthracycline drugs were a gift from Dr. Dick Wolgemuth of Adria Laboratories (Columbus, OH).

Preparation of Rat Liver Microsomal Fraction

Liver microsomal preparations were prepared from 45 day old male Sprague-Dawley rats (150-250 g) that were injected for three days i.p. administration prior to sacrificing with 80 mg/kg phenobarbital or 80 mg/kg arochlor in normal saline. Rats were decapitated and livers removed and homogenized in 0.2 M potassium phosphate buffer, pH 7.5 (teflon-glass homogenizer, 7 stroke at 4°C), the homogenate was centrifuged at 9,000 g for 30 minutes. The 9,000 g supernate
(S-9) was removed and transferred to a polycarbonate tube and spun at 100,000 g for 60 minutes in a Beckman LB-75 ultracentrifuge. The supernate was discarded and microsomal pellet was resuspended in 0.2 M potassium phosphate and placed into NUNC tubes in 1 ml portions and frozen at -80°C until usage. Protein determinations were performed by the Bradford Technique (i.e. Bio Rad kit). Control animals were injected with saline and microsomal fraction were prepared as described. Arochlor induced animals were also prepared as described above.

Preparation of Rat Heart Microsomal Fraction

Rat heart microsomal preparations were prepared from 45 day old Sprague-Dawley rats (150-200 g) that were injected (i.p. administration) daily for three days prior to sacrificing with 80 mg/kg phenobarbital in normal saline. Rats were decapitated and hearts removed and homogenized in 0.2 M potassium phosphate buffer, pH 7.5 using a Tekmar homogenizer. Hearts were homogenized at maximum RPM for 60 seconds at 4°C. The remainder of the isolation condition are as described in the preparation of rat liver microsomal fraction.
Measure of $^{14}\text{CO}_2$ from Decarboxylation (Carboxyl-$^{14}\text{C}$) Benzoate - Trap Method

The metabolic reaction mixture consisted of 2 mM NADPH (final concentration), 4 mg of microsomal protein and 1 or 2 uCi of (Carboxyl-$^{14}\text{C}$) benzoic acid in 4 ml of 0.2 mM potassium phosphate buffer, pH, 7.4. For most experiments the final concentration of benzoate was $1 \times 10^{-5}$M. When present, catalase, superoxide dismutase, and sodium azide were present at the following concentrations 100 ug/ml, 10 ug/ml, and 1 mM (final concentration), respectively. The metabolic reaction mixture was carried out in a 25 ml triple arm distilling flask and incubation was carried out in water bath at $37^\circ\text{C}$ for 30 minutes. Prior to addition of the other components, (carboxyl-$^{14}\text{C}$) benzoate was placed in the triple armed flask that has been sealed with 11 x 17 mM rubber serum stoppers and gassed for 5-10 minutes with compressed air. Upon completion of the incubation, the flasks were then rapidly connected to an ionization chamber electrometer system for $^{14}\text{CO}_2$ measurement from the decarboxylation of benzoate. The experimental details of this system are published elsewhere (Sagone, et. al., 1980a). Briefly, a stirring bar was quickly added through one arm of the flask, immediately followed by a tightly fitted glass adapter which was connected by plastic tubing to a gas...
cylinder containing compressed air. The second outlet flask arm was connected to a 275 ml Cary-Tolbert ionization chamber and a Cary Model 401 vibrating reed electrometer system (Varian Instruments) by a second adapter system. The metabolic reactions were stopped by the addition of 0.5 N HCl through the center arm and rapidly stirred using a magna 4 magnetic stirrer Model 4280-1 (Cole-Palmer Instruments). The evolved $^{14}\text{CO}_2$ was measured directly by passing the gas through the ionization chamber. The resultant time flow curve was integrated using an Apple II computer equipped with a graphic tablet board. The total yield of $^{14}\text{CO}_2$ was calculated from the area under the time flow curve.

Oxidation of $^{14}\text{C}$-Formate By Rat Liver Microsomal Fraction

Incubations for studying the production of $^{14}\text{CO}_2$ from $^{14}\text{C}$-formate by rat liver microsomal fraction were carried out at 37°C and $^{14}\text{CO}_2$ measured using the Cary-Tolbert continuous ionization Chamber-Electrometer method as described previously. The system consisted of 4 mg of liver microsomes in 4 ml of 0.2 M potassium phosphate buffer (pH 7.4). The suspension was placed into a 25 ml triple neck distilling flask containing 1 uCi $^{14}\text{C}$-formate in .1 ml of 0.2 M potassium phosphate, pH 7.4. Cold formate was added to the reaction mixture to obtain a final concentration of
10 mM. The inlet arm of the metabolic flask was connected to a gas cylinder containing compressed air, while the outlet arm was connected to a 275 ml Cary-Tolbert ionization chamber attached to a vibrating reed electrometer. The third arm of the flask was covered with a rubber sleeve-type serum stopper. The rate of gas flow was set by a flowmeter in the system. A duplicate system was used so that $^{14}$CO$_2$ derived from labelled compound could be measured simultaneously from both control and experimental flasks. The reaction mixtures were stirred as previously stated in the decarboxylation of benzoate section. When the effects of various agents were to be assessed, the addition of these agents was made 10-15 minutes prior to the addition of the stimulating agents.

Oxygen Consumption Measurements

Oxygen consumption measurements were performed with the Yellow Spring Oxygen unit and probe (Yellow Spring Instrument, Co., Inc.). Attached to the unit and probe was a Braum Thermomix 1460 circulating water pump (Braum, Inc.) and an A-25 Varian dual pen recorder (Varian Instruments). The operational details of the oxygen consumption measurement are described in the technical bulletin provided by Yellow Springs, Inc. and reports of Bachur, et. al., 1977 and 1978.
The reaction mixtures was added to the probe unit chamber using a 21 gauge spinal needle. The system was initialized with the 0.2 M potassium phosphate buffer, pH 7.4, and was allowed to run for 10-15 minutes to establish a base line of generally 100% O$_2$ saturation. The buffer alone gave a 0% oxygen consumption. After the baseline was established the microsomal fraction (0.1 mg/ml) NADPH (.425 mM final concentration), and drugs were added in that order. The entire system was maintained at 37°C for the entire O$_2$ measurement. Following each addition the system was allowed to run for 10-15 minutes and the oxygen consumption was measured for that individual component prior to the next addition. Oxygen consumption was measured over a 5 minute interval, usually measured from minute 2 to minute 7 for stable measurements and the rate of change was calculated for that component. Substances that were suspected to increase or decrease oxygen consumption were added to the buffer and baseline was established as previously mentioned.

Statistical Analysis

Statistical analysis used the student t-distribution test for data analysis. Data is represented as the mean ± S.D. where indicated.
RESULTS

Decarboxylation of Benzoate by Microsomal System

Earlier we reported the use of (Carboxyl-$^{14}$C) benzoate as a substrate to monitor its decarboxylation as an index of production of hydroxyl radicals. Initially we tested two types of stimulated microsomal fraction to decarboxylate benzoate. The first being phenobarbital stimulated microsomal fraction and second, an arochlor stimulated microsomal fraction. The phenobarbital induced microsomal fraction, in the presence of NADPH, decarboxylated benzoate as depicted in Figure 2. Whereas, the microsomal fraction obtained from arochlor pretreated rats and incubated with NADPH resulted in decarboxylation of benzoate which was not different from that of microsomal fraction from non-treated control (Figure 3). Decarboxylation of benzoate at substrate levels from $10^{-5}$ to $10^{-2}$ M showed a sigmoid curve when plotted against $^{14}$CO$_2$ production (Figure 2). The Km calculated for the kinetic rate in Figure 2 was 5 mM. Addition of 1 mM sodium azide (an inhibitor of catalase) to the system increased the decarboxylation at each benzoate concentration tested as shown in Figure 2 by 220% over the complete microsomal system consisting of benzoate,
microsomal fraction, and NADPH (data not shown). When active catalase (Figure 1) (100 μg/ml) a scavenger of hydrogen peroxide was added to the microsomal system, decarboxylation of benzoate was totally abolished to the background non-enzymatic decarboxylation of benzoate (Figure 4). Addition of heat inactivated catalase showed no effect (Figure 4). In addition, it was demonstrated that when mannitol or dimethylsulfoxide (Figure 1) known scavengers of hydroxyl radicals were added to the system, they decreased the amount of decarboxylation of benzoate in a dose-dependent fashion when compared to control (Figure 4). The presence of SKF-525 A (10^{-5} and 10^{-3} M), a known microsomal inhibitor, had no effect on decarboxylation of benzoate (data not shown). We tested a second microsomal inhibitor, diethyldithiocarbamate on decarboxylation of benzoate. Diethyldithiocarbamate 10^{-5} and 10^{-4} M stimulated decarboxylation of benzoate by 55 and 80% respectively to controls, but at 10^{-3} M the reaction was inhibited by 90% when compared to control (Figure 5). Since it has been suggested that oxygen radicals may serve as the source of hydroxyl radicals, we wanted to elucidate the role of superoxide radicals (Figure 1) in the decarboxylation of benzoate. Therefore, we supplemented the incubations with superoxide dismutase. Superoxide dismutase was found to increase decarboxylation of benzoate by 120% increase over
the control (Figure 5). Similarly, addition of heat inactivated superoxide dismutase showed no effect.
FIGURE 1

Proposed Mechanism of Decarboxylation of (Carboxyl-\textsuperscript{14}C) Benzoate

1. Electron from reduction-oxidation reaction of NADPH, Adriamycin, and Mitomycin C.

2. \[ \text{O}_2 + 1 \text{ e}^- \rightarrow \text{O}_2^- \]

3. \[ 2\text{O}_2^- + 2\text{H}^+ \rightarrow \text{SOD} \rightarrow \text{H}_2\text{O}_2 \]

4. \[ \text{H}_2\text{O}_2 \xrightarrow{\text{Fe}^+2 \text{ or } \text{O}_2^-} \text{Fe}^+3 \rightarrow \text{OH}^- + \text{OH}^- \text{ (Fenton or Haber-Weiss RXN)} \]

5. \[ 2\text{H}_2\text{O}_2 \rightarrow \text{Catalase} \rightarrow 2\text{H}_2\text{O} + \text{O}_2 \]

6. \[ \text{OH}^- + \#\text{Benzoate} \rightarrow \#\text{CO}_2 + \text{Metabolites of Benzoate} \]
   
   \text{or Mannitol}
   \text{or DMSO}
ADDENDUM

In plate 3-6, 10, and 13-15 on the y-axis the units are % of control. In addition, either $^{14}\text{CO}_2$/mg protein/hr or $\text{O}_2$ consumption/min/mg protein was added as a reference source.
FIGURE 2

Effects of varying benzoate concentrations on decarboxylation of (carboxyl-14C) benzoate with rat liver microsomal fraction. The concentrations of microsomal fraction and NADPH are 1 mg/ml and 2 mM (final concentration), respectively. The results are of four experiments and the S.E. for 10^-5, 10^-4, 10^-3, and 10^-2 M are ± .0045, .097, .28, and .95, respectively.
PLATE 1

Effects of varying benzoate concentration on decarboxylation of benzoate with phenobarbital-induced microsomal fraction.
FIGURE 3

Effects of arochlor induced microsomal fraction on decarboxylation of (Carboxyl-$^{14}$C) benzoate. The concentrations of microsomal fraction and NADPH are 1 mg/ml and 2 mM (final concentration), respectively.
PLATE 2

Effects of arochlor induced microsomal fraction on decarboxylation of benzoate.
Effects of catalase, mannitol, dimethylsulfoxide (DMSO) and superoxide dismutase (SOD) on decarboxylation of (carboxyl-\(^{14}\)C) benzoate in the presence of rat liver microsomal fraction. The concentrations of catalase and SOD were 100 ug/ml and 10 ug/ml, respectively. The concentration of mannitol are 10 and 30 mM and DMSO are 3, 10, and 30 mM. The concentrations of benzoate, microsomal fraction, and NADPH were as described in in the Methods section. The yield of \(^{14}\)CO\(_2\) from paired experiments was 0.046 ± 0.003 nanomole \(^{14}\)CO\(_2\)/mg protein/hour for the control (which consisted of benzoate, microsomal fraction, and NADPH) and the additions B–J are expressed as percent of control. The S.E. was > 3% for each agent tested. Inactive catalase and SOD were heat inactivated at 100°C for 30 minutes.
PLATE 3

Effects of catalase, superoxide dismutase, dimethylsulfoxide and mannitol on decarboxylation of benzoate.
FIGURE 5

The effects of diethyldithiocarbamate (DDC) on decarboxylation of (carboxyl-¹⁴C) benzoate in the presence of rat liver microsomal fraction. The control (which consisted of benzoate, microsomal fraction, and NADPH) value for this experiment was 0.063 ± .001 nM ¹⁴CO₂/mg protein/hour. The results are of triplicate paired experiments.
PLATE 4

The effects of diethylthiocarbamate on decarboxylation of benzoate.
EFFECTS OF QUINONE DRUGS ON DECARBOXYLATION OF 
(CARBOXYL-$^{14}$C) BENZOATE

The next series of experiments examined the effect of chemotherapeutic drugs on the rate of decarboxylation of benzoate by rat microsomal fraction. The following agents tested in our system were; adriamycin and its analogs, mitomycin C, 5-Fluorouracil, cyclophosphamide and methotrexate and the concentration of each drug used was 18.4, 30, 78, 39 and 23 uM, respectively. The anthracycline drug, adriamycin, and the quinone drug, mitomycin C, increased decarboxylation of benzoate above the control by 100% and 50%, respectively. Conversely, 5-Fluorouracil, cyclophosphamide, and methotrexate in similar concentration did not stimulate the decarboxylation of benzoate above control and may have decreased the amount of decarboxylation but not to a significant amount (Figure 6). The adriamycin related analogs tested were 5-iminodaurubcin, 4-demethoxydaunomycin, mitozanthron and 4'-deoxyadriamycin. The amount used of these drugs was calculated to equal the molar dose of adriamycin used. 5-Iminodaurucin, 4-demethoxydaunomycin, DHAQ and 4'-deoxyadriamycin stimulated decarboxylation of benzoate by 72, 63, 51, and 63%, respectively (Figure 7). Addition of SKF 525 A, a known microsomal inhibitor, to the reaction containing Mitomycin
C, did not alter the stimulation of benzoate decarboxylation. Further, the effects of superoxide dismutase, catalase, and hydroxyl scavengers on the rate of decarboxylation after the chemotherapeutic agent gave similar results to those obtained with benzoate decarboxylation. Briefly, catalase inhibited the stimulation to background (non-enzymatic decarboxylation) and superoxide dismutase, however, enhanced the rate of decarboxylation above the drug stimulated levels by two fold (data not shown). Also as expected both dimethylsulfoxide and mannitol inhibited the stimulation of benzoate produced by Mitomycin C. These results were similar to the data shown in Figure 4.
The effects of adriamycin, mitomycin C, 5-fluorouracil, cyclophosphamide and methotrexate on decarboxylation of (carboxyl-$^{14}$C) benzoate by rat liver microsomal fraction. Concentrations of the components are as described in the method section and text. The yield of labelled $^{14}$CO$_2$ from six experiments was $0.0275 \pm 0.002$ nanomoles $^{14}$CO$_2$/mg protein/hour for the control (which consisted of benzoate, microsomal fraction, and NADPH). Experiment incubations are expressed as percent of control. The S.E. was less than $\pm 3\%$ for each drug tested.
PLATE 5

Effects of adriamycin, mitomycin C, 5-fluorouracil, cyclophosphamide, and methotrexate on decarboxylation of benzoate.
The effects of adriamycin analogs: 5-Iminodaunurubicin, 4'-Demethoxydaunomycin, Mitozanthron and 4-Deoxyadriamycin on decarboxylation of (carboxyl-\(^{14}\)C) benzoate by rat liver microsomal fraction. Concentration of the components are as described in the method section and text. The yield of \(^{14}\)CO\(_2\) from six experiments was 0.0275 ± 0.002 nanomoles CO\(_2\)/mg protein/hour for the control (which consisted of benzoate, microsomal fraction, and NADPH) and the others are expressed as percent of control. The S.E. was less than ±3% for each drug tested.
PLATE 6

The effects of adriamycin analogs; 5-iminodaunorubicin, 4'-demethoxydaunomycin, mitozantrion and 4'-deoxyadriamycin on decarboxylation of benzoate.
EFFECT OF BENZOATE ON OXYGEN CONSUMPTION

To this point, we have exclusively looked at decarboxylation of benzoate by the generation of $^{14}$CO$_2$ production. It was of interest to examine oxygen consumption under conditions in which the decarboxylation of benzoate occurred to determine whether or not oxygen consumption was also affected by the addition of substrate or drugs. In Figure 8, with increasing benzoate concentration from $10^{-5}$M to $10^{-2}$M, there appeared to be no significant increase in oxygen consumption. Since, SKF 525 A did not increase decarboxylation of benzoate, the effects of this compound on oxygen consumption was studied. SKF 525 A at $10^{-5}$ and $10^{-3}$ M did not alter oxygen consumption (Figure 9). The effects of diethyldithiocarbamate was examined on oxygen consumption in Figure 10. Diethyldithiocarbamate $10^{-5}$, $10^{-4}$, and $10^{-3}$ M stimulated oxygen consumption. Effect of hydroxyl scavengers (tested previously on oxidation of benzoate) on oxygen consumption were studied. Mannitol and DMSO were found not to affect the rate of oxygen consumption compared to control (Figure 11). Effects of superoxide dismutase and catalase were also tested for their effects on oxygen consumption. Both of these agents showed no significant effect on the rate of oxygen consumption (Figure 11). A control was included in which benzoate was deleted and
mannitol or DMSO were present. These agents showed no
difference from the complete system (Figure 11).

The effects of Mitomycin C on oxygen consumption by the
liver microsomal system was tested. A concentration of
Mitomycin C in these experiments was used which stimulated
decarboxylation of benzoate (as indicated in Figure 12). The
enhanced oxygen consumption by liver microsomal fraction, in
the presence of Mitomycin C, was not altered by benzoate,
SKF 525 A, Mannitol, DMSO, catalase, or superoxide
dismutases. In Figure 12, the data with 10 and 30 mM
Mannitol is illustrated to demonstrate that this agent and
the agents discussed above had no effect on the $O_2$
consumption. Diethyldithiocarbamate was examined for its
effect on oxygen consumption with the addition of mitomycin
C. As shown in Figure 13 diethyldithiocarbamate, $10^{-5}$,
$10^{-4}$, and $10^{-3}$ M stimulated Mitomycin C oxygen
consumption.
FIGURE 8

The effects of varying benzoate concentration on oxygen consumption with rat liver microsomal fraction. The concentrations of liver microsomal fraction and NADPH are 0.1 mg/ml and 0.425 mM (final concentration), respectively. The addition of microsomal fraction gave 1% oxygen consumption for all concentrations, so it is represented as a solid line in the figure. The results are of paired experiments and the S.E.M. is 0.5% for the data. The control gave identical results to $10^{-2}$ M concentration of benzoate and is not shown in the figure.
The effects of varying benzoate concentrations on oxygen consumption.
FIGURE 9

The effects of SKF-525 A and benzoate on oxygen consumption by rat liver microsomal fraction. The concentrations of microsomal fraction and NADPH are 0.1 mg/ml and 0.425 mM (final concentration), respectively. The results are of paired experiments and the S.E.M. is ±26%. The control value for the $10^{-5}$ M and $10^{-3}$ M benzoate are 6.57 and 8.13 nanomole O$_2$/min/mg protein, respectively. The control consisted of benzoate, microsomal fraction, and NADPH. The data is given as % of control.
The effects of SKF-525 A and benzoate on oxygen consumption.
The effects of diethyldithiocarbamate (DDC) on oxygen consumption by rat liver microsomal fraction. The concentrations of DDC are $10^{-5}$, $10^{-4}$, and $10^{-3}$ M. Conversely, the concentrations of liver microsomal fraction, NADPH, and benzoate are 0.1 mg/ml, 0.425 M (final concentration) and $10^{-3}$ M, respectively. The addition of microsomal fraction and benzoate with buffer gave 1.5 and 0% oxygen consumption, respectively. The results are of triplicate experiments and the S.E. is $\pm$ 0.5% for the data given.
PLATE 9
The effects of diethyldithiocarbamate on oxygen consumption.
FIGURE 11

The effects of catalase, superoxide dismutase (SOD), Mannitol, and dimethylsulfoxide (DMSO) on oxygen consumption by rat liver microsomal fraction in the presence of benzoate at $10^{-5}$ M concentration. The concentrations of catalase and SOD are 100 ug/ml and 10 ug/ml, respectively. The concentrations of Mannitol and DMSO were 10 and 30 mM. The concentrations of microsomal fraction and NADPH are 0.1 mg/ml and 0.425 mM (final conc.), respectively. Results are of paired experiments and the S.E.M. is ± 26%. The data is given as % of the complete system. The complete system consisted of benzoate, microsomal fraction, and NADPH. The value for the complete system for catalase and superoxide dismutase experiment is 11.94 nanomole O$_2$/min/mg protein. In the complete system system for the Mannitol and DMSO experiment 7.59 nanomole O$_2$/min/mg protein was consumed. Catalase and SOD were heat inactivated for 30 mins. at 100 C. In the Mannitol experiment lines 2 and 3 had a p value of .69 using the Student T distribution test. Line 3 and 4 had a p value of .69 using the similar test. Line 4, 5, 8 and 9 are supplemented only with the agent tested and benzoate is omitted and is labelled as -b then the concentration of agent.
PLATE 10

The effects of catalase, superoxide dismutase, dimethylsulfoxide, and mannitol on oxygen consumption.
The effects of Mannitol on oxygen consumption by rat liver microsomal fraction. The concentration of Mannitol are 10 and 30 mM. Conversely, the concentrations of liver microsomal fraction, NADPH, and benzoate are 0.1 mg/ml, 0.425 mM (final concentration) and $10^{-5}$ M, respectively. The combination of benzoate, buffer, and Mannitol gave zero percent $O_2$ consumption in all experiments and is represented as a solid line. Similarly, the addition of the microsomal fraction gave 1% $O_2$ consumption in all experiments and is also represented as a solid line. The results are of paired experiments and the S.E.M. is 0.5% for the data given.
PLATE 11

The effects of mannitol on oxygen consumption.
FIGURE 13

The effects of diethyldithiocarbamate (DDC) on Mitomycin C stimulated oxygen consumption by rat liver microsomal fraction. The DDC and Mitomycin C concentrations are $10^{-5}$ to $10^{-3}$M and 100 ug/ml, respectively. The concentration of liver microsomal fraction, NADPH and benzoate are 0.1 mg/ml, 0.425 mM (final conc.) and $10^{-5}$, respectively. The results are of paired experiments and the S.E. ± 1.0% for the data given. The control consisted of benzoate, microsomal fraction, NADPH, and mitomycin C (100 ug/ml).
PLATE 12

The effects of diethyldithiocarbamate on mitomycin C stimulated oxygen consumption.
OXIDATION OF $^{14}$C-FORMATE BY DIETHYLDITHIOCARBAMATE (DDC),
DIMETHYL SULFOXIDE AND MANNITOL USING RAT LIVER MICROSOMES

$^{14}$C-formate oxidation was used as an indicator of hydrogen peroxide production by rat liver microsomal fraction. The effects of diethyldithiocarbamate were examined to parallel the decarboxylation of benzoate studies. When diethyldithiocarbamate was supplement to the reaction mixture which consisted of $^{14}$C-formate, NADPH 2 mM final conc. and rat liver microsomal fraction, diethyldithiocarbamate at $10^{-5}$ and $10^{-4}$ M stimulated oxidation of $^{14}$C-formate by 30% in each case and at $10^{-3}$ M inhibited the reaction by 40% (Figure 14). Mannitol and DMSO were tested for their effect on oxidation of $^{14}$C-formate and the results obtained showed that neither of these agents had no effect (Figure 14). Addition of catalase (100 ug/ml) which is required for $^{14}$C-formate oxidation, to the reaction mixture supplemented with the agents being examined, resulted in stimulation of formate oxidation. However, addition of 1 mM sodium azide (an inhibitor of catalase activity) to the reaction mixture and agent being studied, inhibition of $^{14}$C-formate was observed (data not shown).
FIGURE 14

The effects of diethyldithiocarbamate (DDC), dimethyl sulfoxide (DMSO) and Mannitol on oxidation of $^{14}$C-formate by rat liver microsomal fraction. The control consisted of $^{14}$C-formate, NADPH (2 mM final conc.) and liver microsomal fraction. Four milligrams of protein was used per metabolic flask. The control value was $0.146 \pm 0.021$ $^{14}$CO$_2$/mg protein/hour. The results are of triplicate experiments. 1 uCi of labelled formate was used. Total formate in the incubation mixture was 10 uM.
PLATE 13

The effects of diethyldithiocarbamate, dimethylsulfoxide and mannitol on oxidation of $^{14}$C-formate.
The effects of phenobarbital induced rat liver microsomal fraction were compared to non-phenobarbital induced microsomal fraction on decarboxylation of benzoate. It was observed that phenobarbital induced liver microsomal fraction increased the production of decarboxylated benzoate by 40% over non-induced microsomal fractions (Figure 15). Induced microsomal fractions and non-induced microsomal fraction under 100% oxygen atmosphere were compared. The solution were gassed 20 minutes with oxygen, and the reaction mixture was gassed for 20 minutes prior to the addition of NADPH. The results showed that the phenobarbital induced liver microsomal fraction increased decarboxylation of benzoate by 100% over the non-induced microsomal fraction (Figure 15). The effects of rat heart microsomal fraction and supernates (9,000 and 100,000 g supernate) on decarboxylation of benzoate were examined. We were unable to stimulate decarboxylation of benzoate with the addition of NADPH (2 or 4 mM final concentration), Mitomycin C (100 or 200 ug/ml) 5-Fluoruracil (100 ug/ml), and Adriamycin (100 or 200 ug/ml) over their respective control (Figure 16) with heart microsomal fraction. Addition of 1 mM sodium azide to the reaction mixture had no effect. Increasing the benzoate concentration had no effect on decarboxylation of benzoate.
Heart rat microsomal fraction were examined for their effects on oxygen consumption. The results showed that rat heart microsomal fraction showed no activity to stimulate oxygen consumption alone or with the addition of NADPH or Mitomycin C. The 9,000 and the 100,000 g supernates did not stimulate oxygen consumption (data not shown), similar to the microsomal fraction.
FIGURE 15

The effects of phenobarbital induced vs. non-phenobarbital microsomal fraction on decarboxylation of (carboxyl-$^{14}$C) benzoate. The control values for the 100% oxygen and ambient air experiments are .003 and .00295 ± .0004 nM $^{14}$CO$_2$/mg protein/hour, respectively. In the oxygen experiment the complete reaction mixture was gassed for 20 minutes under 100% oxygen and then incubated.
The effects of phenobarbital induced vs. non-induced microsomal fractions on decarboxylation of benzoate.
The effects of the heart microsomal fractions (S-9 fraction, 100,000 g supernate and microsomal fractions) on decarboxylation of (carboxyl-$^{14}\text{C}$) benzoate. The control values are 0.00015 (S-9 exp.), 0.00024 (100,000 g supernate) and 0.00274 (microsomal fraction) nM$^{14}\text{CO}_2$/mg protein/hour. In all cases 4 mg of total protein was used in each reaction mixture. The control consisted of benzoate and respective microsomal fraction.
PLATE 15

The effects of heart microsomal fractions (9,000 and 100,000 g supernate and microsomal fraction) on decarboxylation of benzoate.
DISCUSSION

Earlier it was demonstrated that benzoate undergoes decarboxylation in a number of systems, most likely as a consequence of interaction with hydroxyl radicals. We have confirmed the original observations of Matthews and Sangster that following irradiation, benzoate undergoes decarboxylation. In addition, we have shown that benzoate is decarboxylated by hydroxyl radicals generated by the xanthine-xanthine oxidase enzyme system, by stimulated polymorphonuclear cells and by arachidonic acid stimulated platelets. In our experiments, we have shown that benzoate is decarboxylated by both phenobarbital induced and non-phenobarbital induced liver microsomal fraction that has been supplemented with NADPH. The phenobarbital induced microsomal fractions showed a 40% increase in decarboxylation of benzoate over the non-induced microsomal fraction. Under a 100% oxygen atmosphere the phenobarbital induced microsomal fraction showed an increase in decarboxylation of benzoate compared to non-induced control. Indicating the requirement for oxygen in the decarboxylation of benzoate. The effects of arochlor induced microsomal fraction were examined in our system. Arochlor is an agent which induces the enzyme spectrum that is responsible for metabolism of polycyclic aromatic hydrocarbons. In contrast, phenobarbital induces the
enzymes responsible for drug metabolism. The data indicated that arochlor induced microsomal fraction does not possess the necessary enzyme to cause benzoate to decarboxylate at a sufficient rate. When azide was supplemented to the reaction mixture containing NADPH an enhanced decarboxylation of benzoate was observed. This latter observation suggests that a heme inhibitable compound was present in the preparation and decreased optimal benzoate decarboxylation. In this regard, we suggest that it may be related to the catalase activity present in the microsomal preparation. The decarboxylation of benzoate was not impaired by the microsomal inhibitor SKF 525 A, but was inhibited by hydroxyl scavengers dimethylsulfoxide and mannitol. This suggests that the mechanism by which benzoate is decarboxylated by the microsomal system is independent of the pathway which appears to involve the P-450 microsomal system. A second microsomal inhibitor, diethyldithiocarbamate was examined in our system. It was observed that diethyldithiocarbamate $10^{-5}$ and $10^{-4}$ M stimulated decarboxylation of benzoate, whereas, at $10^{-3}$ M the reaction was inhibited. This observation may suggest that diethyldithiocarbamate stimulates an enzyme at $10^{-5}$ and $10^{-4}$ M concentration, but becomes inhibitory at $10^{-3}$ M. Diethyldithiocarbamate stimulated oxygen consumption at $10^{-5}$, $10^{-4}$, and $10^{-3}$ M. Thus, indicating a possible
relationship at the $10^{-5}$ and $10^{-4}$ M concentration between the effects of diethyldithiocarbamate on decarboxylation of benzoate and oxygen consumption. Yet diethyldithiocarbamate stimulated $O_2$ consumption at $10^{-3}$ M while inhibiting the decarboxylation of benzoate under the same experimental conditions. This experimental finding seems to disassociate the role of oxygen uptake from the decarboxylation reaction and is not readily explained. It may be that at high diethyldithiocarbamate concentrations the increased uptake of oxygen favors other reactions at the expense of the decarboxylation of benzoate. Diethyldithiocarbamate stimulated oxidation of $^{14}$C-formate (which is used as an indicator of hydrogen peroxide production) at $10^{-5}$ and $10^{-4}$ M, whereas the $10^{-3}$ M the reaction was depressed. This finding was similar to that obtained with decarboxylation of benzoate, thus, providing supportive data for the observation with decarboxylation of benzoate. Addition of catalase to the reaction mixture supplemented with the agent being examined, stimulated $^{14}$C-formate oxidation, whereas, addition of sodium azide resulted in inhibition. These results differ from the data obtained from decarboxylation of benzoate, in which catalase inhibited and sodium azide stimulated the reaction. These data with $^{14}$C-formate oxidation, indicates the requirement for catalase to inactivate hydrogen peroxide. Should catalase
levels be deplete, this would favor competing reactions which involves formation of hydroxyl radicals from hydrogen peroxide (reaction 4, Figure 1). Additional experiments were performed to study the effect of varying concentrations of benzoate on oxygen consumption using the microsomal system. In this regard other investigators have suggested that in some cases there may be a feedback inhibition of microsomal enzymes under conditions in which hydroxyl radical is produced. These systems have usually involved the production of prostaglandins from arachidonic acid. The addition of varying amounts of benzoate to the microsomal system was not associated with an enhanced oxygen consumption although the decarboxylation proceeded in direct relationship to substrate concentration. This suggests that the amount of hydroxyl radical generated under these conditions did not cause feedback inhibition in the microsomal system, thus suggesting there is not a direct relationship with $O_2$ consumption. The effect of superoxide dismutase, an enzyme which would decrease the amount of superoxide generated by the microsomes, enhanced the reaction, while catalase depressed the reaction. Heat inactivated enzymes had no effect on control levels of decarboxylation. These observations indicated that superoxide was not required, but hydrogen peroxide was required for the decarboxylation of benzoate. Therefore, in
contrast to the mechanism of benzoate decarboxylation in the xanthine-xanthine oxidase system which appears to require a Haber-Weiss type of interaction (superoxide with hydrogen peroxide), the mechanism in the microsomal system appears to be the production of hydrogen peroxide with the ultimate generation of hydroxyl radical. This provides further evidence that the pathway for the decarboxylation of benzoate by the liver microsomal fraction is different from the pathway which mediates the metabolism of xenobiotics.

Since benzoate decarboxylation demonstrated the feasibility of this reaction system as an assay for the detection of hydroxyl production by microsomal systems, the effect of quinone type drugs was studied. It has been suggested previously that one of the major mechanisms by which these drugs mediate toxicity is by an increased hydroxyl production (Bachur, et. al., 1977 and 1978; Cohen and Cderbaum, 1979). In most of these circumstances, the evidence has been indirect using scavenger studies (Doroshow, 1983), or in other cases the oxidation of substrates such as methional which may not be specific (Komiyana, et. al., 1982; Winston, et. al., 1982).

In this study, adriamycin, 5-iminodaurubicin, 4'-demethoxydaunomycin, DHAQ, 4'-deoxyadriamycin and mitomycin enhanced oxygen consumption and also stimulated decarboxylation of benzoate. This observation suggests that
the stimulation of oxygen consumption was associated with a stimulation of hydroxyl production. In contrast, drugs which are known to be inactive in stimulating oxygen consumption in the microsomal system such as 5-fluorouracil, cyclophosphamide, and methotrexate did not stimulate benzoate decarboxylation, providing some specificity for the reaction. Similar to the decarboxylation of benzoate by the microsomal fraction in the presence of NADPH, the enhanced benzoate decarboxylation induced by the quinone type drugs were impaired by the presence of catalase and hydroxyl scavengers (DMSO, Mannitol). The quinone drugs appear to be augmenting the primary mechanism for the production of hydroxyl radical.

Recently, Komiyama and co-workers reported evidence that methional was oxidized to ethylene in the presence of the microsomal enzyme system and the following drugs: Carbazilquinone, Mitomycin C, Aclacinomycin A, and Adriamycin. Their results with carbazilquinone and aclacinomycin A were similar to this study in that superoxide dismutase enhanced the oxidation of methional to ethylene and catalase inhibited the oxidation of methional. Their results with Mitomycin C and Adriamycin were also similar to this study in that ethylene production from methional was inhibited by catalase. However, superoxide dismutase decreased the oxidation of methional which was not
the case in the present experiments with benzoate decarboxylation. Therefore, Adriamycin and Mitomycin C are capable of producing another reactive oxygen species in addition to hydroxyl having the capacity to oxidize methional to ethylene and which is dependent on $O_2^{-}$ for their production. It appears that oxidation of methional in contrast to benzoate may be mediated by more than one reactive oxygen species generated in the microsomal system and is not a specific indicator of hydroxyl radical production. Also aclacinomycin A and carbazilquinone are capable of only producing one reactive oxygen species (i.e hydroxyl radicals) as compared to Mitomycin C and Adriamycin. One possibility is that methional may also be oxidized by the organic radicals produced by the microsomal enzyme system. In this regard, Winston and Cederbaum (1983) have shown that benzoate was not capable of being decarboxylated by organic radicals.

Since the toxicity of Adriamycin is primarily related to the heart, some experiments were carried out using isolated rat heart microsomal fraction. The fractions examined were the S-9 fraction (9,000 g pellet), 100,000 g supernate and microsomal fraction. The various fractions were examined to obtain data on which of the fraction possessed the activity to decarboxylate benzoate. The data obtained indicated that the various fractions of rat heart did not decarboxylate
benzoate when supplemented with NADPH or after the addition of Mitomycin C and Adriamycin. Thus, heart microsomal preparations isolated by the same methods used for liver did not decarboxylate benzoate. Therefore, it may be necessary to further examine the isolation techniques and characterize the enzyme(s) involved in decarboxylation of benzoate that are present in liver microsomal fraction and may also be present in the heart microsomal fraction.

In summary our results showed that decarboxylation of benzoate can be used as a measure of hydroxyl production in microsomal systems. Using this assay we have further classified the mechanism of the reaction and demonstrated that chemotherapeutic drugs which are active in stimulating the oxygen consumption of microsomal systems are also capable of enhancing benzoate oxidation. These data further support the concept that the hydroxyl radical produced by the activation of these drugs in microsomal system may be related to the cellular toxicity.
SUMMARY

1. This study demonstrated that the decarboxylation of benzoate proceeds by a Fenton reaction rather than the Haber-Weiss reaction and requires hydroxyl radicals.

2. Liver microsomal fractions obtained from arochlor pre-treated rats and incubated with added NADPH resulted in decarboxylation of benzoate which was not different from that of microsomal fractions from non-treated controls. However, phenobarbital induced microsomal fractions, in the presence of NADPH, decarboxylated benzoate at 40% greater than control rates. In the presence of a saturated O₂ atmosphere, phenobarbital-induced microsomes activity was 100% greater than controls.

3. Decarboxylation of benzoate at substrate levels from 10⁻⁵ and 10⁻² M showed a sigmoid curve when plotted against ¹⁴CO₂ produced. Addition of 1 mM sodium azide to the reaction mixture increased ¹⁴CO₂ production 220% at each benzoate concentration tested.

4. In the decarboxylation reaction, the addition of superoxide dismutase to the control (which consisted of
benzoate, microsomal fraction, and NADPH) resulted in a 2-fold increase. Addition of heat inactivated superoxide dismutase resulted in control value. Addition of catalase to control reactions abolished $^{14}$CO$_2$ production to non-enzymatic decarboxylation levels. Heat inactivated catalase gave results similar to controls. Addition of hydroxyl radical scavengers, dimethylsulfoxide and mannitol inhibited the reaction in a dose dependent fashion when compared to control.

5. The presence of SKF-525 A ($10^{-5}$ and $10^{-3}$ M) had no effect on benzoate decarboxylation. Whereas, diethyldithiocarbamate $10^{-5}$ and $10^{-4}$ M stimulated the reaction by 55 and 80% respectively, compared to controls. At $10^{-3}$ M the reaction was inhibited by 90%.

6. Adriamycin, mitomycin, 5-iminodaunorubicin, 4'-demethoxydaunomycin, DHAQ, and 4'-deoxyadriamycin stimulated decarboxylation of benzoate over control (benzoate, microsomes, and NADPH) by 100, 50, 75, 60, 50 and 55%, respectively. Addition of superoxide dismutase to the control reaction having each of the quinones (above) increased decarboxylation 2-fold. Addition of catalase with the presence of the quinones resulted in total inhibition of decarboxylation. Addition of inactive enzymes (catalase and
SOD) was not different from controls. 5-Fluorouracil, cyclophosphamide, and methotrexate were without effect on the decarboxylation.

7. The reaction mixture used for decarboxylation studies was subjected to oxygen consumption determinations. Oxygen consumption in the presence of benzoate ($10^{-5}$ to $10^{-2}$ M) was independent of substrate concentration. SKF 525 A was without effect on control oxygen uptake. However, diethyldithiocarbamate stimulated oxygen consumption at $10^{-5}$ and $10^{-4}$ M. When diethyldithiocarbamate was added at $10^{-3}$ M oxygen uptake was stimulated, yet at this concentration decarboxylation of benzoate was markedly inhibited. Mannitol, dimethylsulfoxide, superoxide dismutase and catalase were each without effect on oxygen uptake. Addition of mitomycin C in the presence of any of these agents resulted in no change in oxygen uptake compared to controls.

8. Diethyldithiocarbamate ($10^{-5}$ and $10^{-4}$ M) stimulated $^{14}$C-formate oxidation, but was inhibitory at $10^{-3}$ M compared to control ($^{14}$C-formate, NADPH and liver microsomal fraction). Dimethylsulfoxide (30 and 100 mM) or mannitol (30 mM) had no effect on $^{14}$C-formate oxidation.
9. The 9,000 and 100,000 g supernates and microsomal fraction of rat heart failed to decarboxylate benzoate in the presence of NADPH. Mitomycin C, adriamycin, and 5-fluorouracil were all without effect regardless of the heart fraction tested. Increasing benzoate (10^{-5} \text{ to } 10^{-2} \text{ M}) and NADPH (2 \text{ mM to } 4 \text{ mM}) had no effect. Addition of 1 \text{ mM} sodium azide did not affect decarboxylation. Addition of NADPH and drugs also failed to stimulate oxygen consumption in the presence of the 9,000 and 100,000 g or microsomal fractions of heart.


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APPENDIX 1

The Structures of Adriamycin and Mitomycin C
ADRIAMYCIN

MITOMYCIN C

PLATE 16
The structures of Adriamycin and Mitomycin C.