1990

Free radicals and the etiology of colon cancer

Charles F. Babbs
Purdue University, babbs@purdue.edu

FREE RADICALS AND THE ETIOLOGY OF COLON CANCER

Charles F. Babbs, M.D., Ph.D.

Biomedical Engineering Center, Purdue University, West Lafayette, Indiana, USA.

(Free Radical Biology & Medicine, Vol. 8, pp. 191-200, 1990)

Abstract

This hypothesis paper reviews diverse evidence suggesting that intracolonic production of oxygen radicals may play a role in carcinogenesis. The hypothesis began to evolve when the author made the chance discovery that 1/10,000 dilutions of feces generated detectable quantities of highly reactive hydroxyl radicals (HO). The rate of HO formation, detected using DMSO as a molecular probe, was quite remarkable, corresponding to that which would be produced by over 10,000 rads of gamma irradiation per day, absorbed in the periphery of the fecal mass adjacent to the mucosa. The relatively high concentrations of iron in feces, together with the ability of bile pigments to act as iron chelators that support Fenton chemistry, may very well permit efficient HO generation from superoxide and hydrogen peroxide produced by bacterial metabolism. Such free radical generation in feces could provide a missing link in our understanding of the etiology of colon cancer: the oxidation of procarcinogens either by fecal HO, or by secondary peroxyl radicals (ROO) to form active carcinogens or mitogenic tumor promotors. Intracolonic free radical formation may explain the high incidence of cancer in the colon and rectum, compared to other regions of the GI tract, as well as the observed correlations of a higher incidence of colon cancer with red meat in the diet, which increases stool iron, and with excessive fat in the diet, which may increase the fecal content of procarcinogens and bile pigments.

Key words: Benzo(a)pyrene, Bile salts, Bilirubin, Fenton reaction, Free radicals, Carcinogenesis, Colon carcinoma, Iron, Hydroxyl radical, Superoxide

Supported by a Focused Giving Grant from Johnson & Johnson.

OVERVIEW OF THE HYPOTHESIS AND RELEVANT CHEMICAL MECHANISMS

My intention in this hypothesis paper is to present a new hypothesis suggesting how oxygen derived free radicals, generated in fecal material next to colonic epithelium, may play a significant role in the etiology of colon cancer. This hypothesis derives from an earlier conjecture by Graf and Eaton [1], coupled with the serendipitous discovery in our laboratory that highly reactive HO radicals can be produced abundantly by suspensions of feces under aerobic conditions. As do many other hypotheses for the pathogenic effects of oxygen centered free radicals in biologic systems[2-6], this one also invokes the superoxide driven Fenton reaction:

$$\text{biologic processes} \longrightarrow O_2^{\cdot-}$$

$$O_2^{\cdot-} + Fe^{+++} \longrightarrow O_2 + Fe^{++}$$

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

In feces a ready biologic source of superoxide is the respiratory activity of bacteria [7-10], notably E. coli [11], perhaps together with spontaneous autoxidation of ferrous iron chelates that have been previously reduced by metabolites of the anerobic subpopulation of fecal flora. Another intriguing source of colonic superoxide is the lipoxygenase activity of normal or sloughed colonic epithelial cells [12]. The necessary iron is provided from the diet, since only a small fraction of dietary iron is absorbed in upper GI tract [13, 14]. Hydroxyl (HO) radicals so formed within the colon could easily trigger a variety of carcinogenic mechanisms. For example, they could participate in aromatic hydroxylation reactions [15,16] to form carcinogenic products [17]; they could abstract hydrogen atoms from indoles to form radicals that subsequently dimerize to produce potential carcinogens [18]; or they could initiate lipid peroxidation leading to formation of lipid hydroperoxides (LOOH) and lipid alcohols (LOH), which have been shown by Bull and coworkers [19, 20] to stimulate DNA synthesis and cell proliferation in colonic epithelium.

In terms of specific chemical mechanisms, however, the work of Marnett and coworkers at Wayne State University, provides the most fascinating possibility. Their research demonstrates, in the case of benzo(a)- pyrene (BP), that free radical oxidations mimic those produced by the cytochrome P450 system in liver, which is classically known to produce active carcinogens from originally less toxic substrates [17, 21-23]. In particular, the metabolic activation of benzo-(a)pyrene hydrodiol to its ultimate carcinogenic form occurs by epoxidation to form the diolepoxide. The resultant diolepoxides are then believed to act as ultimate carcinogens through DNA adduct formation.

Marnett and coworkers, using elegant stereochemical analyses to separate enzymatic from non-enzymatic oxidations, have shown that such epoxidation reactions can and do occur by a free radical mechanism, in which lipid hydroperoxides in the presence of as little as 0.5 μM ferrous iron act as the epoxidizing agents [22-26]. The key step in this novel pathway for activation of polycyclic aromatic hydrocarbons is the nonenzymatic epoxidation of isolated double bonds by lipid peroxyl radicals (LOO). The mechanism is well precedented in the chemical literature and is known to occur effectively at temperatures between 30 ° and 60 °C and at pO_2's as low as 10

mmHg [27, 28]. In addition to polycyclic aromatic hydrocarbons like benzo(a)pyrene, aromatic amines may also be oxidized to mutagenic derivatives by peroxyl radicals [29]. The literature is indeed replete with suggestions that one electron oxidations and epoxidations of procarcinogens by free radical mechanisms can, under certain circumstances, lead to their activation to proximate carcinogenic forms [17].

Free radical mechanisms may represent a significant pathway for metabolic activation of carcinogens in the intracolonic environment in particular. The required unsaturated lipids may derive from dietary sources, from the turnover of epithelial cells, or, as suggested by at least one report [30], from synthesis by fecal microorganisms and/or epithelial cells. Also relevant to the intracolonic environment is the requirement for detergent stabilization of the hydrophobic substrate, such as BP, as well as unsaturated fatty acids derived from dietary or other sources. Marnett used 50 to 100 μM Tween 20 [25]. In the colon, however, the abundance of bile acid salts, such as the salts of cholic acid and chenodeoxycholic acid, could easily provide a similar effect.

As will now be discussed, the necessary conditions may well prevail in the colon, especially near the surfaces of fecal masses containing the right amount of oxygen, the right temperature (30 °C to 60 °C), superoxide producing microorganisms, abundant residual iron from a high iron diet, bile pigments to act as Fenton-promoting iron chelators, organic compounds to form peroxyl radicals, and detergent-like bile salts to emulsify procarcinogenic aromatic compounds and unsaturated lipids.

EVOLUTION OF THE HYPOTHESIS AND RELEVANT PHYSIOLOGICAL MECHANISMS

Our own laboratory has been generously funded by the U.S. Public Health Service to study ischemia/reperfusion injury in myocardium and in cardiac arrest and resuscitation. In this research we have been able to develop a simple and effective means to detect hydroxyl radicals in biologic systems by trapping them with dimethyl sulfoxide (DMSO) and measuring the primary, non-radical product of the trapping reaction, methane sulfinic acid (MSA) [32]. Searching for productive applications of this new method, we tested without success the ability of colon bacteria, obtained from feces, to stimulate free radical production in rat lung when the bacteria were injected into the pulmonary circulation. As a control, we also tested the ability of the dilute fecal suspension alone to produce radicals, and found a small but characteristic signal of hydroxyl radical production.

After ignoring this small signal for weeks, the author suddenly realized its potential biological importance. If highly dilute (1:10,000) feces really do produce these powerful oxidizing radicals, then concentrated feces--especially the well oxygenated periphery of the fecal stream in contact with the colon mucosa--may be the site of free radical oxidations of heretofore unrecognized pathological importance. Carcinoma is the most significant disease of the colon. and although fecal HO radicals would likely react before they could diffuse into epithelial cells, secondary products of hydroxyl radical-initiated free radical reactions could readily prove to be potent

initiators or promotors of carcinogenisis, explaining the high incidence of cancer in the colon compared to other parts of the GI tract.

Fecal iron

The availability of suitably chelated iron is absolutely essential for the formation of HO by the superoxide driven Fenton reaction [33, 34], and it is noteworthy that fecal iron concentrations are substantially higher than iron concentrations in most living systems--including solid tissues and ordinary bacterial cultures. Iron is concentrated in feces because about 90 to 95% of dietary iron is not absorbed, owing to the well known "mucosal block" mechanism [13, 35]. To protect against iron accumulation in the body, which can lead to chronic toxicity (i.e., hemo-chromatosis), iron absorption across the epithelium of the small bowel is limited in adult humans by a saturable carrier protein to about 1 mg of elemental iron per day (maximally, 3 mg/day in anemic states) [36]. Normally an equal amount of iron is lost through shedding of skin surface cells and intestinal epithelial cells, thus maintaining iron balance. The latter process, of course, returns iron to the fecal stream. As a consequence, the iron per unit volume of feces near the output of the GI tract is substantially greater than the amount of dietary iron per unit volume at the input, and also substantially greater than that usually present elsewhere in living systems.

Simple calculations are revealing. Consider a diet containing foods of moderate to high iron content (e.g., sirloin steak 3 mg/100g, spinach 3 mg/100g, liver 12 mg/100 g, kidney beans 7 mg/100 g),37 in which 10 mg of iron are consumed per day. Normal GI iron absorption is only about 1 mg/day, leaving 9 mg/day residue. Assuming a stool volume of 0.5 liters/day, one would expect fecal iron to be approximately (9 mg Fe)/(0.5 L) × (1 mmole)/(56 mg) = 0.32 mM, a value 10-fold greater than that in most tissues, and more than adequate to promote the superoxide driven Fenton reaction, if the iron is appropriately chelated [33, 38].

In living tissues levels of "free" or "low molecular weight, chelate" iron, capable of supporting Fenton chemistry, in the plasma or in tissues are low [39, 40], owing to the presence of iron binding proteins such a transferrin and ferritin. Transferrin, in particular, is able to bind iron tightly and in such a way that it does not participate in the superoxide driven Fenton reaction. Such antioxidant, iron binding proteins that were present in the diet initially are very likely to have been degraded and digested in the upper GI tract by the acidity of the stomach and by digestive enzymes such as pepsin and trypsin, thus making less complex forms of iron available to participate in free radical reactions in the colon.

Role of bile pigments

Although the role of bile acids in colon carcinogenesis has been much discussed [41-44], little attention has yet been paid to bile pigments. Feces are rich in bile pigments, such as bilirubin, biliverdin, and urobilinogen. These compounds, which are derived from iron binding heme pigments in red blood cells, give feces their characteristic brown color. The heme of hemoglobin is degraded within the body by microsomal heme oxidase, which splits the tetrapyrrole ring in the alpha position to give biliverdin, carbon monoxide, and free iron [45]. Biliverdin is reduced to bilirubin, from which multiple secondary products are derived, including urobilinogen, urobilin, stercobilinogen, and stercobilin. In all such compounds the structure of the opened

tetrapyrrole ring, including the four nitrogens that may serve as potential iron chelation sites, is maintained. Although these bile pigments have been proposed to function as chain-breaking antioxidants [46, 47], as will be subsequently shown, they are also excellent iron chelators of the type that both keep iron soluble at near neutral pH and also promote, rather than inhibit, Fenton chemistry.

Role of dicarboxylic amino acids

Other potential low molecular weight chelators of iron that may be quite abundant in the GI tract are the dicarboxylic amino acids, aspartate and glutamate. Deighton and Hider [48] have isolated oxotriiron complexes of these amino acids (e.g., $Fe_3O[glu/asp]_6$) from rat liver, in which the ratio of glutamate to aspartate is typically 7:2. Such dicarboxylic acid complexes with iron may support Fenton chemistry within the intestinal tract with an efficacy similar to that demonstrated in vitro for iron chelates of EDTA, nitrilotriacetate, and citrate [38]. Moreover, it is easy to imagine how amino acid-iron complexes could be formed in the upper GI-tract during the digestive process, as proteases simultaneously break down high molecular weight iron species and liberate free amino acids. Assuming that the resultant amino acid chelates of iron remain in the lumen of the GI-tract, they would naturally be carried downstream and become concentrated in the colon.

Integrated free radical hypothesis

Upon reflection, therefore, it is evident that these biochemical and physiological realities make the feces an especially likely place for free radical chemistry to occur. The availability of suitably chelated iron in high concentrations is coupled with the ability of both aerobic and anerobic bacteria to produce superoxide and hydrogen peroxide [7-10]. Superoxide production by the colonic mucosa itself (exclusive of leukocytes) has been shown to be a potent promotor of mitogenic activity in animal models [12]. Moreover, the metabolic activity of numerous anerobic species in fecal flora is known to generate reductants, such as H_2S, that may tend to keep iron in the ferrous state. Such reduced iron can then readily react with any hydrogen peroxide produced by microaerophilic species to generate significant numbers of hydroxyl radicals by Fenton's reaction in the moderately or weakly aerobic regions of bulk fecal material.

Although feces are usually considered a predominantly anerobic environment [49], aerobic and microaerophilic species, such as E. Coli, thrive. The mucosa is sufficiently well oxygenated to maintain the viability of the colonic epithelium, and the spading and mixing actions of colonic smooth muscle contractions continually bring the mucosa into close contact with fecal material. Accordingly, it is not unreasonable to conclude that the relatively low average levels of oxygen present in feces are nonetheless sufficient to support oxygen radical generation. These insights have led to the following working hypothesis that

1. Dietary iron, liberated from macromolecules during digestion and chelated by bile pigments or amino acids, becomes concentrated in the fecal stream.

2. In the predominantly anerobic environment of the colon a significant fraction of fecal iron is reduced and maintained in the ferrous state.

3. Aerobic or microaerophilic fecal microorganisms in the relatively well oxygenated periphery of fecal masses generate superoxide and hydrogen peroxide. Moreover, additional superoxide and hydrogen peroxide may derive from the lipoxygenase activity of epithelial cells themselves or simply from the autoxidation of reduced iron:

$$[Fe^{+2}A^{-n}] + O_2 \rightarrow [Fe^{+3}A^{-n}] + O_2^{\cdot -},$$

where A^{-n} is a low molecular weight chelator anion.

4. These conditions lead to hydroxyl radical formation via the superoxide driven Fenton reaction.

5. Hydroxyl radicals generated by the superoxide driven Fenton reaction initiate oxidative chain reactions involving unsaturated lipids and dietary pro-carcinogens, which transform a fraction of these species into active carcinogens or tumor promotors, capable of inducing neoplasia and/or stimulating cell proliferation in the colonic epithelium.

To determine if the free radical aspects of this hypothesis are at all meritorious, we conducted some simple preliminary experiments, which are now reported.

PRELIMINARY TESTS OF THE HYPOTHESIS

A key to credibility of the present hypothesis is the confirmation of hydroxyl radical generation in feces. Highly active radical species like HO are difficult to detect, and especially difficult to quantify, owing to their exceedingly short life times, on the order of nanoseconds [5]. Our approach has been to "trap" HO radicals by allowing them to react with the molecular probe, dimethyl sulfoxide (DMSO), to form a stable, nonradical product that can be measured spectrophotometrically.

Because many other molecules that are present in bacterial suspensions or other biological systems compete with DMSO as targets for HO attack [50, 51] the DMSO concentration must be relatively high, on the order of 0. 1 to 1.0 M [32, 52, 53] to trap the major portion of HO radicals generated and thus estimate their true abundance. Fortunately, DMSO is an extremely nontoxic [54, 55] and rapidly penetrating molecular probe that can be tolerated by living tissues in adequately high concentrations [54, 56-60]. It also reacts very rapidly with HO to give a single set of initial products:

$$CH_3-SO-CH_3 + HO\cdot \longrightarrow CH_3-SOOH + CH_3\cdot$$

DMSO	hydroxyl radical	methane sulfinic acid	methyl radical

including a chemically distinctive and stable compound, methane sulfinic acid (MSA), that is not normally present in biological specimens. The colorimetric assay [31] for methane sulfinic acid in biological materials that we used to demonstrate HO generation in fecal materials also produces a similarly shaped absorbance peaks at 420 nm when DMSO is oxidized by known sources of HO radicals. These standard sources of HO include gamma irradiated water [65] Fenton's reaction [65], and UV irradiated hydrogen peroxide [66-67].

Figure 1 shows the visible absorption spectra from a preliminary experiment, in which a 1:100 suspension of rat feces in TRIS Ringer buffer containing 5% (0.7 M) DMSO was incubated for 16 h at 37°C. The upper curve is a spectrum of authentic methane sulfinic acid (30 nanomoles), illustrating the characteristic peak at 420 nm. The middle curve illustrates the spectrum for the fecal suspension. The lower curve is that of a control fecal suspension treated with the iron chelator, deferoxamine. These results are consistent with the interpretation that fecal microorganisms can oxidize DMSO in an iron dependent manner to make methane sulfinic acid. By comparing the absorbance at 420 nm of assayed fecal suspension with that of authentic standard methane sulfinic acid, the apparent cumulative HO generation by the dilute 1:100 fecal suspension was calculated to be approximately 17 nmol/ml in 16 h, or about 1700 nmol per gram of starting fecal material.

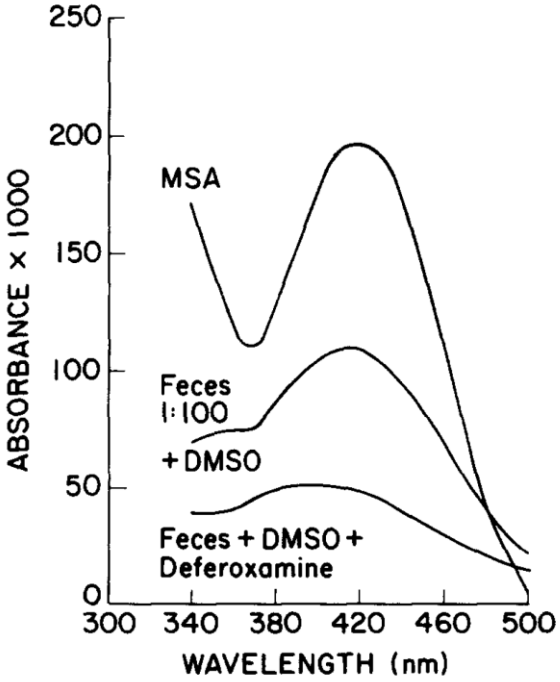

Fig. 1. Visible absorption spectra from a preliminary experiment in which a 1:100 suspension of rat feces in 40 mM TRIS Ringer buffer containing 5% (0.7 M) DMSO was incubated for 16 h at 37°C. The upper curve is a control spectrum of authentic methane sulfinic acid (30 nanomoles). Middle curve illustrates the spectrum for the fecal suspension. Lower curve is that of a control fecal suspension treated with the iron chelator, deferoxamine. Assay procedure described in [31] and [90].

Because TRIS buffer is a known scavenger of HO, tests were also performed to examine the effects of TRIS ($k = 1.5 \times 10^9$ $M^{-1}sec^{-1}$) [68], if any, on the trapping efficiency by DMSO ($k = 7 \times 10^9$ $M^{-1}sec^{-1}$) [51] in the presence of TRIS. In the absence of fecal suspension or antioxidant enzymes, HO generation in the xanthine oxidase/EDTA-iron test system in the presence of 700 mM DMSO was stable and uninfluenced by the presence of TRIS buffer (Table 1).

Table 1. Effect of Added TRIS Buffer on HO Trapping by DMSO Indicated by Colorimetric Assay for Methane Sulfinic Acid (31,32,90) at 425 nm

Tris-HCl (mM)	Abs_{425}	SD
0.2	0.684	0.042
0.5	0.684	0.071
1.0	0.701	0.064
2.0	0.694	0.053
10.0	0.674	0.061
20.0	0.670	0.028
30.0	0.651	0.032

Dilute fecal suspensions are far more convenient for study in the laboratory than undiluted bulk feces, and experimental conditions, such as pH, nutrient concentration, pO_2, etc. in fecal suspensions are much easier and better controlled. However, bacterial growth and metabolism are also likely to be much greater in dilute media (i.e., log phase growth) than in solid feces. Accordingly, we suspected that the HO radical production by the fecal suspensions might over-estimate that in undiluted, bulk feces.

Figure 2 presents mean values for 15 experiments with fecal suspensions, incubated overnight (14 to 17 h), without added iron at dilutions ranging from 1:10 (feces:medium) to $1:10^4$. As expected, the more dilute suspensions produced more HO radicals per gram of starting material than did the more concentrated ones. However, it is nonetheless possible to estimate the value that would be expected in undiluted feces on the log-log plot by extrapolation to the y-axis intercept. This value for Figure 2 corresponds to a cumulative HO generation of 213 nmol/g for the 16 h incubation period in bulk feces, assuming that similar substrate and oxygen availability were to prevail. Such conditions might well be approximated near the fecal surface in contact with blood perfused colon mucosa.

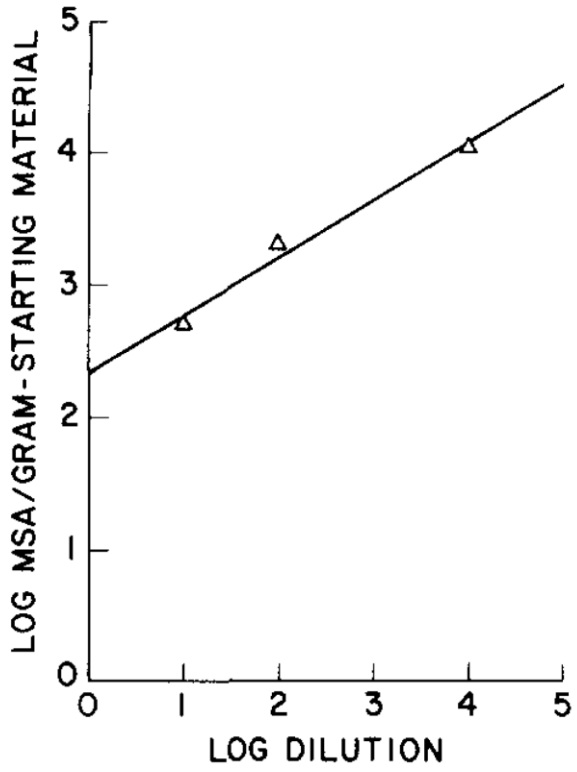

Fig. 2. Mean values for 15 experiments with fecal suspensions incubated overnight (14 to 17 h) at 37 °C in a shaking water bath in the presence of 5% DMSO without added iron at dilutions ranging from 1:10 (feces : medium) to 1:10^4. Please note log scales. Extrapolated value at zero dilution on the log-log plot represents that expected for bulk feces. Other details similar to Figure 1.

This estimated HO generation rate for bulk feces would be biologically significant, as can be appreciated by calculating the dose of gamma irradiation required to generate the same number of HO radicals per unit volume during the radiolysis of water. The number of nascent hydroxyl radicals per unit volume of irradiated water is given by the expression

number of HO radicals = radiation dose × G_{HO} ,

where G_{HO} is the radiochemical yield of hydroxyl radicals, a known constant equal to 2.9 molecules per 100 electron volts of absorbed energy [69]. After converting units (using 100 rads = 1 joule/kg, 1 eV = 1.602×10^{-19} joules, etc.) one obtains the expression for the net yield of hydroxyl radicals in water irradiated by a given dose of high energy gamma rays as

cumulative HO (pmol/g) = 3.01 × dose (rads).

Thus, a yield of 213 nanomoles of hydroxyl radicals per gram (213,000 pmol/g) is equivalent to that caused by a radiation dose of 213,000 / 3.01 or approximately 70,000 rads. For comparison, a dose of gamma irradiation sufficient to kill 97% of cells grown in tissue culture is on the order of 10,000 rads [70] Hence, the numbers of hydroxyl radicals that can be generated in feces would thus appear to be chemically and pathologically significant. The value of 213 nmol/g is about 10 times the largest value we have measured in ischemic and reoxygenated liver or kidney tissue [65], and from our perspective represents a prodigious capacity of colon bacteria to generate oxygen radicals.

To further test the validity of this interpretation, my colleagues and I performed a number of additional experiments with fecal suspensions. To determine if fecal microorganisms oxidize or otherwise degrade sulfinic acid, we measured recovery of standard methane sulfinic acid from fecal suspensions after short and long incubation times. There was minimal degradation of standard MSA by fecal microorganisms (77 ± 6% recovery at 4 h; 71 ± 12% recovery at 16 h). In subsequent experiments, we measured the time course of MSA production by 1:100 suspensions of fecal material (Table 2). When the HO generation rate is computed from the initial slope of accumulated MSA as a function of time, the value obtained from linear regression analysis is 3.74 nmol per ml of 1:100 suspension per hour (r = 0.984), or 374 nmol/g bulk feces. This steady, initial rate of HO generation is abolished in previously autoclaved feces, emphasizing the role of live fecal organisms in producing active oxygen species.

Table 2. Time Course of Cumulative Hydroxyl Radical Production by 1 : 100 Suspensions of Rat Feces in the Presence of 50 /IM Iron--EDTA and 100 mg/dl Glucose

Incubation Time (hr)	Absorbance at 420 nm	Cumulative HO· generation* (nmol MSA/ml)
0	0.061	10.7
1	0.090	15.8
2	0.106	18.7
3	0.112	19.7
4	0.147	25.9
5	0.144	25.4
6	0.181	31.9
7	0.217	38.2
8	0.238	42.0

*Regression line: HO = 10.4 + 3.74 t, $r^2 = 0.97$

Figure 3 compares HO radical production in a standard laboratory model of the superoxide driven Fenton reaction (xanthine oxidase + hypoxanthine) with that of a 1:100 dilution of feces in the presence of varying amounts of EDTA chelated iron (1:1). In the presence of adequate amounts of suitably chelated iron, both fecal microorganisms (lower curve) and the standard

xanthine oxidase system (upper curve) appear to generate sufficient superoxide to drive the iron-catalyzed Fenton reaction to produce HO radicals, as measured by DMSO oxidation. The xanthine oxidase system is a most convenient and reliable source of superoxide for laboratory work and is far more effective in promoting Fenton chemistry than most biological systems that we have tested. The observation that simple dilutions of feces can be as nearly effective as pure xanthine oxidase and hypoxanthine in generating large numbers of oxygen radicals suggests to us that fecal oxygen radical production is a rather robust phenomenon.

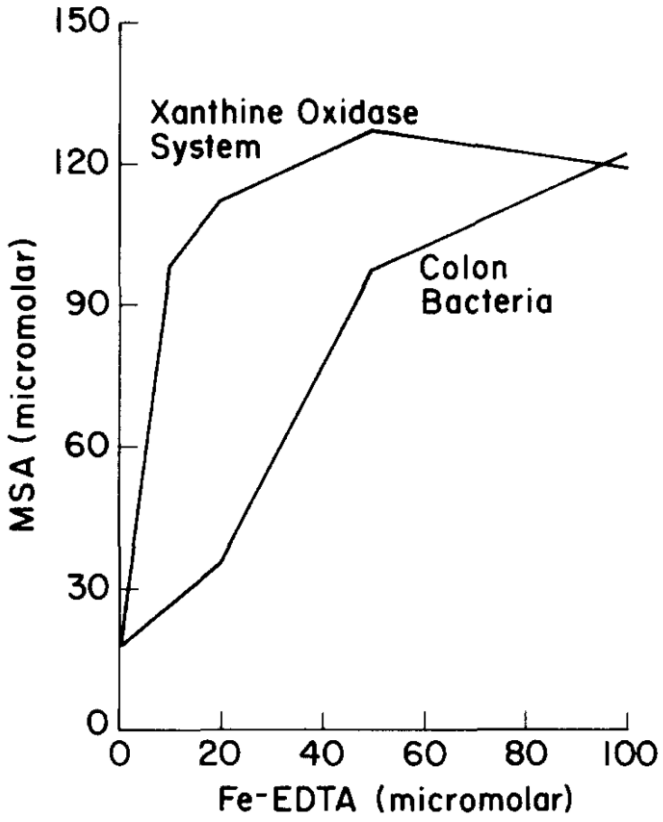

Fig. 3. HO radical production in a standard laboratory model of the superoxide driven Fenton reaction (xanthine oxidase 48 mU/ml, hypoxanthine 100 μM, Fe--EDTA 0 to 100 μM, and DMSO 5%) compared with that of a 1:100 dilution of feces in 5% DMSO

The EDTA:Fe (1:1) complex utilized in the xanthine oxidase system is most effective in promoting Fenton's reaction in vitro, but EDTA is not present in normal feces. We wondered, therefore, whether bile pigments would substitute as suitable Fenton-promoting chelators. This issue is important because HO radical production by Fenton chemistry at near neutral pH is highly dependent on the nature of the iron chelator [33, 71, 72]. Some iron chelates, such as Fe--EDTA 1:1 support Fenton chemistry readily [71]. Others such as deferoxamine-- Fe^{+++} do not [34]. Ferric iron itself is very poorly soluble in aqueous solutions above pH 3, the ferric iron--

hydroxide solubility product being 1.1 x 10^{-36} [73]. Even though feces tend to be acidic with respect to most body fluids, fecal iron must nonetheless be chelated to participate in Fenton chemistry.

Figure 4 compares the effects of various chelators in our standard xanthine oxidase test system. Each curve represents HO production by the xanthine oxidase test system as a source of superoxide in the presence of increasing concentrations of iron and 100 μM of the chelator. Clearly, both the absolute amount of iron and the nature of the chelator are important. Iron in phosphate buffer supports little HO formation, while same amount of iron chelated to EDTA or to bile pigments permits the production of abundant hydroxyl radicals in the xanthine oxidase system.

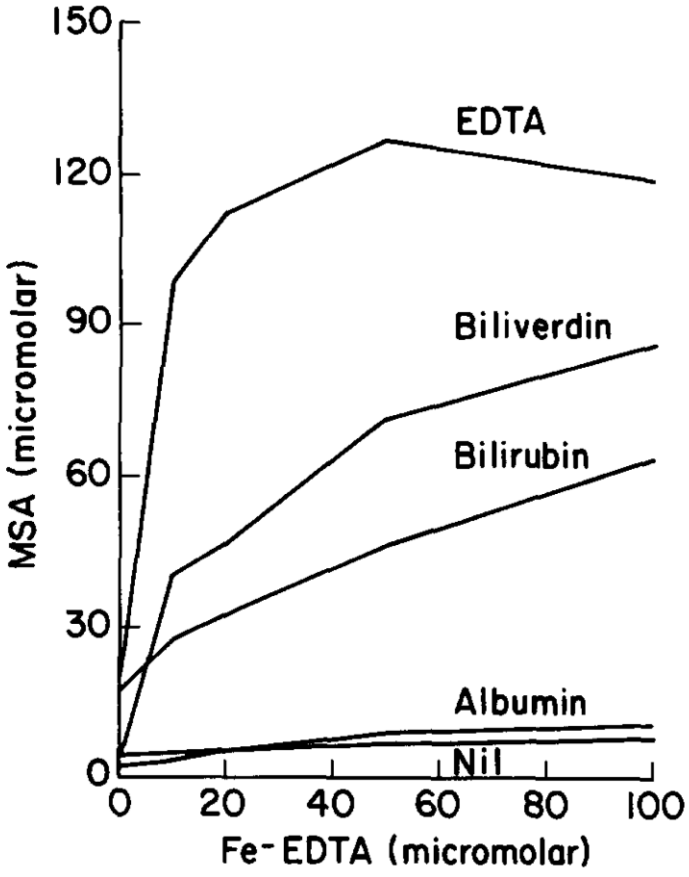

Fig. 4. HO radical production using the DMSO trapping technique by the standard xanthine oxidase test system (as in Fig. 3) in the presence of iron and various chelators. Each curve represents HO production by the xanthine oxidase test system as a source of superoxide in the presence of increasing concentrations of iron and 100 μM of the chelator. Nil indicates phosphate buffer only.

Of particular interest in the present context are results obtained with the hemoglobin breakdown products, bilirubin and biliverdin, which are abundant in feces as glucuronic acid conjugated bile pigments. Iron is released from hemoglobin during its initial catabolism in reticulo-endothelial organs and retained in the body complexed with ferritin, while the heme moieties are excreted in the form of bile pigments. Bilirubin and biliverdin, however, retain the ability to chelate iron, and the resulting chelates appear to be quite capable of supporting Fenton chemistry. The presence of these lyso-porphyrin chelates of iron in the colon may make the intracolonic environment particularly congenial to formation of HO radicals by the superoxide driven, iron catalyzed Fenton reaction.

Having obtained initial supportive evidence for the relevant chemistry, we have begun to develop a simple biological model to test and extend the hypothesis of free radical mediated activation of procarcinogens by colonic bacteria. Fecal suspensions are placed inside a bag of dialysis tubing. The surface of the bag thus simulates the epithelial surface of the colon, and the contents of the bag simulate the contents of the colon. The bag and its contents are in turn placed in a flask containing Salmonella tester strains utilized in the Ames mutagenesis assay. Bacteria in the two compartments are adequately separated by the dialysis membrane (in the absence of leaks) so that there is no cross contamination.

The Ames test [74] is based upon the principle that there is a high correlation between chemical carcinogenicity and chemical mutagenicity, since both mutagens and carcinogens must be capable of interacting with and modifying DNA. In the classical Ames test Salmonella typhimurium mutants, incapable of synthesizing histidine, are exposed to a potential mutagen and grown on histidine deficient media. If the test compound is a mutagen, some of the microorganisms will back-mutate to the wild type and grow rapidly. The number of such viable microorganisms is a rough indicator of the mutagenicity of the test compound. Compounds positive in this test can then be further studied as putative carcinogens.

In the dialysis bag model, Ames tester strains are inoculated into Oxoid nutrient broth No. 2, which surrounds the dialysis bag containing the colon bacili. The rationale of the model is that the genetic material of the tester bacteria simulates that of the colonic epithelial cells. The bag and flask are incubated overnight at 37 °C in a shaking water bath. Experimental conditions, such as iron concentration, oxygen content of the headspace gas, or the concentration of antioxidant drugs can be easily varied. After incubation the dialysis bag is discarded and the tester strain bacteria are separated by centrifugation, resuspended in fresh, low histidine media, added to top agar, and plated in the usual way for the Ames plate incorporation assay (without S-9 activator) [74]. Results are expressed in terms of revertants per plate, which form visible colonies that can be counted to quantify the results.

We have begun to study this simple biological test system, using the amino acid, tryptophan, as a prototype procarcinogen, based upon the early work of Dunning, Bryan, Pipkin, Nishimura, and their respective coworkers [75-78] suggesting that tryptophan may be oxidized to mutagenic products, perhaps by free radical mechanisms. Very preliminary work in progress with this model, using Salmonella tester strain TA-102, suggests that a roughly 10-fold greater number of revertants per plate are found in a complete system including dialysis bags filled with feces,

EDTA-iron, bile, tryptophan, and glucose, compared with control systems minus iron or minus feces. When the test is performed with autoclaved feces, counts are not different from background. This relatively simple and inexpensive model may prove valuable in further exploring the role of free radicals in colon carcinogenesis.

IMPLICATIONS OF THE HYPOTHESIS

The free radical hypothesis proposed herein, if proved correct, may provide a useful missing link in our knowledge of the etiology of colon cancer, complementing, and not refuting, other theories. It is consistent with many established facts of the epidemiology of the disease [79-82] including increased incidence of colon cancer in people who

1. ingest a high meat diet (which provides more iron),

2. ingest a high fat diet (which provides more procarcinogens and stimulates bile secretion),

3. ingest a low fiber diet (which allows for greater fecal concentration of both iron, procarcinogens, oxidizable lipids, and bile salts), or

4. suffer from chronic ulcerative colitis (which provides peri-mucosal superoxide from activated leukocytes, heme iron from chronic bleeding, and which also may dictate supplementation of the diet with iron to correct for chronic blood loss and consequent iron deficiency anemia).

The free radical hypothesis is also consistent with the demonstrated decreased incidence of colon cancer in animals fed antioxidants such as vitamin E, vitamin A, butylated hydroxy anisole (BHA), butylated hydroxy toluene (BHT), ascorbic acid, and selenium [79, 83-88]. The report [89] of the ability of phytic acid (inositol phosphate), to reduce the number and volume of azoxymethane induced colon tumors, as well as the mitotic activity of colonic crypts in rats, is also explained by the concept of intraluminal Fenton chemistry. Although phytic acid is a putative HO radical scavenger, present in high fiber diets, it may also chelate ferric iron, rendering it inert in the superoxide driven Fenton reaction and thereby inhibiting iron mediated HO formation [89]. This point was originally made by Graf and Eaton [1], who may have been the first to propose a connection between hydroxyl radicals and colon carcinogenesis.

In these ways, then, the free radical hypothesis is a unifying concept, which explains and relates a number of diverse observations in epidemiology and in clinical and laboratory medicine. In addition to being of academic interest, however, the free radical hypothesis, if proved correct, would also seem to have significant public health implications. These include the desirability of achievable and practical measures to reduce the risk of colon cancer, such as

1. attempting to screen high-risk patients by stool analysis to determine the relative propensity of the feces to produce oxygen radicals,

2. recommending a lower iron diet and fewer iron supplements in adult men and postmenopausal women, who are at greater risk of developing colon cancer and do not have high physiologic requirements for iron,

3. developing and testing of non-absorbable antioxidants such as Vitamin E-like polymers or phytic acid, capable of quenching free radical reactions in the feces of high risk patients,

4. recommending a diet low in foods containing procarcinogens identified by subsequent research to be specifically susceptible to activation by free radical mechanisms, and

5. attempting to colonize the colons of high risk patients with genetically engineered, antioxidant-producing bacteria.

These potential practical implications of the free radical hypothesis, which focus on prevention, would seem to lend considerable public health significance to the further investigation of free radical mechanisms in the etiology of colon cancer.

Acknowledgement--The cheerful and competent technical assistance of Ms. Joanne C. Cusumano and Ms. Allison Morrison is gratefully acknowledged.

REFERENCES

1. Graf, E.; Eaton, J. W. Dietary suppression of colonic cancer-- fiber or phytate? Cancer 56:717-718; 1985.

2. Bulkley, G. B. The role of oxygen free radicals in human disease processes. Surgery 94:407-411; 1983.

3. Babbs, C. F. Role of iron ions in the genesis of reperfusion injury following successful cardiopulmonary resuscitation: preliminary data and a biochemical hypothesis. Annals Emerg. Med. 14:777-783; 1985.

4. McCord, J. M. Oxygen-derived free radicals in postischemic tissue injury. New Engl. J. of Med. 312:159-163; 1985.

5. Slater, T. F. Free radical mechanisms in tissue injury. London: Pion Limited; 1972.

6. Halliwell, B.; Gutteridge, J. M. C. Oxygen toxicity, oxygen radicals, transition metals and disease. Biochem. J. 219:1 14: 1984.

7. Stephenson, M. Bacterial metabolism. 2nd ed. London: Longmans, Green, and Co.; 1939.

8. Davis, B. D.; Dulbecco, R.; Eisen, H. N.; and Ginsberg, H. S. Microbiology. 3rd ed. Philadelphia: Harper and Row; 1980.

9. Pandalai, N. G. A textbook of microbiology. New York: Asia Publishing House; 1961.

10. Joklik, W. K.; Willett, H. P.; Amos, D. B. Zinsser microbiology. 18th ed. Norwalk, CT: Appleton-Century-Crofts; 1984.

11. Hassan, H. M,; Fridovich, I. Paraquat and Escherichia coil: mechanism of production of extracellular superoxide radical. J. of Biologic. Chem. 254:10846-10852; 1979.

12. Craven, P. A.; Pfanstiel, J.; DeRubertis, F. R. Role of reactive oxygen in bile salt stimulation of colonic epithelial proliferation. J. Clin. Invest. 77:850-859; 1986.

13. Spiro, T. G.; Saltman, P. Inorganic chemistry. In: Jacobs. A.; Worwood, M., eds. Iron in biochemistry and medicine. New York: Academic Press; 1974, 1-28.

14. Henry, J. B.; Clinical diagnosis and management by laboratory methods. 16th ed. Philadelphia: W.B. Saunders Company; 1987:294-298.

15. Grootveld, M.; Halliwell, B. Aromatic hydroxylation as a potential measure of hydroxyl radical formation in vivo. Biochem. J. 237:499-504; 1986.

16. Richmond, R.; Halliwell, B.; Chauhan, J.; Darbre, A. Superoxide dependent formation of hydroxyl radicals: detection of hydroxylation of aromatic compounds. Analyt. Biochem. 118:328-335; 1981.

17. Cavalieri, E.; Rogan, E.; Roth, R. Multiple Mechanisms of Activation in Aromatic Hydrocarbon Carcinogenesis. In: Floyd, R. A., ed. Free radicals and cancer. New York: Marcel Dekker; 1982.

18. Osawa, T.; Namiki, M.; Suzuki, K.; Mitsuoka, T. Mutagen formation by intestinal bacteria. Mutat. Res. 122:299-304; 1983.

19. Bull, A. W.; Nigro, N. D.; Golembieski, W. A.; Crissman, J. D.; Marnett, L. J. In vivo stimulation of DNA synthesis and induction of orinithine decarboxylase in rat colon by fatty acid hydroperoxides, autoxidation products of unsaturated fatty acids. Cancer Res. 44:4924-4928; 1984.

20. Bull, A. W.; Nigro, N. D.; Marnett, L. J. Structural requirements for stimulation of colonic cell proliferation by oxidized fatty acids. Cancer Res. 48:1771-1776; 1988.

21. Nagata, C.; Kodama, M.; loki, Y.; Kimura, T. Free radicals produced from chemical carcinogens and their significance in carcinogenesis. In: Floyd, R. A., ed. Free radicals and cancer. New York: Marcel Dekker; 1982.

22. Dix, T. A.; Marnett, L. J. Metabolism of polycyclic aromatic hydrocarbon derivatives to ultimate carcinogens during lipid peroxidation. Science 221:77-79; 1983.

23. Marnett, L. J.; Hydroperoxide-dependent oxygenation of polycyclic aromatic hydrocarbons and their metabolites. In: Harvey, R. G., ed. Polycyclic hydrocarbons and carcinogenesis. Washington DC: American Chemical Society; 1985:307- 326.

24. Marnett, L. J.; Reed, G. A.; Dennison, D. J.; Prostaglandin synthetase dependent activation of 7,8-dihydro-7,8-dihydroxybenzo(a)pyrene to mutagenic derivatives. Biochem. Biophys. Res. Commun. 82:210-216; 1978.

25. Dix, T. A.; Marnett, L. J. Free radical epoxidation of 7,8- dihydroxy-7,8-dihydrobenzo[a]pyrene by hematin and polyunsaturated fatty acid hydroperoxides. J. Am. Chem. Soc. 103:6744-6746; 1981.

26. Eling, T.; Curtis, J.; Battista, J.; Marnett, L. J. Oxidation of ±7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene by mouse keratinocytes: evidence for peroxyl radical- and monoxygenase- dependent metabolism. Carcinogenesis 7:1957-1963; 1986.

27. Walling, C. Free radicals in solution. New York: John Wiley & Sons; 1957.

28. Mayo, F. R. Free-radical autoxidations of hydrocarbons. Accounts Chem. Res. 1:193-201; 1968.

29. Robertson, I. G. C.; Sivarajah, K.; Eling, T. E.; Zeiger, E. Activation of some aromatic amines to mutagenic products by prostaglandin endoperoxide synthetase. Cancer Res. 43:476-480; 1983.

30. Sammons, H. G.; Vaughan, D. J.; Frazer, A. C. Synthesis of long-chain fats by bacteria isolated from human feces. Nature 177:237; 1956.

31. Babbs, C. F.; Gale, M. J. Colorimetric assay for methanesulfinic acid in biological samples. Anal. Biochem. 163:67-73; 1987.

32. Babbs, C. F.; Griffin, D. W. Scatchard analysis of methane sulfinic acid production from dimethyl sulfoxide: a method to quantify hydroxyl radical formation in physiologic systems. Free Radical Biol. Med. 6:493-503; 1989.

33. Aust, S. D.; Morehouse, L. A.; Thomas, C. E. Hypothesis paper--role of metals in oxygen radical reactions. J. Free Radicals Biol. Med. 1:3-25; 1985.

34. Gutteridge, J. M. C.; Richmond, R.; Halliwell, B. Inhibition of the iron-catalyzed formation of hydroxyl radicals from superoxide and lipid peroxidation by desferrioxamine. Biochem. J. 184:469-472; 1979.

35. Guyton, A. C. Textbook of medical physiology. 6th ed. Philadelphia: W. B. Saunders Co.; 1981:61-64.

36. Wheby, M. S. Disorders of iron metabolism, in: Thorup, O. A., ed. Leavell and Thorup's fundamentals of clinical hematology. 5th ed. Philadelphia: W. B. Saunders; 1987:212-250.

37. Diem, K., eds. Documenta Geigy scientific tables. 6th ed. Ardsley, NY: Geigy Pharmaceuticals; 1962.

38. Smith, J. B.; Cusumano, J. C.; Babbs, C. F. Quantitative effects of iron chelators on hydroxyl radical production by the superoxide- driven Fenton reaction. Free Radical Res. Commun. 8:101-106, 1990.

39. Krause, G. S.; Joyce, K. M.; Nayini, N. R.; Zonia, C. L.; Garritano, A. M.; Hoehner, T. J.; Evans, A. T.; Indrieri, R. J.; Huang, R. R.; Aust, S. D.; White, B. C. Cardiac arrest and resuscitation: brain iron delocalization during reperfusion. Ann. Emerg. Med. 14:1037-1043; 1985.

40. Holt, S.; Gunderson, M.; Joyce, K.; Nayini, N. R.; Eyster, G. F.; Garitano, A. M.; Zonia, C.; Krause, G. S.; Aust, S. D.; White, B. C. Myocardial tissue iron delocalization and evidence for lipid peroxidation after two hours of ischemia. Annals Emerg. Med. 15:1155-1159; 1986.

41. Joossens, J. V.; Hill, M. J.; Geboers, J. Mechanisms of colorectal carcinogenesis. In: Diet and human carcinogenesis. Amsterdam, New York, Oxford: Excerpta Medica; 1985.

42. Bruce, W. R. Recent hypotheses for the origin of colon cancer. Cancer Res. 47:4237-4242; 1987.

43. Wargovich, M. J.; Eng, V. W. S.; Newmark, H. L.; Bruce, W. R. Calcium ameliorates the toxic effect of deoxycholic acid on colonic epithelium. Carcinogenesis 4:1205-1207; 1983.

44. Reddy, B. S.; Wynder, E. L. Large-bowl carcinogenesis: fecal constituents of populations with diverse incidence rates of colon cancer. J. Natl. Cancer Inst. 50:1437-1442; 1973.

45. White, A.; Handler, P.; Smith, E. L.; Hill, R. L.; Lehman, I. R. Principles of biochemistry. 6th ed. Philadelphia; W. B. Saunders; 1987.

46. Stocker, R.; Glazer, A. N.; Ames, B. N. Antioxidant activity of albumin-bound bilirubin. Proc. Natl. Acad. Sci. USA 84:5918-5922; 1987.

47. Stocker, R.; Yamamoto, Y.; McDonagh, A. F.; Glazer, A. N.; Ames, B. N. Bilirubin is an antioxidant of possible physiological importance. Science 235:1043-1046; 1987.

48. Deighton, N.; Hider, R. C. Intracellular low molecular weight iron. Biochem. Soc. Transac. (London) 17:490; 1989.

49. Wilkins, T. D.; Microbiological considerations in interpretation of data obtained with experimental animals, pp. 3-10 In: Bruce, W. R., ed. Banbury report 7. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory; 1981.

50. Walling, C.; Fenton's Reagent Revisited. Accounts of Chem. Res. 8: 125-131; 1975.

51. Dorfman, L. M.; Adams, G. E. Reactivity of the hydroxyl radical in aqueous solutions. (National Standard Reference Data Series 46, NSRDS-NBS46). Washington DC: U.S. Government Printing Office, U.S. National Bureau of Standards; 1973.

52. Repine, J. E.; Pfenninger, O. W.; Talmage, D. W. Berger, E. M.; Pettijohn, D. E. Dimethyl sulfoxide prevents DNA nicking mediated by ionizing radiation or iron/hydrogen peroxide-generated hydroxyl radical. Proc. Natl. Acad. Sci. 78:1001-1003; 1981.

53. Yamauchi, N.; Kuriyama, H.; Watanabe, N.; Neda, H.; Maeda, M.; Niitsu, Y. Intracellular hydroxyl radical production induced by recombinant human tumor necrosis factor and its implication in the killing of tumor cells in vitro. Cancer Res. 49:1671- 1675; 1989.

54. Mason, M. M. Toxicology of DMSO in animals. In: Jacob, S. W.; Rosen, E. E.; Wood, D. C. eds. Dimethyl sulfoxide. New York: Marcel Dekker; 1971.

55. Smith, E. R.; Hadidian, Z.; Mason, M. M. The single and repeated dose toxicity of dimethyl sulfoxide. Ann. New York Acad. Sci. 141:96-109; 1967.

56. Benville, P. E.; Smith, C. E.; Shanks, W. E. Some toxic effects of dimethylsulfoxide in salmon and trout. Toxicol. Appl. Pharmacol 12:156-187; 1968.

57. Maurer, R. R. In: Ciba Foundation Symposium 52: the freezing of mammalian embryos, Amsterdam: Elsevier; 1977:116.

58. Wilmut, I.; Rowson, L. E. A. Experiments on the low temperature preservation of cow embryos. Vet. Rec. 92:686 690: 1973.

59. Willadsen, S. M. Factors affecting the survival of sheep embryos during deep-freezing and thawing. In: Ciba Foundation Symposium 52: the freezing of mammalian embryos, Amsterdam: Elsevier, 1977:175-201.

60. Ashwood-Smith, M. J. Current concepts concerning radioprotective and cryoprotective properties of dimethyl sulfoxide in cellular systems. Ann. NY Acad. Sci. 243:246-256; 1975.

61. Lagercrantz, C.; Forshult, S., Trapping of short-lived free radicals as nitroxide radicals detectable by ESR spectroscopy. The radicals formed in the reaction between OH-radicals and some sulphoxides and sulphones. Acta Chemica Scand. 23:811-817; 1969.

62. Dixon, W. T.; Norman, R. O. C.; Buley, A. L. Electron spin resonance studies of oxidation. Part II. Aliphatic acids and substituted acids. J. Chem. Soc. 3625-3634; 1964.

63. Klein, S. M.; Cohen, G.; Cederbaum, A. I. The interaction of hydroxyl radicals with dimethylsulfoxide produces formaldehyde. FEBS Letters 116:220-222; 1980.

64. Klein, S. M.; Cohen, G.; Cederbaum, A. I. Production of formaldehyde during metabolism of dimethyl sulfoxide by hydroxyl radical generating systems. Biochem. 20:6006-6012: 1981.

65. Babbs, C. F.; Gale, M. J. Methane sulfinic acid production from DMSO as an indicator of hydroxyl radical production in vivo. In: Rice-Evans, C.; Halliwell, B., eds. Free radicals: methodology and concepts. London: Richelieu Press; 1988:91 121.

66. Floyd, R. A.; West, M. S.; Eneff, K. L.: Hogsett, W. E.: Tingey, D. T. Hydroxyl free radical mediated formation of 8-hydroxyguanine in isolated DNA. Archiv. Biochem. Biophys. 262:266-272; 1988.

67. Steiner, M. G. Detection and quantitation of hydroxyl radicals in post-ischemic rat kidney. Doctoral thesis, Purdue University West Lafayette, IN; August, 1989.

68. Buxton, G. V.; Greenstock, C. L.; Helman, W. P.; Ross, A. B. Critical review of rate constants for reactions of hydrated electrons, hydrogen atoms and hydroxyl radicals in aqueous solution. Radiation Chemistry Data Center, Radiation Laboratory University of Notre Dame, Notre Dame, IN, 46556, USA: June, 1986.

69. Spinks, J. W. T.; Woods, R. J. An introduction to radiation chemistry. 2nd ed. New York: Wiley-Interscience; 1976.

70. Suit, H. D. Radiation biology: a basis for radiotherapy. In: Textbook of radiotherapy. Philadelphia: Lea & Febiger; 1973.

71. Tien, M.; Morehouse, L. A.; Bucher, J. R.: Aust, S. D. The multiple effects of ethylenediaminetetraacetate in several model lipid peroxidation systems. Arch. Biochem. Biophys. 218:450- 458; 1982.

72. Tien, M.; Aust, S. D. Comparative aspects of several model lipid peroxidation systems. Lipid Perox. in Biol. Med. 23 39; 1982.

73. Weast, R. C., ed. CRC handbook of chemistry and physics. 60th ed. Boca Raton, FL: CRC Press: 1979.

74. Hassan, H. M. ; Moody, C. S. Determination of the mutagenicity 5 of oxygen free radicals using microbial systems. Methods in Enzymol. 105:254-263; 1984.

75. Dunning, W. F.; Curtis, M. R.: Maun, M. E. The effect of added dietary tryptophane on the occurrence of 2-acetylaminofluorene-induced liver and bladder cancer in rats. Cancer Res. 10:454-459; 1950.

76. Bryan, G. T.; Brown, R. R.; Price, J. M. Mouse bladder carcinogenicity of certain tryptophan metabolites and other aromatic nitrogen compounds suspended in cholesterol. Cancer Res. 24:596-602; 1964.

77. Nishmura, R.; Pipkin, G. E.: Duke. G. A.; Schlegel, J. I . Nonenzymatic formation of cinnabarinic acid in urine of patients with tumors of the urinary bladder. Investigative urology 7:206-214; 1969.

78. Pipkin, G. E.; Nisbimura, R.: Banowsky, L.; Schlegel. J. U. Stabilization of urinary 3-hydroxyanthranilic acid by oral administration of L-ascorbic acid. Proc. Soc. Exp. Biol. Med. 126:702-704: 1967.

79. Sherlock, P.; Lipkin, M.: Winawer, S. J. The prevention of colon cancer. Amer. J. Med. 68:917-931, 1980.

80. Creasey, W. A. Diet and cancer. Philadelphia: Lea & Febiger: 1985.

81. Reddy, B. S. Diet and colon cancer: Evidence from human and animal model studies. In: Reddy, B. S.; Cohen, L. A.. ed., Diet, nutrition, and cancer: a critical evaluation. Volume 1 Macronutrients and cancer. Boca Raton, FL: CRC Press:1986, 47-65.

82. Wynder, E. L.; Shigematsu, T. Environmental factors of cancer of the colon and rectum. Cancer 20:1520-1561; 1967.

83. Wattenberg, L. W. Inhibition of chemical carcinogenesis. J. Natl. Cancer Inst. 60:11 18; 1978.

84. Wattenberg, L. W. Brief communication: inhibition of dimethylhydrazine-induced neoplasia of the large intestine by disulfiram. J. Natl. Cancer Inst. 54:1005-1006; 1975.

85. Sporn, M. B.; Dunlop, N. M.: Newton, D. L.: Smith, J. M. Prevention of chemical carcinogenesis by vitamin A and its synthetic analogs (retinoids). Nutr. and Cancer 35:1332-1338; 1976.

86. Newberne, P. M.; Rogers, A. E. Rat colon carcinomas associated with aflatoxin and marginal vitamin A. J. Natl. Cancer Inst. 50:439-448; 1973.

87. Cook, M. G.; McNamara, P. Effect of vitamin E on dimethylhydrazine-induced colonic tumors in mice. Cancer Res. 40:1329; 1980.

88. Jacobs, M. M.; Forst, C. F.; Beams, F. A. Biochemical and clinical effects of selenium on dimethylhydrazine-induced colon cancer in rats. Cancer Res. 41:4458; 1981.

89. Shamsuddin, A. M.; Elsayed, A. M.; Ullah, A. Suppression of large intestinal cancer in F344 rats by inositol hexaphosphate. Carcinogenesis 9:577-580; 1988.

90. Babbs, C. F.; Steiner, M. G. Detection and quantitation of hydroxyl radical using dimethyl sulfoxide as a molecular probe. Meth. Enzymol. 186:137-147, 1990.