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Ascorbic Acid, Lipid Peroxidation, and Aging

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    The role of ascorbic acid with regard to lipid

peroxidation and aging has been examined. A thorough

literature analysis indicates that free radical-induced

lipid peroxidation is a plausible biochemical explana-

tion for aging. Lipid peroxidation causes cellular
damage due to altered enzyme activities, error-prone
nucleic acid metabolism, and membrane dysfunction, as
well as the accumulation of aging pigments in the lyso-
somes. Ascorbic acid, a water soluble free radical
quencher, was examined with regard to carbon tetra-
chloride-induced lipid peroxidation and in vivo
aging. Carbon tetrachloride, a well-known free
radical inducer, caused marked increases in the ratio
of oxidized/reduced vitamin C only in the organs which
metabolize carbon tetrachloride to the free radical
form. Vitamin C treatment, 250 mg% in the drinking
water, reduced the extent of carbon tetrachloride-
induced lipid peroxidation. Aging is associated with
marked increases in the ratio of oxidized/reduced
vitamin C in all organs examined with the exception
of thymus. In organs exposed to high oxygen tensions,
or in those exposed to high levels of free radicals,
the ratio of oxidized/reduced vitamin C exceeded the
in vitro ratio, indicating extensive lipid peroxida-
tion. Vitamin C treatment reduced the extent of
lipid peroxidation in vivo as determined by the ratio
of oxidized/reduced vitamin C.

ASCORBIC ACID, LIPID PEROXIDATION, AND AGING

by

BRIAN EVAN LEIBOVITZ

A thesis submitted in partial fulfillment

of the **requirements** for a degree of

MASTER OF SCIENCE

in

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TO THE OFFICE OF GRADUATE STUDIES AND RESEARCH:

The members of the Committee approve the
thesis of Brian Evan Leibovitz presented May 10,1979.

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CHAPTER I

INTRODUCTION

A current explanation for aging at the bio-
chemical level is based on the production of free
radicals with subsequent free radical chain reac-
tions leading, ultimately, to membrane damage and
cross-linking of biomolecules. The net result is
a decline in cellular integrity caused by reduced
enzyme activities, error-prone nucleic acid meta-
bolism, damaged membrane functions, and accumula-
tion of 'aging pigments' in lysosomes. Ascorbic
acid (vitamin C) is a water soluble free radical
quencher, and thus possesses potential for re-
tardation of the aging process. Studies described
herein pertain to the role of vitamin C as a free
radical quencher using the murine model system.

CHAPTER II

REVIEW OF THE LITERATURE

The initiating event in most biological free radical reactions is the production of superoxide radical $(O_2\cdot)$(SOR), which is formed from molecular oxygen by addition of a single electron (Figure 1). SOR is acted upon by superoxide dismutase (SOD) yielding hydrogen peroxide, which is capable of reacting with another molecule of SOR to produce a more potent radical, the hydroxyl radical (Figure 1). SOR-mediated decomposition of hydrogen peroxide is catalyzed by ferric ion (Haber-Weiss reaction). The subject of the biology of oxygen radicals has been treated in a number of recent reviews (1-3).

Respiring cells are a major source of SOR, in spite of tight binding of oxygen by cytochrome oxidase. The extent of SOR production during respiration in eukaryotes is not known. However, in extracts of Streptococcus faecalis in which SOD activity had been inhibited by specific antibody, a total of 17 percent of the oxygen consumed

$0_2 \xrightarrow{\hspace{1cm} e^- \hspace{1cm}}$ (electron transport, drugs, enzymes, pollutants, radiation) $\hspace{1cm} 0_2 \cdot$ (SOR)

$0_2 \cdot \hspace{1cm} 2 H^+$

superoxide
dismutase

0_2

$H_2 0 \xleftarrow{\text{catalase}}$

$H_2 0_2$
(hydrogen
peroxide)

2 GSH

GSSG

$H_2 0$ $\hspace{0.5cm}$ GSH
peroxidase $\hspace{0.5cm} 0_2 \cdot$

Fe^{+++}

Haber-Weiss
Reaction

0_2

$OH^- + OH \cdot$
(hydroxyl
radical)
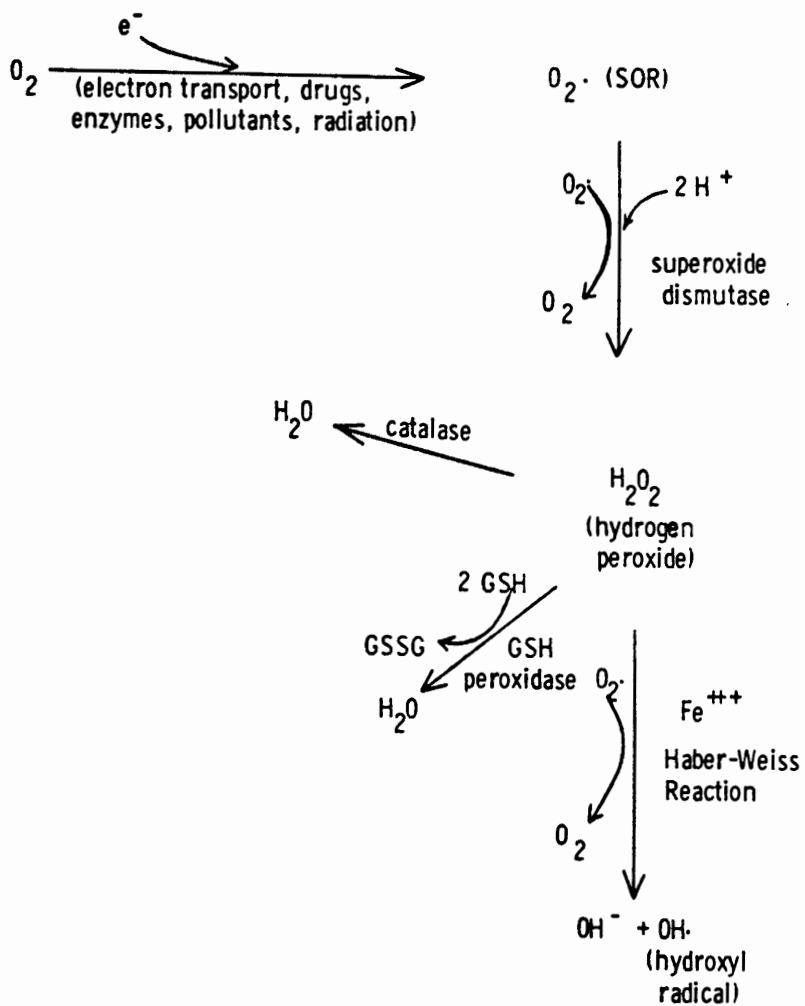
Figure 1. Production of superoxide radical, hydroxyl radical, and hydrogen peroxide.

resulted in SOR production (4). The importance of
SOR production during respiration was demonstrated
by Loschen et al. (5), who found that the production
of hydrogen peroxide was tightly coupled to energy
conservation in rat liver mitochondria. The as-
sociation of SOR production and respiration has led
to the suggestion that the mitochondrion is the
"molecular clock" in eukaryotic systems, and that
the maximal life span of a species is related to
the rate of oxygen consumption (6). In this regard,
low oxygen concentrations have been shown to extend
the life span of cultured human diploid cells in
vitro (7).

Respiration is not the only source of SOR,
however, and its production has been demonstrated
to be associated with, or required for, the
activity of a number of enzymes, including xanthine
oxidase, aldehyde oxidase, flavin dehydrogenase,
diamine oxidase, and ribulose-1,5-diphosphate
carboxylase (1,8,9). SOR production has also been
shown during the autooxidation of a variety of
molecules, including hydroquinones, flavins,
catecholamines, tetrahydropterins, ferrodoxins,
and hemoglobin (1,10,11,12,13). The autooxidation
of various chemicals, including herbicides (paraquat),
medicines (adriamycin), and alkyl halides (carbon

tetrachloride) also produce SOR (14-18).

One consequence of free radicals is the pro-
duction of lipid peroxides. Free radical attack
on polyunsaturated fatty acids yields lipid radicals
with allylic double bonds, which may add to mole-
cular oxygen to produce lipid peroxy radicals,
which by abstraction of a proton and electron
ultimately produce lipid peroxides (Figure 2).
Lipid peroxides are known to decompose and yield
a variety of products, including ethane, pentane,
and various aldehydes. The most important al-
dehyde produced is malondialdehyde ($CHO-CH_2-CHO$),
which forms Schiff base cross-links with amines
of proteins, phospholipids, and nucleic acids.
The product of this reaction is a biomolecule
many times the size of the original, which because
of unusual bonds, is not digested in the lysosome
and accumulates there with age. These biopolymers
are fluorescent, with a characteristic excitation
maxima at 360-380 nm, and an emission maxima at
440-470 nm. The accumulation of these pigments,
termed aging pigments, ceroid, or lipofuscin, is
directly related to the age of the animal (19,20).
The Schiff base products of the condensation of
malondialdehyde with the primary amines of proteins,
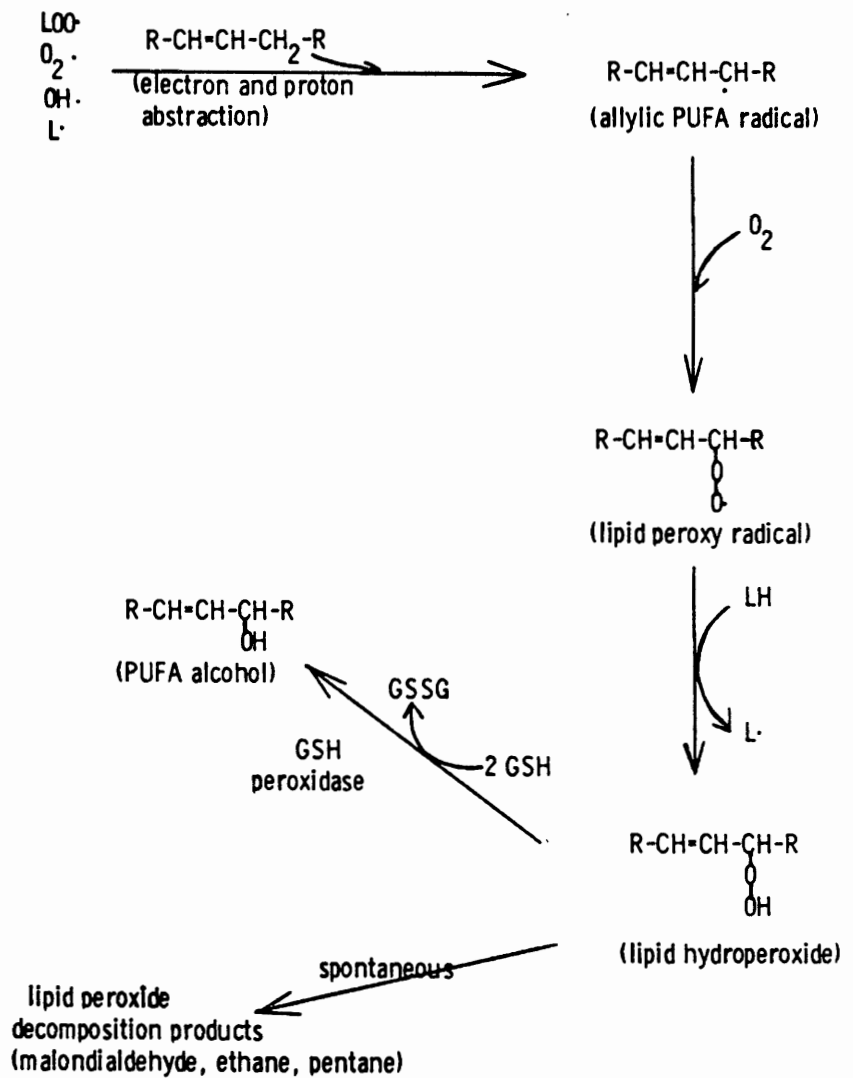
Figure 2. Free radical-induced lipid peroxidation.

nucleic acids, or phospholipids has fluorescent
properties similar to those observed for native
lipofuscin (21). Further, peroxidation of sub-
cellular organelles (lysosomes, mitochondria, and
microsomes) in vitro results in the formation of
aging pigments with spectra similar to that of
native lipofuscin (22).

The significance of lipid peroxidation and
cross-linking is the reduction in cellular inte-
grity caused by such events. Lipid peroxides have
been noted to inactivate various enzymes, including
isocitrate dehydrogenase (23), and to activate
potentially damaging enzymes, such as guanylate
cyclase (24). Lipid peroxidation damages integrated
membrane functions, the best example being the in-
hibition of oxidative phosphorylation of rat liver
mitochondria by methyl linoleate hydroperoxide (25).
Lipid peroxidation causes an increased ratio of
oxidized/reduced glutathione (via glutathione
peroxidase), which may inactivate important re-
gulatory enzymes, such as adenylate cyclase (26),
as well as decreasing the amount of transcription-
ally active genetic material (27).

Although free radicals are ubiquitous, they
are by no means omnipotent. Elaborate enzymatic

systems have been evolved by aerobic organisms to
reduce the fluxes of SOR and hydrogen peroxide, which
in turn will reduce the production of hydroxyl radicals
(Figure 1). Superoxide dismutase (SOD) is the major
defense system which protects against elevations in
the levels of SOR. SOD catalyzes the dismutation
of two molecules of SOR to yield hydrogen peroxide
and molecular oxygen (Figure 1). Hydrogen peroxide,
while less toxic than either SOR or hydroxyl radical,
is nevertheless damaging to biological systems, and
must therefore be detoxified. Glutathione peroxidase
utilizes the reducing power of glutathione (γ-L-
glutamyl-L-cysteinylglycine) to reduce hydrogen
peroxide to water (Figure 1), and contains selenium
as a coordinating mineral. Glutathione peroxidase
also acts on lipid peroxides, reducing these to
fatty acid alcohols (28). Glutathione peroxidase
reacts with a wide variety of lipid hydroperoxides,
and is specific for the hydroperoxyl group and not
for attached alkyl or aryl groups (28).

Ascorbic acid appears to play a protective
role against free radical damage. Ascorbic acid
has been shown to be oxidized by SOR generated from
a xanthine-xanthine oxidase system, with a second
order rate constant of 2.7×10^5 $M^{-1}sec^{-1}$ (29). At
cellular pH, the rate constant for the reaction

between SOR and SOD is about 8×10^8 $M^{-1}sec^{-1}$ (1),
or about 3,000 times faster than the ascorbate-
catalyzed reaction. The level of SOD in most tissues,
however, is approximately 10^{-6} M, about 1000 times
less than the approximate concentration of ascorbic
acid (29). The actual rate of reaction between SOR
and SOD is, therefore, only about 3 times that for
the ascorbate-catalyzed reaction, which indicates
that ascorbic acid may play a supplementary role
against SOR-mediated toxicity. In support of this
protective role of ascorbic acid, the production of
SOR by guinea pig macrophages in vitro was inhibited
by various antiinflammatory agents including
ascorbic acid (30). Ascorbic acid has also been shown
to quench the hydroxyl radical (31).

Ascorbic acid may play a primary role as an
extracellular defense against free radicals in such
tissues as the lung, where approximately 50 percent
of the ascorbic acid is found in the fluid lining of
the respiratory epithelium in the rat (32) and the
guinea pig (33). Scorbutic guinea pigs have been shown
to maintain the extracellular pool of ascorbic acid in
the lung, and in fact, the percentage of extracellular
ascorbic acid increases from 57 percent in controls to
78 percent in the scorbutic guinea pig (33). Thus,

even in scurvy, a substantial proportion of the lung
vitamin C is found in the respiratory lining in spite
of an overall decrease in lung ascorbic acid content.
This is of considerable interest in that enzymatic
defense systems including SOD and glutathione per-
oxidase are found only intracellularly, and would not
be available for reaction with extracellular free
radicals or oxidants.

The toxicity of ozone and oxygen is due to pro-
motion of free radical reactions (34), and ascorbic
acid (as well as other antioxidants) has been shown
to protect against oxidant damage. Ascorbic acid was
found to be particularly effective against lung damage
in rats exposed to 4-5 atmospheres of oxygen, while
its effect on prolonging survival in mice exposed
to 5 atmospheres of oxygen was less striking (35).
Vitamin C also decreased mortality in mice exposed
to various concentrations of ozone, and was respon-
sible for a decrease in pulmonary edema which follows
such exposure (36).

Dietary ascorbic acid has also been shown to
reduce the extent of lipid peroxidation in both the
mouse (37) and the guinea pig (38). In the latter
study, dietary ascorbic acid increased the level of
vitamin E in the plasma of vitamin E-deficient guinea
pigs, which indicates an important nutritional inter-

relationship with respect to free radical quenchers.
Ascorbic acid has also been demonstrated to reduce
mortality due to carbon tetrachloride in the rat (15).
This is of considerable interest in that carbon tetra-
chloride is a well known free radical inducer, and
studies described herein explore the role of ascorbic
acid in carbon tetrachloride-induced lipid peroxidation.

A curious feature of the molecular biology of
ascorbic acid is that very low concentrations appear
to induce lipid peroxidation in brain and liver micro-
somes in vitro, while higher, physiologic concentrat-
ions prevent such peroxidation (39-41). The mechanism
of this effect is that ascorbic acid can reduce ferric
ion to the ferrous state, which can then catalyze
the decomposition of hydrogen peroxide to the hydroxyl
radical, as in the Haber-Weiss reaction (Figure 1).
The observed inhibition of lipid peroxidation at
higher concentrations of ascorbic acid is probably
due to its free radical quenching action. Despite
this in vitro induction of lipid peroxidation by
ascorbic acid, no evidence has been presented for
the production of free radicals by ascorbic acid
either in vivo or in vitro. Halliwell and Foyer (42)
have shown that the autooxidation of ascorbic acid
does not generate SOR. Reaction of ascorbic acid

with free radicals produces a partially oxidized form
of ascorbate known as the ascorbate free radical (AFR),
which may react with another radical to produce de-
hydroascorbate (Figure 3). It is theoretically
possible that AFR itself may initiate free radical
chain reactions: however, it has been demonstrated
that AFR is not reactive with a variety of intermed-
iary metabolites, does not react with oxygen, and
quenches itself extremely rapidly (43). Further,
an enzyme system has been isolated from rat liver
which catalyzes the reduction of AFR to ascorbic
acid (NADH: semidehydroascorbate oxidoreductase)
(Figure 3), which should further lessen the pos-
sibility of AFR-induced lipid peroxidation (44).
The complete oxidation of ascorbic acid to the
dehydroascorbate form results in the quenching of
two moles of free radicals per mole of ascorbate
oxidized. Reduced glutathione reacts directly
with dehydroascorbic acid and results in the for-
mation of ascorbic acid, which may provide an ef-
fective recycling system for ascorbic acid-mediated
free radical quenching (45). Ascorbic acid also
reacts directly with hydrogen peroxide, and may
be an ancillary mechanism to protect against toxicity
due to various hydroperoxides (46). Ascorbic acid
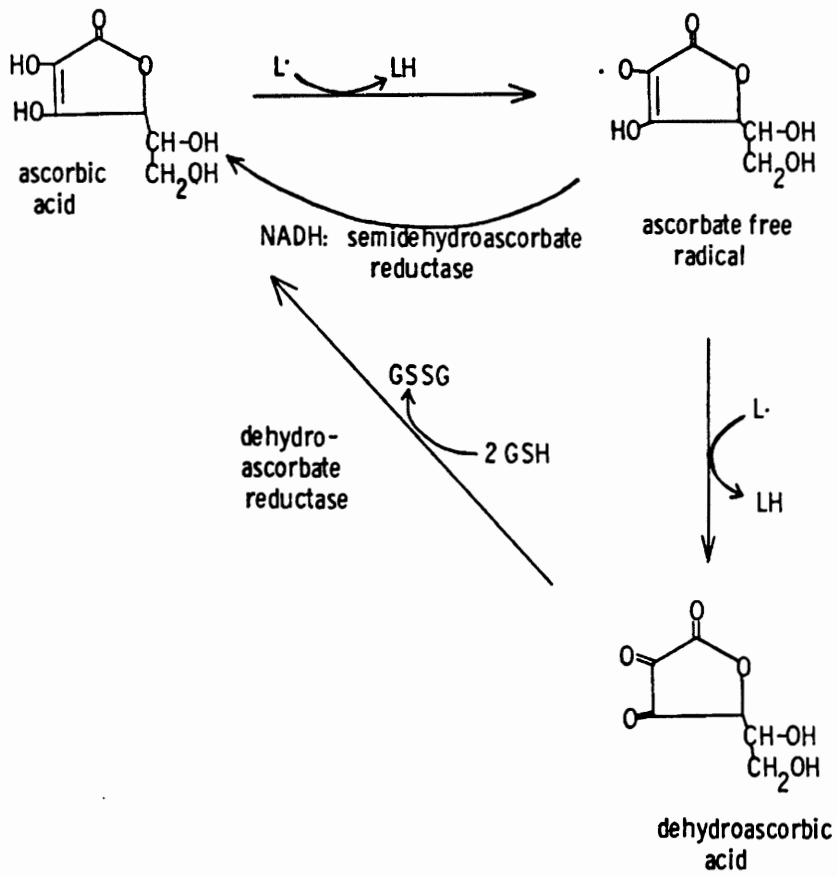
Figure 3. Ascorbic acid and free radical quenching.

appears, therefore, to be a molecule which may quench free radicals without leading to further free radical production, and may thus play a protective role against free radical reactions in vivo.

The ability of antioxidants to quench free radical reactions should, theoretically, lead to enhanced longevity, but to date very few invest-igations of this nature have been reported. Harman (47) first reported that synthetic antioxidants, when added to the diets of mice, were able to extend the life span. Addition of 2-mercaptoethylamine (2-MEA) (1.0%), cysteine HCl (1.0%), or 2,2'-di-aminodiethyldisulfide dihydrochloride (0.5%) in-creased the half survival time of AKR mice by ap-proximately 20%. In a subsequent study by Harman (48), antioxidants were shown to prolong the life span of both AKR and C3H mice. 2-MEA (1.0%) was very effective in this regard, and resulted in an increase of 26% in the mean life span of C3H mice. Hydroxylamine HCl showed a slight effect on long-evity, increasing the mean lifespan of C3H mice by about 7%. In the AKR strain, cysteine HCl (1.0%) and hydroxylamine HCl (1.0%) increased the half survival time by about 15%. Harman (49) has also reported that addition of synthetic antioxidants

to the diet of LAF_1 mice reduced the rate of mortality.
2-MEA resulted in a dose-dependent increase in mean
lifespan; at 0.5% an increase of 12.8% in the mean
lifespan was noted, and at 1.0% an increase of 29.3%
was observed. Butylated hydroxytoluene, at 0.5% of
the diet, increased the mean lifespan of LAF_1 mice
by 45%. A pilot study on the effect of ascorbic acid
on the lifespan of guinea pigs has been reported
by Davies et al. (50). Guinea pigs given 1.0% L-
ascorbic acid in the drinking water showed no increase
in mean lifespan compared to guinea pigs given 5 mg
ascorbic acid per kilogram body weight (by oral
intubation). These results are difficult to in-
terpret, however, since the high and low vitamin C
groups received ascorbic acid by different routes
of administration.

CHAPTER III

MATERIALS AND METHODS

MICE

Mice used in these studies were of the fol-
lowing strains: BALB/c, DBA/2, NZB, BALB/c x DBA/2
F_1 hybrids, and BALB/c x NZB F_1 hybrids. BALB/c and
DBA/2 breeding stocks were obtained from Jackson
Laboratory, Bar Harbor, Maine. NZB breeding stocks
were obtained in 1966 from the Otago University
Medical School, Dunnadin, New Zealand. All mice
used in these studies were bred in our own colony
from stocks described above.

DIETS

All mice were maintained on Purina Rodent chow
(Ralston-Purina Co., Saint Louis, Mo.), which was
given ad libitum. Mice not given vitamin C re-
ceived tap water ad libitum. Vitamin C-treated mice
received tap water containing 250 mg% L-ascorbic acid
ad libitum. This regimen of 250 mg% L-ascorbic acid
in the drinking water resulted in a daily intake of
about 400 mg ascorbic acid per kilogram body weight.
Ascorbic acid-containing water was stored in brown

bottles, and changed every other day in order to avoid oxidation.

CHEMICALS

2,4-dinitrophenylhydrazine was obtained from U.S. Biochemical Corporation, Cleveland, Ohio. Thiourea was obtained from Sigma Chemical Company, Saint Louis, Missouri. Cupric sulfate was purchased from Matheson, Coleman and Bell Company, Cincinnati, Ohio. Carbon tetrachloride was obtained from Malinckrodt Inc., Saint Louis, Missouri. All chemicals used in these studies were reagent grade.

PREPARATION OF REAGENTS

The 2,4-dinitrophenylhydrazine-thiourea-cupric sulfate (DTC) mix was prepared as follows: 1.00 g   of 2,4-dinitrophenyl hydrazine, 0.125 g   of thiourea, and 0.015 g   of cupric sulfate were added to 50 ml of 9 N H_2SO_4, stirred on a magnetic stirrer, and filtered through a sintered glass filter funnel. The DTC mix thus obtained was stored at 2° C, and was prepared every week. The 2,4-dinitrophenylhydrazine-thiourea (DT) mix was prepared as follows: 1.00 g   of 2,4-dinitrophenylhydrazine and 0.250 g   of thiourea was added to 50 ml of

9 N H_2SO_4, stirred with a magnetic stirrer, and fil-
tered through a sintered glass filter funnel. The
DT mix was stored at 2° C, and was prepared every
week. 65% sulfuric acid was prepared by adding
560 ml of concentrated sulfuric acid to 240 ml of dis-
tilled water. 9 N sulfuric acid was prepared by
adding 300 ml distilled water to 100 ml of con-
centrated sulfuric acid.

TISSUE PREPARATIONS

Blood was obtained by orbital sinus puncture,
allowed to clot at room temperature for 10 minutes,
and centrifuged at 2,000 rpm for 10 minutes in an
International centrifuge (model HN). Serum thus
obtained was diluted with 9 volumes cold 10% tri-
chloroacetic acid (TCA) and mixed thoroughly.
Tissues were removed immediately following ex-
sanguination, weighed on a Mettler H6 electronic
balance, and homogenized in 9, 19, 39, or 79 volumes
of cold 10% TCA, depending on the weight of the
tissue. Total volumes of TCA homogenates were be-
tween 3 and 8 ml. Samples were centifuged at 3,000
rpm for 10 minutes in a PR-2 International refriger-
ated centrifuge, and the supernatants pipetted into
a 13 x 100 mm borosilicate test tube. Aliquots of

the protein-free supernatant were used immediately for determination of oxidized and total vitamin C, as described below.

ASSAY OF TOTAL VITAMIN C

Total vitamin C levels were determined by the Lowry (51) modification of the method of Roe and Keuther (52). 400 μl of the protein-free supernatant was added to labeled 13 x 100 mm borosilicate test tubes, followed by addition of 130 μl of the DTC mix. All samples were run in triplicate. Tubes were vortexed, and incubated in a 37° C water bath for exactly four hours. Samples were then placed in an ice bath, and 670 μl of 65% sulfuric acid was added. Samples were vortexed, and color development allowed to proceed at room temperature for 45 minutes. Percent transmittance was recorded on a Beckman model B spectrophotometer at 520 nm. Blanks were prepared from 400 μl of 10% TCA plus all reagents; standards were prepared from 400 μl of a 0.2 mg/100 ml ascorbic acid solution plus all reagents. Ascorbic acid standards (1.0 mg/ml) were prepared in batch and frozen at -20° C, and on the day of assay, were diluted 1:500 (volume:volume) with 10% TCA, to yield the working standard (0.2 mg ascorbic acid/100 ml).

ASSAY OF OXIDIZED VITAMIN C

Oxidized vitamin C was determined essentially by the method of Stevenson and Brush (53). Samples were prepared exactly as for determination of total vitamin C, except that 130 μl of the DT mix was added instead of the DTC mix. The exclusion of cupric sulfate from the DT mix prevented the oxidation of the ascorbic acid, and therefore results obtained with this procedure yielded values of "oxidized" vitamin C (dehydroascorbic acid and 2,3-diketo-gulonic acid). Since ascorbic acid is easily ox-idized, even in 10% TCA, all samples to be assayed for oxidized vitamin C were incubated in the DT mix within 30 minutes after cervical dislocation.

CALCULATIONS

Percent transmittance was converted to ab-sorbance using standard tables from the Handbook of Analytical Chemistry. Both total and oxidized vitamin C levels were calculated in the following manner:

$$\frac{\text{Absorbance (unknown)}}{\text{Absorbance (standard)}} \times D = \text{mg/100 grams wet weight}$$

or mg/100 ml serum

Homogenization volumes of 9,19,39, and 79 required

use of 2, 4, 8, and 16 for the D factor, respectively.
Values were obtained for both total and oxidized
vitamin C, and reduced vitamin C levels were cal-
culated by subtraction.

STATISTICAL ANALYSIS

Analysis of the data obtained was performed
on a Monroe programmable computer, using the student
't' test. P values of greater than 0.05 were con-
sidered insignificant.

EXPERIMENTAL PROTOCOLS

Baseline Studies

DBA/2 and BALB/c mice were taken at various ages,
and their tissues and serum vitamin C levels deter-
mined as described above, in order to determine the
effect of age on tissue and serum vitamin C levels
in the mouse. Mice used for baseline studies were
not given supplemental ascorbic acid. Groups of from
3-11 mice were used.

In Vitro Oxidized/Reduced Ratio

In order to determine the equilibrium between
oxidized and reduced vitamin C in vitro, 25 mg asc-
orbic acid was dissolved in 100 ml of 0.1 M phosphate

buffer, pH 7.4, and the time course of its oxidation followed during incubation in a 37°C water bath. Total and oxidized vitamin C levels were assayed as described above.

In Vivo Oxidized/Reduced Ratio

These studies were designed in order to determine the effect of age on the ratio of oxidized/reduced vitamin C in tissues of mice not given supplemental ascorbic acid. Mice were sacrificed at various ages, as described in the results section, and tissues prepared and assayed for total and oxidized vitamin C as described above. Groups of 4 to 7 mice were utilized.

Effect Of Vitamin C On In Vivo Oxidized/Reduced Ratios

This experiment was carried out in order to determine the effect of supplemental ascorbic acid on the ratio of oxidized/reduced vitamin C in vivo. BALB/c female mice, 15 months of age, were employed in this study. The vitamin C-treated group were placed on a regimen of 250 mg% L-ascorbic acid in the drinking water at 3 months of age ; the control group received tap water. Serum and tissues were prepared and assayed for total and oxidized vitamin C as described above. Four mice were used in each group.

Time Course Of CCl_4-Induced Lipid Peroxidation

Carbon tetrachloride (CCl_4) is a well known lipid peroxidizer, and it was of interest to determine the time course of CCl_4-induced lipid peroxidation, as determined by the ratio of oxidized/ reduced vitamin C. For this experiment, BALB/c x DBA/2 F_1 male hybrids, 7 to 9 months of age, were employed. No supplemental ascorbic acid was given. Groups of 4 to 5 mice were injected intraperitoneally with 0.05 ml of CCl_4, and were sacrificed at 1, 2, and 3 days post-injection. Four mice served as controls, and were not treated with CCl_4. Liver, kidney, and spleen were removed following cervical dislocation, and total and oxidized vitamin C determined as described above. Spleen weights and hematocrits were also obtained since CCl_4 is known to effect these parameters.

Effect Of Ascorbic Acid On CCl_4-Induced Lipid Peroxidation

The effect of supplemental ascorbic acid on CCl_4-induced lipid peroxidation was determined using BALB/c x NZB F_1 males, 10 to 12 months of age. Fourteen control mice, and 14 vitamin C-treated mice, were injected with 0.05 ml CCl_4 intraperitoneally, and sacrificed two days post-injection. Vitamin C-treated

mice had been on the ascorbic acid regimen for 5 weeks prior to CCl_4 injection. Liver, kidney, and spleen were removed following cervical dislocation; and total and oxidized vitamin C determined as described above.

CHAPTER IV

RESULTS

EFFECT OF AGE ON TOTAL VITAMIN C LEVELS IN
BALB/c AND DBA/2 MICE

The effect of age on the levels of vitamin C in
serum, liver, and spleen for DBA/2 and BALB/c mice is
shown in Table I. In the DBA/2 strain, males as well
as females manifested depressed serum levels with ad-
vancing age, approximately 35% over a period of 27
months. BALB/c mice also displayed decreases in serum
vitamin C levels with advancing age, 41% over a 28
month period in females and 54% in males after 8
months, the last month available in our colony. In-
terestingly, both DBA/2 and BALB/c mice showed major
depressions of serum vitamin C levels during the first
two months of life, which parallels the decline in
liver vitamin C levels during this age period.
Another depression in serum vitamin C occurred in
DBA/2 females at 14 months of age and in BALB/c
females at 21 months. DBA/2 males continued to show
a relatively steep decline up to 8 months, mainly
leveling off thereafter.

TABLE I

EFFECT OF AGE ON TOTAL VITAMIN C LEVELS

IN TWO INBRED STRAINS OF MICE

Strain and sex	Age (mo.)	Number of mice	Vitamin C (mg/100 grams)±S.E.M. [a]		
			Serum	Liver	Spleen
DBA/2 females	1	11	2.78±0.06	35.0±1.40	28.8±1.21
	2	6	2.42±0.04	31.8±1.48	41.3±1.17
	4	4	2.86±0.06	20.7±0.32	38.1±1.36
	14	5	2.83±0.48	17.8±0.37	41.0±3.20
	21	5	2.09±0.09	15.8±0.58	40.6±1.24
	27	4	1.76±0.18	n.d. [b]	49.5±2.10
DBA/2 males	1	5	2.67±0.25	35.7±1.22	27.4±1.14
	2	4	2.30±0.04	22.8±1.98	34.7±1.40
	8	6	1.95±0.06	22.6±0.44	34.6±1.46
	9	3	1.83±0.21	23.0±0.09	n.d.
	13	4	n.d.	23.8±0.62	42.0±0.28
	27	4	1.76±0.18	22.8±0.49	44.4±2.44
	29	5	1.86±0.33	18.6±0.88	47.6±4.78
BALB/c females	1	7	2.41±0.06	35.5±1.03	31.7±1.03
	2	6	2.12±0.06	27.1±0.55	32.4±1.13
	16	8	2.07±0.09	27.2±1.14	45.6±1.95
	20	8	2.08±0.11	19.6±0.83	43.5±2.20
	23	9	1.39±0.06	19.9±0.99	44.4±0.98
	28	3	1.42±0.03	18.5±0.64	46.4±2.04
BALB/c males	1	6	3.25±0.16	27.4±1.33	29.3±0.93
	2	9	1.90±0.10	21.6±1.04	38.1±1.00
	6	5	1.83±0.20	22.0±2.06	39.6±1.45
	8	5	1.51±0.06	15.3±0.98	40.0±1.22

[a] Values expressed as mg vitamin C per 100 ml serum and mg vitamin
C per 100 grams wet weight of liver and spleen.

[b] Not determined.

Liver vitamin C levels were observed to decrease in both the DBA/2 and BALB/c strains with advancing age. DBA/2 females showed a 55% reduction in liver vitamin C over a period of 21 months, while DBA/2 males exhibited a 48% reduction in 29 months. Similar results were obtained with BALB/c mice, in which females showed a 48% reduction and males a 44% reduction over a period of 28 and 8 months, respectively. In both the DBA/2 and BALB/c strains, very high liver vitamin C levels of the order of 27-35 mg/100 grams wet weight of tissue were observed at 1 month of age, suggesting extensive synthesis of ascorbic acid.

In the case of the spleen there was marked accumulation of vitamin C with aging in the DBA/2 and BALB/c strains, the steepest rise occurring from 1 to 2 months of age. In the DBA/2 strain, females exhibited a 72% increase, and males a 74% increase, during 27 and 29 months of life, respectively. In the BALB/c strain, females showed a 46% increase, and males a 36% increase, during 28 and 8 months of life, respectively.

RATIO OF OXIDIZED/REDUCED VITAMIN C IN VITRO

The results of this experiment are shown in Figure 4. The ratio of oxidized/reduced vitamin C
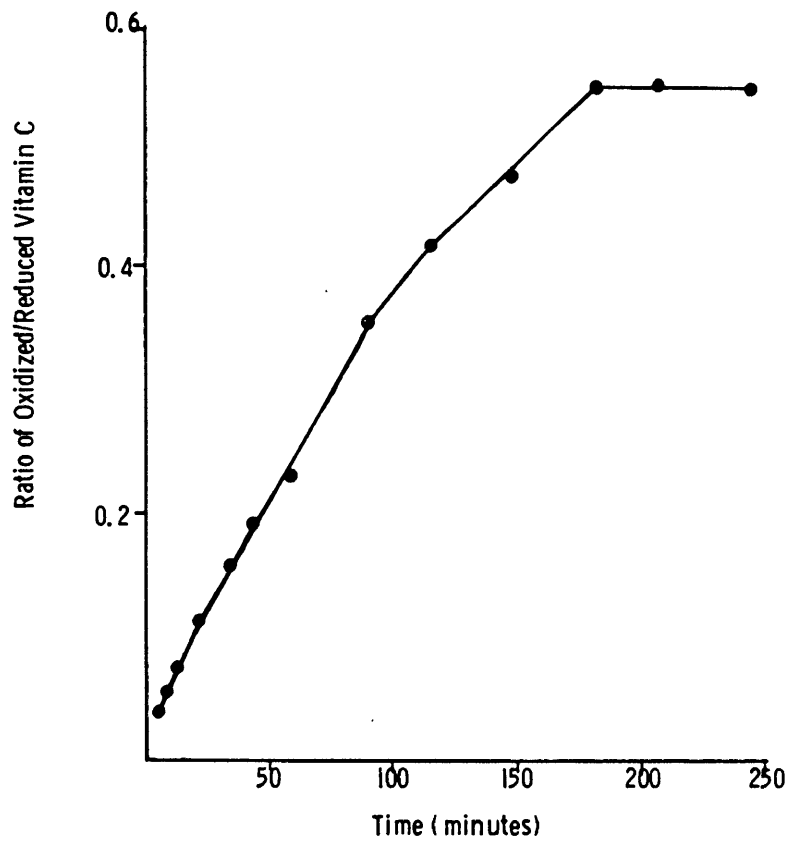
Figure 4.   Time course of the oxidation of ascorbic acid in vitro.

increased from a value of 0.05 at 1 minute to 0.56
at 180 minutes, after which there was no further
increase. These data demonstrate that ascorbic acid
is oxidized under simulated physiological condidtions
to an _in vitro_ equilibrium ratio of 1:2 (oxidized:
reduced). This _in vitro_ ratio will be used as a mar-
ker for _in vivo_ studies, in which the ratio is noted
to increase during aging and during carbon tetra-
chloride-induced lipid peroxidation.

EFFECT OF AGE ON THE RATIO OF OXIDIZED/
REDUCED VITAMIN C IN VIVO

DBA/2 males

The effect of age on the ratio of oxidized/
reduced vitamin C in DBA/2 males is shown in Table
II. In all tissues examined a significant increase
was observed in the ratio with advancing age in
liver, spleen, lung, and kidney. Marked increases
were observed in kidney oxidized/reduced ratios,
which rose from a 2 month value of 0.12 to a 17
month value of 0.42, an increase of 3.5 fold. It
is of interest that in the lung, the ratio increased
to a value greater than the _in vitro_ equilibrium ratio
described above, suggesting that the extent of lipid
peroxidation exceeds the capacity of the lung to

TABLE II

EFFECT OF AGE ON THE RATIO OF OXIDIZED/
REDUCED VITAMIN C IN DBA/2 MALES[a]

Age (mo.)	No. of mice	Ratio of Oxidized/Reduced Vitamin C ± S.E.M.			
		Liver	Spleen	Lung	Kidney
2	5	0.10 ± 0.01	0.33 ± 0.01	0.49 ± 0.01	0.12 ± 0.01
		$p < 0.001$	$p < 0.001$	$p < 0.025$	$p < 0.001$
17	5	0.19 ± 0.01	0.49 ± 0.01	0.65 ± 0.04	0.42 ± 0.02

[a] DBA/2 males, not given supplemental ascorbic acid, were maintained as described in the text. Tissues were removed and assayed for total and oxidized vitamin C as described in the text.

reduce dehydroascorbic acid to ascorbic acid.

BALB/c females

The effect of age on the ratio of oxidized/
reduced vitamin C in BALB/c females is shown in
Table III, for mice aged 1, 8, and 15 months. In
the liver, a small but significant increase was ob-
served between 1 and 8 months, and a marked increase
observed between 8 and 15 months. A four-fold in-
crease in the liver ratio was observed between 1 and
15 months of age. In the spleen, a significant in-
crease was observed between 1 and 8 months, after
which there was no further rise. The lung exhibited
a three-fold increase between 1 and 15 months, with
significant increases at all age periods studied.
The 8 and 15 month ratios of oxidized/reduced vitamin
C in the lung exceeded the in vitro equilibrium ratio,
suggesting extensive lipid peroxidation in vivo.
Values for the thymus did not change between 1 and
8 months, and the thymus tissue was not obtainable
from 15 month-old mice due to thymic involution. The
kidney exhibited marked increases at all age periods
examined, and showed a six-fold increase between 1
and 15 months of age.

TABLE III

EFFECT OF AGE ON THE RATIO OF OXIDIZED/REDUCED
VITAMIN C IN BALB/c FEMALES[a]

Age (mo.)	No. of mice	Ratio of Oxidized/Reduced Vitamin C ± S.E.M.				
		Liver	Spleen	Lung	Thymus	Kidney
1	4	0.12 ± 0.01	0.24 ± 0.03	0.36 ± 0.05	0.18 ± 0.04	0.08 ± 0.01
		$p < 0.005$	$p < 0.05$	$p < 0.001$	$p > 0.2$[b]	$p < 0.001$
8	5	0.16 ± 0.01	0.34 ± 0.02	0.69 ± 0.07	0.23 ± 0.02	0.28 ± 0.02
		$p < 0.001$	$p > 0.2$	$p < 0.001$	---	$p < 0.005$
15	4	0.47 ± 0.05	0.38 ± 0.02	0.96 ± 0.04	---	0.46 ± 0.04

[a] BALB/c females, not given supplemental ascorbic acid, were maintained as described in the text. Tissues were removed and assayed for total and oxidized vitamin C as described in the text.

[b] $p > 0.05$, and therefore insignificant.

COMPARISON OF OXIDIZED/REDUCED VITAMIN C RATIOS IN
4-5 WEEK OLD BALB/c AND NZB FEMALES

The ratios of oxidized/reduced vitamin C in tissues from .4 week old BALB/c females are compared to the ratios observed in 5 week old NZB females in Table IV. NZB females exhibited a significantly increased ratio in spleen, lung, and kidney, and a significantly decreased ratio in liver, when compared to age-matched BALB/c females.

EFFECT OF VITAMIN C-TREATMENT ON OXIDIZED/REDUCED
RATIOS IN 15 MONTH OLD BALB/c FEMALES

The effect of supplemental ascorbic acid on tissue oxidized/reduced vitamin C ratios in 15 month old BALB/c females is shown in Table V. Vitamin C-treated mice were placed on a regimen of 250 mg% L-ascorbic acid in the drinking water at 3 months of age, while controls recieved tap water. Vitamin C-treated mice exhibited significantly reduced ratios in serum, kidney, liver, spleen, and lung, while the ratio in heart was unaffected by such treatment.

TIME COURSE OF CCl_4-INDUCED LIPID PEROXIDATION

The time course of CCl_4-induced lipid peroxidation is shown in Figure 5 for BALB/c x DBA/2

TABLE IV

COMPARISON OF OXIDIZED/REDUCED VITAMIN C RATIOS IN

4 TO 5 WEEK OLD BALB/c AND NZB FEMALE MICE[a]

Group	Age (weeks)	No. of mice	Ratio of Oxidized/Reduced Vitamin C ± S.E.M.			
			Liver	Spleen	Lung	Kidney
BALB/c females	4	4	0.12 ± 0.01	0.24 ± 0.03	0.31 ± 0.02	0.08 ± 0.01
			$p < 0.001$	$p < 0.05$	$p < 0.025$	$p < 0.05$
NZB females	5	7	0.06 ± 0.01	0.34 ± 0.02	0.43 ± 0.02	0.16 ± 0.02

[a] BALB/c and NZB females, not given supplemental ascorbic acid, were maintained as described in the text. Tissues were removed and assayed for total and oxidized vitamin C as described in the text.

TABLE V

EFFECT OF VITAMIN C TREATMENT ON THE RATIO OF

OXIDIZED/REDUCED VITAMIN C IN

15 MONTH OLD BALB/c FEMALES[a]

Group	No. of mice	Ratio of Oxidized/Reduced Vitamin C ± S.E.M.					
		Serum	Heart	Kidney	Liver	Spleen	Lung
Control	4	1.06±0.11	0.58±0.06	0.46±0.04	0.47±0.05	0.38±0.02	0.96±0.04
		$p < 0.005$	$p > 0.5$[b]	$p < 0.025$	$p < 0.05$	$p < 0.05$	$p < 0.01$
Vitamin C-treated	4	0.56±0.01	0.58±0.01	0.31±0.03	0.30±0.03	0.30±0.03	0.78±0.01

[a] Female BALB/c mice were maintained as described in the text. Vitamin C-treated mice were placed on a regimen of 250 mg% L-ascorbic acid in the drinking water at 3 months of age; controls received tap water. At 15 months of age, all mice were sacrificed and tissues prepared and total and oxidized vitamin C assayed as described in the text.

[b] $P > 0.05$, and therefore insignificant.

F_1 males injected i.p. with a large dose of CCl_4 (0.05 ml/mouse). A presumptive estimate of lipid peroxidation was determined by the vitamin C ratio. In both the liver and the kidney, significant increases in the ratio were observed at 24 and 48 hours post-injection, with the peak ratio observed at 48 hours. By 72 hours post-injection, the ratios appeared to fall toward baseline values. In the spleen, however, no effect of CCl_4 was noted, as the ratio remained constant at about 0.44. Total vitamin C levels, spleen weights, and hematocrits for these CCl_4-treated mice are shown in Table VI. Liver vitamin C values, initially at 17.1 mg%, fell to 8.82 at 24 hours, and 6.19 at 48 hours, and then increased to 8.22 at 72 hours. Liver vitamin C levels in CCl_4-treated mice were significantly lower than controls at all times. Kidney values showed the same trends, that is, a drop in vitamin C levels occurred at 24 and 48 hours post-injection, with a rise toward baseline values at 72 hours. However, only the 48 hour value obtained statistical significance in the ease of the kidney. Spleen vitamin C levels were unaffected by CCl_4 treatment. Hematocrits were significantly increased, while spleen weights were significantly decreased, at all times following CCl_4 administration.
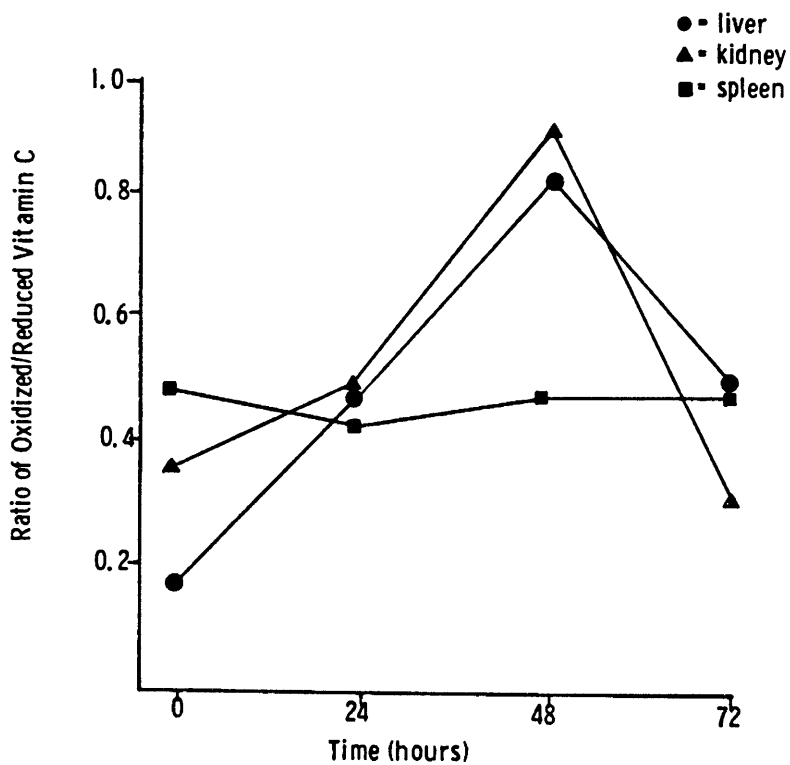
Figure 5. Time course of CCl_4-induced alteration in the oxidized/reduced vitamin C ratio in BALB/c x DBA/2 F_1 male hybrids.

TABLE VI

EFFECT OF CCl_4-TREATMENT ON TOTAL VITAMIN
C LEVELS, SPLEEN WEIGHTS, AND HEMATOCRITS
IN 7 TO 9 MONTH OLD BALB/c x DBA/2
MALE F_1 HYBRIDS[a]

Time post injection	No. of mice	Hct. ±S.E.M.	Spleen Wt. (g)±S.E.M.	Total Vitamin C (mg%)± S.E.M.		
				Liver	Kidney	Spleen
0 hours (control)	4	48.8 ±0.92	0.0980 ±0.0100	17.1±1.0	10.4±0.52	34.1±0.43
24 hours	5	61.5 ±2.66	0.0659 ±0.0070	8.82±1.87	9.63±0.81	35.2±2.23
48 hours	4	57.6 ±1.90	0.0557 ±0.0170	6.19±0.38	4.23±0.45	36.0±1.58
72 hours	4	61.0 ±2.04	0.0311 ±0.0054	8.22±0.26	10.3±0.64	33.7±1.83
Statistical Evaluation						
24 hours vs. controls		$p<0.005$	$p<0.025$	$p<0.025$	$p>0.5$	$p>0.5$
48 hours vs. controls		$p<0.005$	$p<0.025$	$p<0.001$	$p<0.001$	$p>0.4$
72 hours vs. controls		$p<0.005$	$p<0.005$	$p<0.001$	$p>0.5$	$p>0.5$

[a] BALB/c x DBA/2 F_1 males were maintained as described in the text, without supplemental ascorbic acid. Tissues were prepared and vitamin C assayed as described in the text.

EFFECT OF SUPPLEMENTAL ASCORBIC ACID ON CCl_4-INDUCED LIPID PEROXIDATION IN BALB/c x NZB F_1 MALE HYBRIDS

The effect of supplemental ascorbic acid (250 mg% in the drinking water) on CCl_4-induced lipid peroxidation in BALB/c x NZB F_1 males is shown in Table VII. Ascorbic acid treatment resulted in a significantly elevated level of vitamin C in the liver and kidney, but not in the spleen of these mice. Ascorbic acid treatment was observed to decrease the ratio of oxidized/reduced vitamin C in liver, kidney, and spleen, with a 50% reduction in the ratios observed.

TABLE VII

EFFECT OF ASCORBIC ACID TREATMENT ON TOTAL VITAMIN C LEVELS AND

RATIOS OF OXIDIZED/REDUCED VITAMIN C IN BAL3/c x NZB

F_1 MALES INJECTED WITH 0.05 ml OF CCl_4[a]

Group	No. of mice	Total Vitamin C (mg%)±S.E.M.			Ratio of Oxidized/Reduced Vit. C±S.E.M.		
		Liver	Kidney	Spleen	Liver	Kidney	Spleen
Control	9	7.16±0.53	8.16±0.49	34.0±1.74	0.59±0.04	0.56±0.05	0.60±0.04
		$p<0.001$	$p<0.005$	$p>0.2$[b]	$p<0.001$	$p<0.001$	$p<0.001$
Vitamin C-treated	7	13.4±0.32	10.9±0.66	36.6±1.61	0.25±0.01	0.30±0.01	0.32±0.01

[a] BALB/c x NZB F_1 males were maintained as described in the text. Vitamin C-treated mice were placed on a regimen of 250 mg% L-ascorbic acid in the drinking water 5 weeks prior to CCl_4 injection. Mice were injected with 0.05 ml CCl_4 i.p., and sacrificed two days post-injection for total and oxidized vitamin C determinations, as described in the text.

[b] $p>0.05$, and therefore insignificant.

CHAPTER V

DISCUSSION

AGE-RELATED CHANGES IN TOTAL VITAMIN C LEVELS

Aging in the mouse has been demonstrated to be
associated with a fall in serum and liver vitamin C
levels. These results are in agreement with those
obtained from human and guinea pig studies. In
humans, significant depressions have been observed
in plasma, platelet, leucocyte, and buffy coat
vitamin C levels with advancing age (54-60). Very
little information exists on variations in tissue
vitamin C levels with age in humans, however. Schaus
(61), in 1957, reported decreases in all tissues which
he examined, including some 70 specimens obtained
at autopsy. A depression of 49%, 77%, 33%, and 60%
was observed for pituitary, cerebral cortex, myocar-
dium, and pectoral muscle, respectively, between 1
and 80 years of age. Hughes and Jones (62) have in-
vestigated the effects of age on tissue vitamin C
levels in the guinea pig, and have found that older
animals showed significantly lower concentrations of
ascorbic acid in spleen, adrenal glands, and eye lens,

with elevated levels of ascorbic acid observed in brain.

The decrease in serum vitamin C parallels the drop in liver vitamin C, and it is likely that the serum levels merely reflect the rate of liver synthsis of ascorbic acid, since the serum is not a storage organ for vitamin C. The drop in the rate of synthesis of ascorbic acid (as evidenced by a decrease in liver vitamin C levels) is of considerable interest in that, in the unsupplemented mouse, the liver is the only source of vitamin C. It is possible that, with advancing age, the transcription of genes coding for enzymes involved with ascorbic acid synthesis declines. No evidence, however, has been presented to support this contention. Lipid peroxides have been shown to decrease the rate of ascorbic acid synthesis (63), suggesting that an increase in lipid peroxidation would shut off vitamin C synthesis. Since lipid peroxidation is known to increase with advancing age (19,20), it is very likely that the observed decrease in liver vitamin C levels is secondary to an increase in liver lipid peroxidation. A vicious cycle could then ensue, in which lipid peroxidation causes a decrease in the vitamin C biosynthetic rate, which reduces the amount of

ascorbic acid available for quenching free radical reactions, which would lead to enhanced lipid per-oxidation. This cycle of events may, in part, account for the observed increase in the accumulation of aging pigments with advancing age.

In the spleen, the marked accumulation of vita-min C observed in these studies may represent either a storage mechanism for vitamin C, or may represent an increased requirement of the spleen for vitamin C with advancing age. The spleen is a highly oxidative organ due to its content of phagocytes, which produce superoxide radical during intracellular killing, and it is possible that the storage of vitamin C observed in the mouse is in response to oxidative damage med-iated by neutrophils and macrophages. In either case, the spleen accumulates vitamin C in spite of a drop in liver and serum levels, indicating that an active transport system (or facilitated transport system), operating against a concentration gradient, must be functioning. These results are not in accord with those obtained in the guinea pig (62), in which a decrease in vitamin C was observed with age. These differences may reflect species specific variances in vitamin C sequestered by the spleen, or may simply reflect variances in the serum levels of vitamin C.

RELIABILITY OF THE RATIO OF OXIDIZED/REDUCED

VITAMIN C AS AN ASSAY FOR LIPID PEROXIDATION

The CCl_4 experiments were performed in order
to evaluate the reliability of the ratio of oxidized/
reduced vitamin C as an assay for lipid peroxidation.
CCl_4 was chosen because it is the most standard ex-
perimental technique for inducing lipid peroxidation
(64). CCl_4 is metabolized exclusively in organs
containing the microsomal cytochrome P-450 system,
and the initiating event has been suggested to be the
homolytic cleavage of the CCl_3-Cl bond, causing a
trichloromethyl radical ($CCl_3 \cdot$) to be formed (65).
This causes free radical-mediated lipid peroxidation
in the microsomal lipids surrounding cytochrome P-450,
with chain reactions leading to lipid peroxidation
in much of the liver cell membranes. Lipid per-
oxidation caused by CCl_4 causes an inhibition in
the secretion of lipoproteins and triglycerides,
which results in the characteristic fatty liver
of CCl_4-treated animals (66).

Lipid peroxidation should result in an increase
in the ratio of oxidized/reduced vitamin C by the
following mechanism:

lipid peroxides ⟩ ⟨ GSH ⟩ ⟨ dehydroascorbate
lipid alcohols ⟵ ⟶ GSSG ⟶ ⟵ ascorbic acid
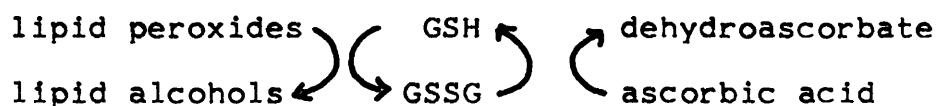
The observed increase in liver and kidney oxidized/
reduced ratios (Figure 5) suggests this mechanism,
and strongly suggests that this ratio may be useful
as an estimation of lipid peroxidation in vivo.
Since lipid peroxidation is known to increase with
advancing age (19,20), the determination of oxidized/
reduced vitamin C ratios may represent a novel es-
timation of aging.

At no time was the spleen ratio effected by
CCl_4 treatment. This is in accord with present
knowledge concerning the metabolism of CCl_4 in
that the spleen, lacking a cytochrome P-450 system,
is not involved in CCl_4 metabolism, and therefore no
change in the ratio of oxidized/reduced vitamin C is
observed. This tissue specificity demonstrates that
the CCl_4-induced increase in the ratio in liver and
kidney is not a result of non-specific toxicity, but
possibly of tissue specific free radical-induced
lipid peroxidation.

It is of interest that during CCl_4 treatment,
the kidney evidenced a dramatic increase in the
oxidized/reduced ratio at 24 hours post-injection,
despite no change in the total vitamin C concentration
at that time (Table VI). This finding indicates that
the change in the ratio is due to a drop in the level

of reduced vitamin C, which suggests that reduced vitamin C is either being utilized in order to quench free radicals produced by CCl_4, or is being utilized to reduce GSSG (oxidized glutathione), in order to accelerate the glutathione peroxidase-mediated lipid peroxide quenching activity. In either case, reduced vitamin C is being consumed as a defense against CCl_4-induced lipid peroxidation.

Total vitamin C decreases in liver and kidney (at 48 hours) suggesting metabolism of vitamin C beyond the 2,3-diketogulonic acid stage. The lack of effect of CCl_4 on spleen total vitamin C is further proof of tissue specificity. Of interest is the finding that, in spite of a marked drop in spleen weights (Table VI), spleen vitamin C concentrations remained constant. Previously, an inverse relationship between spleen weight and spleen vitamin C was found (67), and it was expected that, as the spleen weights decreased following CCl_4, the spleen vitamin C would increase. The finding that spleen vitamin C remained constant indicates a reduction in the amount of vitamin C per spleen, and this suggests that the spleen may be playing the role of a storage organ, capable of

donating its vitamin C to other organs when the need
arises.

These results, taken together, indicate that
the ratio of oxidized/reduced vitamin C is a sensitive
indicator of lipid peroxidation in vivo, and may
therefore be applied to other situations involving
lipid peroxidation. This novel estimation of lipid
peroxidation has technical advantages over existing
methods, which involve laborious histological ex-
aminations, electron microscopy, or extraction of
tissues with toxic organic solvents (68). The ratio
of oxidized/reduced vitamin C is a sensitive assay
(note standard error of the mean in Tables II, III,
IV,V, and VII), is reliable from day to day, does
not require use of organic solvents, and is relative-
ly easy to perform. This ratio has advantages over
existing methods in that it theoretically measures
both lipid peroxidation as well as cytosolic free
radical damage, whereas lipofuscin, ethane, pentane,
or malondialdehyde measure only lipid peroxidation,
and as has been mentioned, free radicals are produced
in the cytosol by numerous enzymes, drug auto-
oxidations, and x-irradiation. Further, the for-
mation of aging pigments reflects only oxidative
damage because of a lack of degradation of these

pigments in vivo, whereas the ratio of oxidized/
reduced vitamin C reflects both oxidative damage as
well as reductive capacity of the cell. The signifi-
cance of this lies in the fact that lipofuscin ac-
cumulation is a unidirectional process, while the
ratio of oxidized/reduced vitamin C is a steady-
state condition, which is more likely to reflect
the current extent of oxidative damage and repair
in the cell.

APPLICATION OF THE RATIO OF OXIDIZED/REDUCED
VITAMIN C TO IN VIVO AGING

Assuming that the ratio of oxidized to
reduced vitamin C reflects lipid peroxidation in a
model system, it was of interest to determine the
effect of age on in vivo ratios. These experiments
were carried out in two different strains of mice,
in order to be certain that the results were not
merely a strain anomaly. The results (Table II,
Table III) indicate an age-associated increase in
the ratio of oxidized/reduced vitamin C in all tis-
sues examined, except in the thymus, and except in
the spleen of BALB/c females between 8 and 15 months
of age. The increase in the ratio was often dramatic:
the liver ratio role 4 fold, and the kidney ratio 6

fold, in BALB/c females between 1 and 15 months of age.

It is of interest that organs exposed to the most oxidative damage yielded the highest ratios. Thus the lung, exposed to chronic high oxygen tensions, showed the highest ratios of oxidized/reduced vitamin C in all tissues examined,with the exception of serum. Lung ratios frequently exceeded the _in vitro_ equilibrium ratio of 0.55, suggesting that lipid peroxidation was proceeding to a greater extent than the reductive capacity of the lung. For example, a ratio of 0.96 was observed for lung tissue from 15 month old BALB/c females, which is well above the _in vitro_ equilibrium ratio.

Serum oxidized/reduced ratios were the highest observed for any tissue (Table 5), demonstrating that vitamin C transported in the serum is approximately 50% in the oxidized form. Thus, tissues are exposed to a large amount of oxidized vitamin C, which is the form of vitamin C absorbed preferentially by such cells as leucocytes and erythrocytes (69). The high serum ratios do not reflect free radical activity, but rather a lack of reductive capacity of this tissue.

An organ exhibiting high oxidized/reduced

ratios is the spleen, which is exposed to oxidative damage due to superoxide radical production during intracellular killing in macrophages and granulocytes. Spleen values were generally higher than those observed in liver, kidney, or thymus, but did not exceed the in vitro equilibrium ratio of 0.55. The lack of increase in the spleen ratio of BALB/c females between 8 and 15 months is very important, because during this period the spleen accumulates vitamin C dramatically (Table I). It is possible that the increased vitamin C content of the spleen at this age period provides protection against free radical-induced lipid peroxidation.

Kidney and liver oxidized/reduced ratios, initially low at 1 to 2 months of age, increased dramatically with advancing age, particularly with regard to the kidney. However, ratios did not exceed the in vitro equilibrium ratio of 0.55, suggesting that cellular reductive capacity was at least equivalent to cellular oxidative damage.

Of interest is the observation that ratios of oxidized/reduced vitamin C in heart tissue (Table V) were greater than the in vitro equilibrium ratio, suggesting extensive lipid peroxidation in that organ. This is in agreement with present knowledge

on lipid peroxidation in cardiac muscle, in that
heart contains the highest number of mitochondria
per cell of any organ, and accumulates aging pigments
to a greater extent than any other organ except brain
and testes (68).

Thymus is the only organ which did not show
age-associated changes in the oxidized/reduced ratio.
This is likely due to the low level of oxidative
processes which occur in the thymus. T-lympho-
cytes do not produce much superoxide radical, and
there are no phagocytes located in the thymus. Thus,
the ratio of oxidized/reduced vitamin C may be an
indicator of cellular aging only in those tissues
which exhibit oxidative metabolism, or are exposed
to oxygen or to superoxide radical.

Finally, the ratios of oxidized/reduced vitamin
C in the lung are higher than published values for
the ratio of oxidized/reduced glutathione in the
lung of the rat (70), which was observed to be 0.06.
This would indicate that the ascorbic acid-dehydro-
ascorbic acid system is a backup redox system for a
more important function; namely the GSSG/GSH ratio, which
regulates enzymatic activities, genetic transcription,
and the enzyme glutathione peroxidase. It would be
of interest to determine age-related alterations in

the ratio of GSH/GSSG in animal tissues in order to determine the relationship between the GSH/GSSG system and the oxidized/reduced vitamin C ratio.

Further work should extend these studies on the effect of age on the ratio of oxidized/reduced vitamin C to other species, including humans. It is possible that in the future, an assay for aging based on this ratio may be routinely applied to human cells, such as leucocytes, which are readily available and which store vitamin C to a much greater extent than does serum.

THE NZB MOUSE AS A MODEL FOR RAPID AGING

A comparison of the ratios of oxidized/reduced vitamin C between NZB and BALB/c female mice (Table IV) demonstrates significant increases in the ratio in the spleen, lung, and kidney of NZB females. In the liver, however, NZB's exhibited a significantly lower ratio than their BALB/c counterparts. These results suggest that lipid peroxidation is occurring at an elevated rate in NZB tissues (except for liver), which is of considerable interest in that the NZB mouse is a model for rapid aging and autoimmune disease. The mean lifespan of NZB mice in our laboratory is about 14 months, which is half that for mice of

the BALB/c strain, and it is conceivable that the accelerated aging in the NZB mouse is caused by an elevated rate of oxidative damage. In this regard, we have previously shown that NZB mice between 16 and 20 months of age have significantly lower vitamin C levels in spleen, liver, and serum compared to either BALB/c or DBA/2 mice (67). Further, NZB mice do not accumulate vitamin C in their spleens with advancing age (67), as was demonstrated for BALB/c and DBA/2 mice (Table I). These data, taken together, indicate that lowered vitamin C levels in the NZB may be, in part, responsible for the accelerated aging phenomenon, and that the ratio of oxidized/reduced vitamin C appears to reflect this rapid aging. The decrease in liver oxidized/reduced ratios, which would suggest a lower rate of lipid peroxidation, is not in accord with data obtained for the other organs. The reason for this discrepancy is currently unknown.

EFFECT OF ASCORBIC ACID-TREATMENT ON LIPID PEROXIDATION DURING AGING AND DURING CCl_4 ADMINISTRATION

Assuming that the ratio of oxidized to reduced vitamin C is a reliable indicator of lipid

peroxidation (and therefore aging), it was of interest to examine the possible protective effect of ascorbic acid against age- and CCl_4-induced increases in this parameter. With regard to aging, the experiment was designed in order to evaluate the effect of long-term ascorbic acid treatment (13 months out of 15 months of life) on in vivo lipid peroxidation. These results (Table V) indicate that ascorbic acid treatment reduced the extent of oxidative damage in all organs examined except the heart. These data suggest that the aging process may be retarded by dietary antioxidant treatment, as has been amply demonstrated for vitamin E and selenium, as well as various synthetic antioxidants, including butylated hydroxytoluene and 2-mercaptoethylamine (see Chapter II). The lack of effect of ascorbic acid treatment on ratios in heart may be due to the low storage capacity of the heart for vitamin C. Zannoni (71) has found that heart contains the lowest amount of vitamin C of any of the 10 organs studied, with the exception of plasma. Spleen, for example, contains 9 times the amount of vitamin C found in heart (71). Since lipid peroxidation has been implicated as a risk factor in coronary heart

disease (72), the value of dietary ascorbic acid supplements in the management of this condition appears dubious.

One potential criticism of this protocol is that dietary ascorbic acid may artificially effect the ratio of oxidized/reduced vitamin C. In depth examination, however, reveals that this criticism is not of significance. In the serum, which supplies the organs with vitamin C, a significant fraction of the vitamin C is in the oxidized form, and in fact, in the serum of animals not supplemented with ascorbic acid, the ratio of oxidized/reduced vitamin C is about 1:1. Even during ascorbic acid supplementation, the ratio is still high, about 1:2, indicating that tissues are receiving a relatively large amount of oxidized vitamin C. Further, it has been demonstrated that oxidized vdtamin C is preferentially taken up by such cells as lymphocytes, granulocytes, and erythrocytes (69, 73), rather than the reduced form. Inside these cell types, the amount of reduced vitamin C depends on the activity of the enzyme dehydroascorbate reductase (73). It is of interest that the ability of granulocytes to withstand ionizing radiation has been attributed to their high levels of dehydroascorbate

uptake and reduction (73), when compared to the radiosensitive lymphocyte. Also of interest is the fact that, despite our precautions with the preparation and storage of vitamin C-containing water, a considerable proportion (about 30%) of the ascorbic acid is oxidized before it is consumed by the mice. It is unlikely, therefore, that the ascorbic acid treatment should artifactually lower the ratio of oxidized/reduced vitamin C. However, as an added precaution, experiments are now in progress to determine the effect of selenium and vitamin E supplements on the ratio of oxidized/reduced vitamin C in vivo, since any decrease in the ratio due to selenium or vitamin E treatment is definitely a result of a decrease in lipid peroxidation (both selenium and vitamin E have been observed to reduce lipid peroxidation in numerous animal studies).

The results of this aging study, if they are confirmed, present the possibility of a reduction in the rate of aging by dietary antioxidants, which has tremendous implications for human nutrition. The ultimate proof would reside in studies on the effect of vitamin C on longevity. These studies are similar in nature to those discussed in Chapter II, in which

an extension of the mean life span was observed for mice fed synthetic antioxidants.

With regard to CCl_4 intoxication, administration of ascorbic acid resulted in a significant protective effect against CCl_4-induced lipid peroxidation (Table VII). Ascorbic acid treatment reduced the extent of lipid peroxidation by approximately 50% in both liver and kidney. The spleen ratio was also reduced dramatically by ascorbic acid treatment, but since it was previously determined that the spleen was unaffected by CCl_4, this decreased ratio probably reflects the reduction in age-associated lipid peroxidation in the spleen, as was shown for the spleen of BALB/c females given long-term supplements of ascorbic acid (Table V). It is of great interest that even though the total vitamin C levels in the spleen were not affected by ascorbic acid treatment, the ratios of oxidized/reduced vitamin C decreased dramatically (Table VII), indicating that the ratio is not merely reflective of the total vitamin C concentration of the organ, but rather reflective of the extent of oxidative damage.

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