Free Rad. Res. Comms., Vol. 6, No. 6, pp. 359–367
Reprints available directly from the publisher
Photocopying permitted by license only

FREE RADICAL GENERATING MECHANISMS IN THE COLON: THEIR ROLE IN THE INDUCTION AND PROMOTION OF COLORECTAL CANCER?

M.H. BLAKEBOROUGH[1], R.W. OWEN[2] and R.F. BILTON[1]†

[1] *Biochemistry Division, School of Natural Sciences, Liverpool Polytechnic, Byrom Street, Liverpool L3 3AF.*

[2] *PHLS Centre for Applied Microbiology Research, Pathology Division, Bacterial Metabolism Research Group, Porton Down, Salisbury, Wiltshire SP4 0JG*

(Received November 23rd 1988, in revised form March 20th 1989)

A hypothesis is presented to account for the dietary induction and promotion of colorectal cancer. The principal agents are the secondary bile acids, lithocholic and deoxycholic acids, the vitamin K group and ferrous iron complexes. These metabolites may interact to subvert the normal free radical generating mechanisms involved in mucosal defence. Diets high in fat and red meat and low in fibre support a Bacteroides-dominated colonic microflora, which both synthesises and utilises vitamin K_2 isoprenalogues or menaquinones as enzyme co-factors. Iron(II) complexes such as haemin from the breakdown of dietary haemoglobin and myoglobin also serve as growth factors for these bacteria and provide a rich source of haem-iron for intestinal uptake. Biliary secretion is stimulated by dietary fat and bile acids are essential for the intestinal uptake of vitamin K and possibly of iron complexes such as haemin.

In the mature colonocyte, vitamin K and haemin may initiate redox cycling reactions which liberate superoxide ($O_2^{-}\cdot$). Bile acids can activate the membrane bound phospholipase to liberate arachidonate and diacylglycerol. This leads in turn to the production of more $O_2^{-}\cdot$ which can enter the microcirculation and acts as a potent chemoattractant for the neutrophils that line the lamina propria. The released diacylglycerol can activate protein kinase C in the neutrophil membrane to switch on the respiratory burst oxidase system generating yet more $O_2^{-}\cdot$ and may stimulate the proliferation of transformed stem cells by a similar protein kinase C mediated mechanism. The additive effects of bile acids, vitamin K and iron(II) in oxygen radical generation may overcome the antioxidant defence mechanisms of the stem cell leading ultimately to semiquinone and hydroxyl radical mediated DNA damage and tumour induction.

KEY WORDS: Colorectal cancer, bile acid, menaquinone, iron.

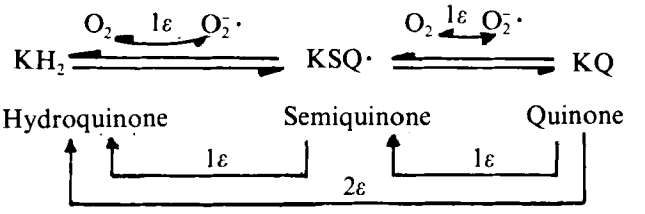
INTRODUCTION

It is proposed that lithocholic acid and deoxycholic acid, bacterial menaquinones and iron(II) complexes interact to induce an oncogenic effect in the colon by the generation of free radicals which subvert the normal mucosal defence mechanisms.

Endogenous menaquinones are synthesised primarily by *Bacteroides fragilis* strains[1] which predominate in the colon of subjects on diets rich in animal fat and red meat and low in fibre.[2] A rich source of haem-iron is available for uptake from the breakdown of dietary haemoglobin and myoglobin, or from intestinal bleeding. A lack of dietary fibre is crucial to this model since phytate, a component of vegetable fibre, is a potent chelator of iron and inhibitor of iron-mediated free radical reactions.[3] The elevated bile secretion resulting from high fat intake results in increased produc-

† To whom correspondence should be addressed.

tion of the co-mutagenic[4] and co-carcinogenic[5] secondary bile acids by the microflora of the distal colon. It has been postulated that the Bacteroides strains synthesise and utilise menaquinones and possibly vitamin K_1 in the oxidation of bile acid (and other) substrates to yield reduced K vitamins (KH_2).[6] These could be transported into the mature coloncyte, together with iron complexes and bile acids as mixed micelles, where superoxide ($O_2^-\cdot$) generating redox cycling reactions may occur.



(1)

This scheme involves $O_2^-\cdot$ production by a non-enzymic route and regeneration of KH_2 by either 1 electron (ε) flavoprotein-mediated reduction or a 2ε reduction by DT-diaphorase.[7] Additional O_2^- may be generated and diffuse into the microcirculation following bile acid activation of the mucosal phospholipase which releases arachidonate and diacylglycerol from tissue lipid stores,[8] resulting in neutrophil chemotaxis,[9] respiratory burst oxidase activation[10] and ultimately the proliferation of transformed stem cells by a protein kinase C mediated mechanism.[11]

Redox cycling (Eq. 1) in the stem cell nucleus can lead to depletion of the antioxidant pool and additional free radical generating reactions via superoxide dismutase (SOD) induction

$$2O_2^-\cdot + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2 \qquad (2)$$

and hydroxyl radical ($\cdot OH$) formation via the iron-catalysed Haber–Weiss reaction.

$$Fe^{2+} + H_2O_2 \longrightarrow OH^- + \cdot OH \qquad (3)$$

The consequence of liberating $KSO\cdot$, $O_2^-\cdot$, H_2O_2 and $\cdot OH$ could be that these highly reactive species can generate DNA damage and hence act as initiators and promoters in colorectal cancer (CRC).

Previous reports by Hill *et al.*[12,13] favour the nuclear steroid-dehydrogenase-positive *Clostridium paraputrificum* strains as aetiological agents in CRC. In view of the low numbers of these Clostridia in faeces (10^6/gm) and their inability to synthesise menaquinones,[1] we suggest that the bile tolerant Bacteroides strains, with their high numbers (10^{10}–10^{11}/gm) and potent nuclearsteroid dehydrogenase and 7α-dehydroxylase activity,[6] are more likely to be the major aetiological agents.

RESULTS AND DISCUSSION

A positive correlation between the intraluminal concentration of lithocholate and deoxycholate and the incidence of colorectal cancer[14] indicates that these bile acids may serve as selective agents in the development of a menaquinone producing Bacteroides dominated colonic microflora. To date none of the natural K vitamins have been shown to possess mutagenic activity. However, one report[15] indicated that menadione and several hydroxylated naphthoquinones lacking a prenyl side-chain

were mutagenic in the Ames pre-incubation assay.[16] Frame-shift mutagenesis was detected using *Salmonella typhimurium* strains TA2637 and T98 and there was an absolute requirement for the addition of phenobarbital induced rat liver microsomes. Since the *Salmonella* mutagenicity assay does not detect many chemicals that generate active oxygen species[17] we have used the more sensitive recently constructed tester

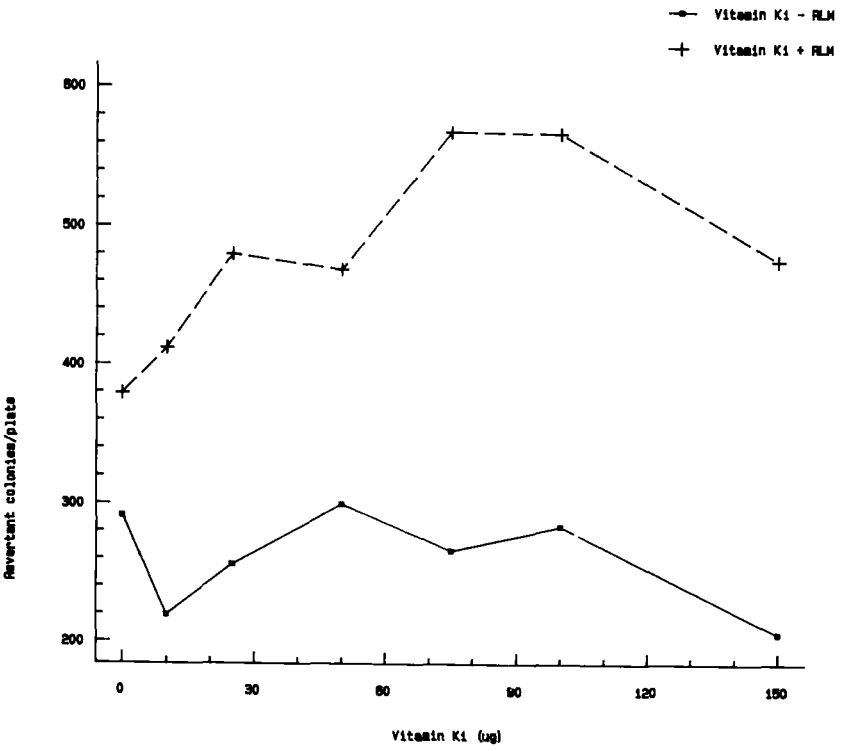


FIGURE 1 Dose Response data for vitamin K_1 ± rat liver microsomes with *Salmonella typhimurium* TA102 in the Ames Preincubation Assay. Key: – + – – Vitamin K_1 (in DMSO) + Arachlor 1254 induced rat liver microsomes (S9-mix); – ■ – – Vitamin K_1 (in DMSO) minus S9-mix. *Experimental*: RLM fractions from Arachlor 1254 induced rats were kindly provided by the Safety of Medicines Group, ICI Pharmaceuticals, Alderley Park, Cheshire, and incorporated into the S9-mix according to the procedure of Venitt *et al.* (1984)[38]. S9-mix was prepared on the day of use and kept sterile on ice prior to the assay. S9 additions (μmoles/ml) KCl, 33; $MgCl_2.6H_2O$, 8; glucose-6-phosphate 5; $NADP^+$, 4; and sodium phosphate buffer pH 7.4, 100. The above ingredients were filtered through a 0.22 μm filter into a sterile bottle placed in crushed ice before the RLM fraction was added. RLM fractions were thawed and added to the above to give a final RLM concentration of 4% (v/v). *Ames Preincubation assay*: The method used was essentially that of Maron and Ames (1983)[16] modified as follows to include a preincubation step. 0.5 ml of S9-mix was added to capped culture tubes in an ice-bath. 0.1 ml bacterial culture and 10 μl of test solution (vitamin K_1 in DMSO) were added in strict sequence to avoid placing the bacteria in direct contact with undiluted test compound and solvent. The tubes were gently vortexed and incubated at 37°C for 20 mins in an L.H. Fermentation Mark X shaking incubator at 120 rpm. 2.0 ml of top agar maintained at 45°C was then added to each tube, the contents vortexed for 3 secs and quickly poured onto minimal glucose agar plates. The test plates were then treated as in the Ames Plate incorporation assay. After 48 hours incubation at 37°C, duplicate test and control plates were examined for the presence of a confluent background lawn and revertant colonies were counted.

strains TA102 and TA104 which detect a variety of oxidative mutagens.[18] TA102 can detect oxidative mutagens such as X-rays, bleomycin, hydrogen peroxide and other hydroperoxides, streptonigrin and other quinones, and phenylhydrazine.[17] TA104 is useful for the detection of some mutagenic amines, quinones and hydroperoxides.[18] These strains have been genetically modified to detect A·T base pair mutations initiated by oxidative mutagens.

Using these new strains in the Ames pre-incubation assay we have been able to detect weak mutagenic activity of K_1 in the presence of Arachlor-induced rat liver microsomes (RLM) (Figure 1) with TA102. TA104 did not respond to K_1 in a dose dependent manner, but when lithocholate was added at a concentration of 30 μg/plate there was a sharp increase in revertant colonies over the K_1 dose range 0–40 μg (Figure 2).

The addition of iron complexes in the form of haemin and haemolysed rat blood gave low but consistent increases in reversion frequency (data not shown).

When the SOS chromotest[19] was used to detect DNA damage rather than mutation, we observed a dose dependent increase in the SOS response to K_1 in the absence of

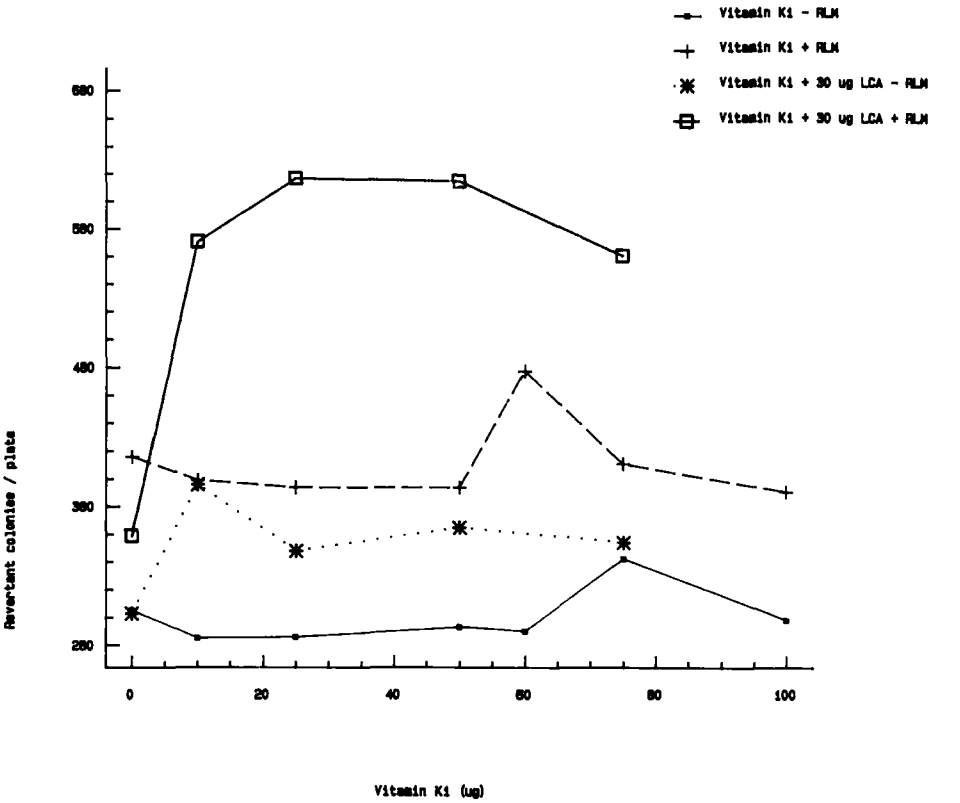


FIGURE 2 Dose response data for vitamin K_1 using the Ames preincubation assay in the presence and absence of sodium lithocholate and S-9 mix with *Salmonella typhimurium* TA104. Key: + – Vitakin K_1 + S9-mix; ■ – Vitamin K_1 – S9-mix; * – Vitamin K_1 + 30 μg lithocholate – S9-mix; □ – Vitamin K_1 + 30 μg lithocholate + S9-mix. Experimental: As for fig. 1 but with the addition of sodium lithocholate as an aqueous solution to the pre-incubation mixture.

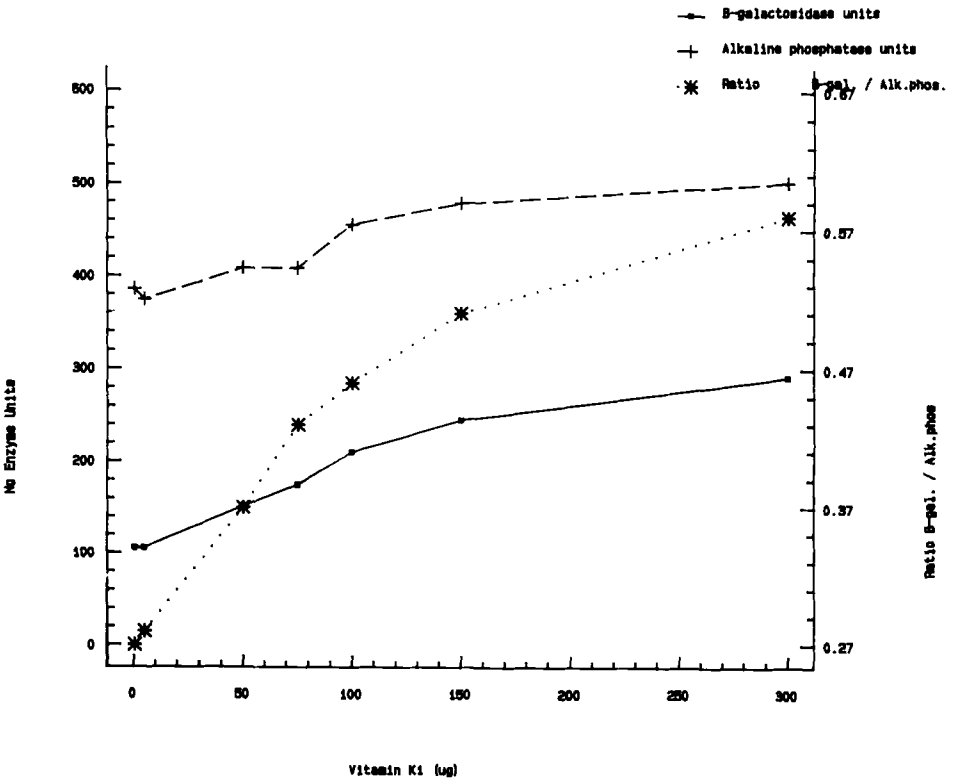


FIGURE 3 Dose response data for vitamin K_1 using the SOS-chromotest. Key: + – alkaline phosphate units; ■ – β-galactosidase units; * – Ratio of β-gal.(u)/Alk.Phos.(u) activity. The protocol followed that of Quillardet *et al.* (1982)[19] where 0.1 ml of a stock culture was inoculated into 10 ml of L-broth/Ampillin (Lamp) medium and incubated at 130 rpm. 0.25 ml of overnight culture was transferred to 10 ml of fresh Lamp medium at 37°C. After approximately 2–3 hours growth at 37°C with shaking (130 rpm) the culture had reached an optical density of 0.4 at 600 nm yielding about 2×10^8 cells/ml. The culture was then diluted 10-fold into fresh Lamp medium and the OD_{600} read and recorded. Aliquots of 0.6 ml were distributed into 20 ml flow screw cap glass tubes containing 20 μl of test compound. After 2 hours shaking (130 rpm) at 37°C, 0.3 ml fractions were withdrawn and dispensed into a separate set of tubes. The two sets of tubes were then assayed for β-galactosidase and alkaline phosphatase respectively. *Controls for the test included*: (i) Negative controls containing appropriate solvents for the test compounds. (ii) Positive controls were always included to maintain the sensitivity of the assay, the most commonly used being 4-nitroquinoline-1-oxide in DMSO and aqueous nitrofurantoin. *Expression of Results*: From the absorbance readings taken, the number of enzyme units for each dose of test compound were calculated using the equation

$$\text{Enzyme units} = \frac{OD_{420} \times 1000}{t \times v \times OD_{600}}$$

OD_{42} = Optical density at 420 nm read at the end of the assay.
OD_{600} = Optical density at 600 nm of the cell culture read just before the assay.
t = Incubation time with substrate (o-nitrophenylgalactoβDpyranoside and p-nitrophenylphosphate).
v = Volume of culture used in assay (0.6 ml).

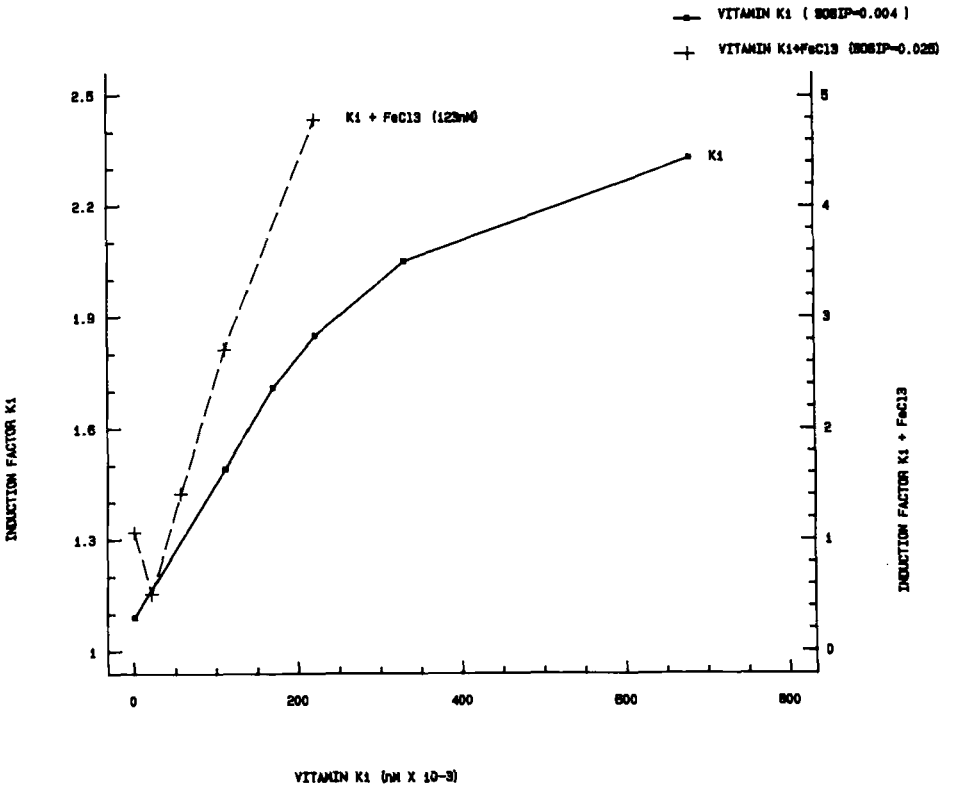


FIGURE 4 Dose response curve of Induction Factor versus nmole dose of vitamin K_1 in the presence and absence of ferric chloride. Key: ■ – vitamin K_1 alone; + – vitamin K_1 + $FeCl_3$ (123 nmoles); From the graph the SOS inducing potencies can be calculated for vitamin K_1 alone = 0.004 and vitamin K_1 + $FeCl_3$ = 0.025.

metabolic activation (Figure 3), as measured by the increase in the ratio of β-galactosidase activity to alkaline phosphatase activity. This activity ratio (R_1) is normalised to its value in the absence of test substance (R_0); this value $R_1 R_0$ is referred to as the induction factor (IF). When IF is plotted against K_1 dose (nmole) the slope of the linear region of the graph is taken as a measure of the SOS inducing potency (SOSIP) of the test compound, i.e. the DNA damaging potential of the compound. Figure 4 shows the effect of K_1 alone and in the presence of 123 uM $FeCl_3$. The addition of iron has resulted in an apparent 6-fold enhancement of the SOSIP.

Studies with lithocholate[20] indicate that its genotoxic mode of action may be via covalent attachment to the ε-amino group of lysine in histone which serves to dissociate the DNA from its stabilising histone core.

The synthetic vitamin K_3 (menadione) exerts powerful cytotoxic effects on isolated hepatocytes in the presence of Warfarin which blocks the protective clearance enzyme DT-diaphorase[7] indicating the involvement of a semiquinone free radical. Vitamin K_1 has a much lower cytotoxicity.[21] Side effects which may potentiate DNA damage

include depletion of the NAD(P)H and glutathione pools and loss of intracellular calcium.

Warfarin has been shown to reduce the incidence of microadenomas in azoxymethane treated rats.[22] Microadenomas may resemble certain benign tumours (B16) in having a warfarin resistant vitamin K-2,3-epoxide reductase[23] and are therefore susceptible to vitamin K mediated oxidative damage amplified to cytotoxic levels by warfarin. Since the natural K vitamins are probably involved in the above phenomenon, it is not unreasonable to suggest that they may act as genotoxins in the colon.

Iron can be involved in 3 types of oxidative stress, namely, decomposition of lipid peroxides, $O_2^-\cdot$ and H_2O_2 generation and the formation of $\cdot OH$.[24] Support for this concept lies in the increased cancer incidence in situations where persistent bleeding occurs. Large bowel cancer is enhanced in animal model systems by mechanical tissue damage,[25] is increased 3-fold in Ulcerative Colitis[26] and is common at surgical anastomoses.[27] It has been shown that 'labile' iron can be released from haemoglobin by lipid peroxides or H_2O_2 and may be responsible for the free radical reactions implicated in cartilage damage in the rheumatoid joint[28] and in promoting reperfusion injury after tissue ischaemia.[29] Furthermore, recent work indicates that iron released from myoglobin may stimulate $OH\cdot$ formation from H_2O_2.[30] Such reactions, if moderated, could lead to sublethal DNA damage and mutation in the stem cells of the colonic mucosa.

Several workers have reported that faecal extracts cause chromosomal damage which was enhanced by the addition of transition metals and phenobarbitone induced rat liver microsomes.[31,32] Addition of catalase reduced this effect. Phenobarbitone is a potent inducer of 1ε-flavoprotein dehydrogenases such as cytochrome P_{450} reductase which is present in the nucleus[33] and is known to convert quinones to their respective semiquinones.[34] That some faecal extracts required anaerobic incubation for activation[35] also supports our contention that these extracts probably contained lithocholate, deoxycholate, reduced menaquinones and FeII complexes as the active ingredients.

Our own evidence for this proposal arises from the observation that an impure bacterial transformation product of lithocholate yielded strong positive results in the Ames Test.[36] The pure chemically synthesised compound (3-oxochola-1,4-dien-24-oic acid) gave negative results. Extraction of the impure material with hexane yielded a yellow oil with the UV spectrum characteristic of a 2,3-substituted naphthoquinone.[37] We have recently extracted similar menaquinones from Bacteroides fermentations with the aim of including them in free radical generating systems for genotoxicity testing.

Our hypothesis provides a candidate set of radicals which can cause DNA damage directly by adduct formation ($KSQ\cdot$) and strand scission ($\cdot OH$) or indirectly via secondary radical species (e.g. lipid peroxides). Bile acids or their bacterial metabolites may occupy a crucial role in:

1. Establishment of a bile-tolerant, menaquinone synthesising colonic microflora.
2. Formation of micelles with menaquinone and Fe(II) complexes for genotoxin uptake by colonic mucosa.
3. Activation of mucosal phospholipase.
4. Direct activation of the neutrophil respiratory burst oxidase system.

Acknowledgements

MHB thanks the SERC for the postgraduate studentship which funded this work, and the Cancer Research Campaign for providing additional support for chemicals and equipment.

References

1. Ramotar, K., Conly, J.M., Chubb, H. and Louie, T.J. Production of menaquinones by intestinal anaerobes. *J. Infect. Dis.*, **150,** 213–218 (1984).
2. Maier, B.R., Flynn, M.A., Burton, G.C., Tsutakawa, R.K. and Hentges, D.J. Effects of a high-beef diet on bowel flora: a preliminary report. *Amer. J. Clin. Nutr.*, **27,** 1470–1474 (1974).
3. Graf, E., Empson, K.L., Eaton, J.W. Phytic Acid a natural anti-oxidant. *J. Biol. Chem.*, **262,** 11647–11650 (1987).
4. Wilpart, M., Mainguet, P., Maskens, A. and Roberfroid, M. Mutagenicity of 1,2-dimethylhydrazine towards *Salmonella typhimurium*; co-mutagenic effect of secondary bile acids. *Carcinogenesis*, **4,** 45–48 (1983).
5. Narisawa, T., Magadia, N.E., Weisburger, T.H. and Wynder, E.L. Promoting effect of bile acids on colon carcinogenesis after intrarectal instillation of N-methyl-N′-nitrosoguanidine in rats. *J. Natn. Cancer Inst.*, **53,** 1093–1097 (1974).
6. Owen, R.W., Bilton, R.F. and Tenneson, M.E. The degradation of cholic and deoxycholic acids by Bacteroides spp under strict anaerobic conditions. *Biochem. Soc. Trans.*, **5,** 1711–1713 (1977).
7. Thor, H., Smith, M.T., Hartzell, P., Bellomo, G., Jewell, S.A. and Orrenius, S. The metabolism of menadione (2-methyl-1,4-naphthoquinone by isolated hepatocytes. *J. Biol. Chem.*, **257,** 12419–12425 (1982).
8. De Rubertis, F.R., Craven, P.A. and Saito, R. Bile salt stimulation of colonic epithelial proliferation: evidence for involvement of lipoxygenase products. *J. Clin. Invest.*, **74,** 1614–1624 (1984).
9. Halliwell, B. and Gutteridge, J.M.C. Oxygen toxicity, oxygen radicals, transition metals and disease. *Biochem. J.*, **219,** 1–14 (1984).
10. Babior, B.M. The respiratory burst oxidase. *Trends in Biochem. Sci.*, **12,** 241–243 (1987).
11. Nishizuka, Y. Protein kinases in signal induction. *Trends in Biochem. Sci.*, **9,** 163–166 (1984).
12. Hill, M.J. Bacteria and the aetiology of colon cancer. *Cancer*, **34,** 815–818 (1974).
13. Goddard, P. and Hill, M.J. The dehydrogenation of the steroid nucleus by human gut bacteria. *Biochem. Soc. Trans.*, **1,** 1113–1116 (1973).
14. Owen, R.W., Dodo, M., Thompson, M.H. and Hill, M.J. Faecal steroids and colorectal cancer. *Nutr. Cancer*, **9,** 73–80 (1987).
15. Tikkanen, L., Matsushima, T., Natori, S. and Yoshihira, K. Mutagenicity of natural naphthoquinones and benzoquinones in the Salmonella/microsome test. *Mutation Research*, **124,** 25–34 (1983).
16. Maron, D.M. and Ames, B.N. Revised methods for the Salmonella mutagenicity testing. *Mutation Res.*, **113,** 173–215 (1983).
17. Levin, D.E., Hollstein, M., Christian, M.F., Schwiers, E.A. and Ames, B.N. Detection of oxidative mutagens with a new Salmonella tester strain (TA102). *Methods in Enzymology*, **105,** 249–254. Academic Press Inc. (1984).
18. Marnet, L.J., Hurd, H.K., Hollstein, M.C., Levin, D.E., Esterbauer, H. and Ames, B.N. Naturally occurring carbonyl compounds are mutagens in *Salmonella typhimurium* tester strain TA104. *Mutation Research*, **148,** 25–34 (1985).
19. Quillardet, P., Huisman, O., d'ari, R. and Hofnung, M. The SOS chromotest. *Proc. Natl. Acad. Sci. USA*, **79,** 5971–5975 (1982).
20. Nair, P.P., Mendelofff, A.I., Vocci, M., Bankofski, J., Gorelik, M., Herman, G. and Plapinger, R. Lithocholic acid in the human liver: identification of ε-lithocholyllysine in tissue protein. *Lipids*, **12,** 922 (1977).
21. Akman, S.A., Dietrich, M., Chlebowski, R., Limberg, P. and Block, J.B. Modulation of menadione sodium bisulphite versus Leukemia L1210 by the acid-soluble thiol pool. *Cancer Research*, **45,** 5257–5262 (1985).
22. Cooke, T., Kirkham, N., Stamthorp, D.H., Inman, C. and Goeting, N. The detection of early neoplastic changes in experimentally induced colorectal cancer using scanning electron microscopy and cell kinetic studies. *Gut*, **25,** 748–755 (1984).
23. Uitendall, M.P., Thijssen, H.H., Drittij-Reijnders, M.J. and Hoeijmakers, M.J. B16 tumour cells contain a Warfarin sensitive vitamin K_1 2,3-epoxide reductase. *Biochem. Biophys. Res. Commun.*, **137(3),** 1015–1020 (1986).

24. Gutteridge, J.M.C., Richmond, R. and Halliwell, B. Inhibition of iron-catalysed formation of hydroxyl radicals from superoxide and of lipid peroxidation by desferrioxamine. *Biochem. J.*, **184,** 469–472 (1979).
25. Schmahl, D. Colon carcinogenesis and co-carcinogenesis. In Molt, R.A. and Williamson, R.C.N. eds. Colonic carcinogenesis. MTP Press Ltd., Lancaster, Boston, The Hague, 235–241 (1982).
26. Kewenter, J., Ahlman, H. and Hulton, L. Cancer risk in extensive ulcerative colitis. *Ann. Surg.*, **188,** 824–832 (1970).
27. Williamson, R.C.N. and Rainey, J.B. The relationship between intestinal hyperplasia and carinogenesis. *Scand. J. Gastroenterology*, **19,** 57–76 (1984).
28. Gutteridge, J.M.C. Iron promoters of the Fenton reaction and lipid peroxidation can be released from haemoglobin by peroxides (1986) *Febs. Letters*, **201,** 291–295 (1986).
29. McCord, J.M. and Roy, R.S. The pathophysiology of superoxide: Roles in inflammation and ischaemia. *Can. J. Physiol. Pharmacol.*, **60,** 1346–1352 (1982).
30. Puppo, A. and Halliwell, B. Formation of hydroxyl radicals in biological systems. Does myoglobin stimulate hydroxyl radical formation from hydrogen peroxide? *Free Radical Res. Comm.*, **4,** 415–422 (1988).
31. Stich, F.H. and Kuhnlein, U. Chromosome breaking activity of human faeces and its enhancement by transition metals. *Int. J. Cancer*, **24,** 284–287 (1979).
32. Ehrich, M., Aswell, J.E., Tan Tassel, R. and Wilkins, T.D. Mutagens in the faeces of three South African populations at different levels of risk for colon cancer. *Mutation Res.*, **64,** 231–240 (1979).
33. Cohen, G.M., d'arcy Doherty, M. Free radical mediated cell toxicity by redox cycling chemicals. *Brit. J. Cancer*, **55,** suppl. VIII, 46–52 (1987).
34. Kappus, H. Overview of enzyme systems involved in the bioreduction of drugs and in redox cycling. *Biochem. Pharmacol.*, **35,** 1–6 (1986).
35. Lederman, M., Van Tassel, R., West, S.E.H., Ehrich, M.N.F. and Wilkins, T.D. *In vitro* production of a human faecal mutagen. *Mutation Res.*, **79,** 115–124 (1980).
36. McKillop, C.A. Mutagenicity studies with novel steroids and their associated metabolites produced by the bacterial transformation of bile acids. PhD Thesis (CNAA) (1981).
37. Blakeborough, M.H. Genotoxicity studies with potential large bowel carcinogens. PhD Thesis (CNAA) (1987).
38. Venitt, S., Crofton-Sleigh, C., and Forster, R. In Venitts, S., and Parry, J.M. eds. Mutagenicity testing a practical approach. IRL Press Oxford, Washington DC. Chapter 3 45–98 (1984).

Accepted by Prof. B. Halliwell