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ANTIMUTAGENICITY OF SORGHUM GRAIN POLYPHENOLS

Heidi Renate Grimmer

A thesis submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Doctor of Philosophy.

October 1993

ABSTRACT

Polyphenols, suspected of being mutagenic, were extracted from sorghum cultivars SNK3144, NK222, NK300, PNR8311, SSK30, SSK52 and Barnard Red with 80% (v/v) aqueous acetone. The crude extracts from these various cultivars were all separated into three fractions (F_1, F_2 and F_3) on a Sepharose CL-6B column. Each of these fractions was tested for mutagenicity in the Ames assay, both with and without the S9 metabolic activating system. The concentrations tested ranged from 1 µg/ml to 10 mg/ml. Results obtained from the Ames test were analysed by the Students t-test and showed that none of the phenolic fractions were mutagenic. To confirm these results, fractions from SSK52, a popular bird-resistant sorghum, were fed to *Drosophila melanogaster* in the somatic mutation and recombination test (SMART). There was no significant increase (by the Poisson null-test hypothesis) in single multiple wing hair spots. To check for possible mutagenicity in a product derived from sorghum viz. sorghum beer, powder from a brew-pack was tested in SMART, and at 20mg/ml and 50mg/ml levels there was no positive indication of mutagenicity. Since some of the results from the Ames test indicated that the reversion frequency in polyphenol treated plates had decreased when compared to control plates (no toxicity was observed), the phenolics were thought to maybe have an antimutagenic role. Polyphenols tested with the standard mutagens sodium azide, daunomycin and 2-aminofluorene, caused a reduction (significant by an analysis of variance) in the potency of these mutagens.

The mechanism of this reduction in mutagenic potency was investigated using polyphenols from SSK52 and radioactively labelled daunomycin. From fluorescence spectrophotometry and the change in ^{3}H-daunomycin to ^{14}C-thymidine ratio, it would appear that the polyphenols reduce mutagen binding to DNA possibly by preventing daunomycin uptake into the cells or by effectively decreasing the concentration of free mutagen by forming a polyphenol-mutagen complex unable to enter the cell.

DECLARATION

I declare that this thesis is my own, unaided work and has not been submitted for any degree at any other University.



Heidi Renate Grimmer

13th Day of October 1993.

. . . . may Science find its rightful place in a new and peaceful South Africa.

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ABBREVIATIONS

SMART	=	Somatic Mutation and Recombination Test
SLRT	=	Sex-linked Recessive Test
2AF	=	2-Aminofluorene
2AAF	=	2-Acetylaminofluorene
B(a)p	=	Benzo(a)pyrene
P450	=	Cytochrome P450
S9	=	Mammalian Metabolic Activation System Containing the Cytochrome P450 Enzymes
ER	=	Endoplasmic Reticulum
MNNG	=	N-methyl-N'-nitro-N-nitrosoguanidine
Trp	=	Tryptophan

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Chapter 1. GENERAL INTRODUCTION

1. GENERAL INTRODUCTION

Polyphenols are chemically very reactive and are widely distributed throughout the plant kingdom. The aim of the present work was to establish whether there could be a link between the consumption of foods rich in polyphenols (in particular sorghum grain) and the incidence of oesophageal cancer in the high risk black population in South Africa. According to Warmick and Harington (1973) the high incidence of oesophageal cancer in Transkei, South Africa, was first recorded in 1955. Since then similar high cancer incidence areas in other parts of the world such as Iran and China have been noted (Morton, 1970 and 1989; Muñoz et al., 1982 and 1985; Oterdoom, 1985). Morton postulated that there could possibly be a link between the occurrence of digestive tract cancer and the consumption of foods high in tannin. Stich and Powrie (1979) concluded from the collective work of several laboratories that plant phenolics could on their own have genotoxic effects and, in combination with mutagens, could act as modulators of their mutagenic potency. Simple phenolics (C6), phenolic acids (C6-C1) and cinnamic acids (C6-C3) from a variety of fruits and grains were found to have clastogenic (causing chromosomal abberations) and carcinogenic effects. The carcinogenic effects of individual phenolics could be enhanced when certain combinations of these compounds were tested in various animal assays. Combinations found to be synergistic were (i) tannic acid, quercetin, and eugenol and (ii) chlorogenic acid and quercetin. The latter mixture is found in betel nut, providing a

possible explanation for the high incidence of oral mucosa cancer among the chewers of this nut. Furthermore, it was found that a combination of gallic acid, pyrogallol, vanillin and hydroquinone prevented the formation of several mutagenic nitrosamines (Gray and Dugan, 1975: Kawabata *et al*., 1979 and Groenen, 1977). From these experiments, it was noted that phenolics could act both as enhancers of carcinogenesis and as inhibitors of mutagenesis. It would appear that polyphenols are a very diverse group and include flavonoids which on their own are also mutagenic (Brown, 1980; Brown and Dietrich, 1979; Elliger *et al*., 1984; Hardigree and Epler, 1978 and Watson, 1982). Elliger and coworkers (1984) have shown that flavonoids tested in the Ames assay and in Drosophila were active mutagens. In fact, it was shown that the hydroxylation pattern, and in particular the 3 position hydroxyl, and metabolic activation were critical features required for mutagenic expression (Brown and Dietrich, 1979). In addition to acting as substrates for various enzymes, polyphenols have been used by Matsushima *et al*. (1979) to increase the level of mutagen-activating (cytochrome P450's) enzymes in rat liver. Sorghum phenolics have been shown to increase the protein content of rat liver and subsequent benzo(a)pyrene activation in the Ames assay (Grimmer and McGrath, 1991 and Parhboo, 1993). Brown and Dietrich (1979) demonstrated that flavonol glycosides shown not to have mutagenic activity could be activated by the breakage of the glycosidic bond by the addition of rat caecal bacteria glycosidases.

Although there is abundant evidence for the mutagenicity of polyphenols, evidence is also available for their protective effects (Grimmer *et al.*, 1992; Kada *et al.*, 1985 and Ogawa *et al.*, 1985). Miyamoto *et al.* (1987) showed that intraperitoneally administered hydrolysable tannins (here ellagitannins) acted as antitumour agents in rats. For maximal activity a dimeric structure with several galloyl groups on a glucose core was found to be essential. Ogawa *et al.* (1985) found that quercetin (itself a mutagenic flavonoid) could be both antimutagenic when coincubated with benzo(a)pyrene or comutagenic in the presence of 2-acetylaminofluorene. Grimmer *et al.* (1992) found that quercetin was especially mutagenic in the presence of the cytochrome P450 enzymes, and that it acted as an antimutagen when coincubated with 2-aminofluorene. Gallic, chlorogenic and caffeic acid when coadministered with aflatoxin B_1, reduced the metabolism and mutagenicity of this toxin (San and Chan, 1987). Fungal toxins on stored sorghum grain in the Transkei have been shown to be mutagenic (van Rensburg *et al.*, 1985) and as such could be in part responsible for the observed cancer incidence.

In addition to the mutagenic and antimutagenic effects seen for phenolics, these compounds could be reducing the nutritional quality of the sorghum grain which is consumed in large quantities by the Transkeians, both as a staple food and sorghum beer. The antinutritional effect could predispose the victims, by damaging the mucosal lining of the oesophagus, upon which other mutagens such as

those found in cigarette smoke could act (Bradshaw and Harington, 1986). Evidence for the antinutritional effect was evident in rats feed a tannic acid rich diet (Glick and Joslyn, 1969; Price *at al.*, 1980). Not only do polyphenols increase the loss of protein from the body, but they also bind the nutrients available in the foods in which they occur.

The question then is why study sorghum grain. As has been mentioned, sorghum forms an integral part of the diet of a population amongst whom the incidence of cancer is high. Sorghum cultivars grown in these parts of the world contain a high polyphenol content, making them drought and fungal resistant and thus ideally suited for growth in these arid regions. The polyphenols in sorghum include simple phenolics, flavonoids and tannins (proanthocyanidins) and as such are a source of potential mutagens, comutagens and antimutagens. To determine whether sorghum, and in particular the polyphenols present, could provide a possible explanation of cancer incidence, it was decided to investigate various cultivars of sorghum grain for biological activity. Hahn *et al.* (1983) had shown that light sorghum grain (low tannin content) contained water soluble phenolics, which have subsequently been shown by others (Stich and Powrie, 1979 and Brown, 1980) to be mutagenic.

Chapter 2. POLYPHENOL - CHEMISTRY & FRACTIONATION

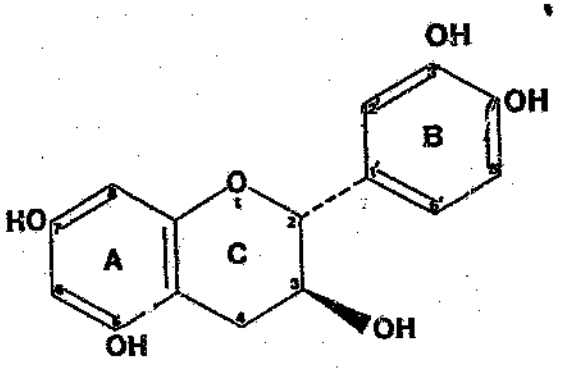
2 POLYPHENOL - CHEMISTRY AND FRACTIONATION

2.1 INTRODUCTION

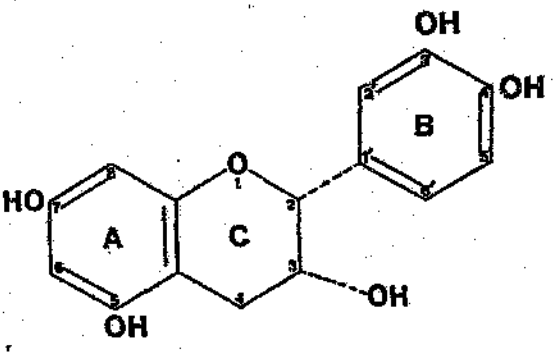
Polyphenols, or vegetable tannins as they were more commonly known, have the tendency to complex with proteins, carbohydrates, alkaloids and nucleic acids (Spencer *et al.*, 1988; Haslam and Lilley, 1988; Haslam, 1989). These compounds are responsible for the astringent properties of many of the foodstuffs in which they occur. This astringency is due to the binding of polyphenols to the lubricating glycoproteins in the mouth. Vegetable tannins are secondary metabolites and are postulated to act as a defensive mechanism for plants. Fungal contamination of crops rich in tannins is reduced due to polyphenol binding of fungal enzymes needed to penetrate the seed coat. The presence of polyphenols makes the leaves unpalatable and unpleasant to herbivores. Plants rich in polyphenols have been used medicinally in Chinese remedies for many years. It is only recently that the negative aspect of excessive polyphenol intake has become evident. It is thought that the regular intake of herbal infusions could be linked to gastric and oesophageal cancer (Morton, 1989).

Plant polyphenols have been divided into two major classes, the non-hydrolysable (condensed) and the hydrolysable tannins (Haslam, 1989). The condensed tannins (proanthocyanidins) are large polymers with molecular weights up to 20 000 (Haslam, 1989), though sizes of up to 50 000 have been reported by McGrath and Smith (1990). The

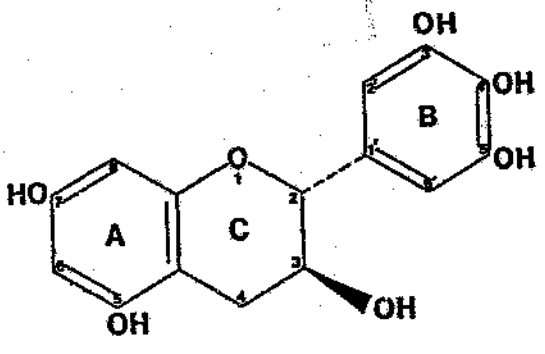
hydrolysable tannins fall in the 3000 M.W. range. Proanthocyanidins are composed of flavan-3-ol units, linked by linear 4 → 8 additions of further flavan-3-ol units. The principal flavan-3-ols units are (+)-catechin, (-)-epicatechin, (+)-gallocatechin and (-)-epigallocatechin (Fig. 2.1). The major dimers of these are referred to as procyanidins B1, B2, B3 and B4, depending on the following combinations of the flavan-3-ols; B1: epicatechin-(4β → 8)-catechin; B2: epicatechin-(4β → 8)-epicatechin; B3: catechin-(4α → 8)-catechin and B4: catechin-(4α → 8)-epicatechin. Since the nomenclature of the proanthocyanidins according to the IUPAC system is quite complex, Porter *et al.* (1982) proposed naming proanthocyanidins in a similar way to that used for oligo- and polysaccharides. The configuration at the interflavonoid bond at C-4 is indicated by the appropriate αβ nomenclature, in accordance with IUPAC rules. Oligomeric proanthocyanidins are produced by a stereoselective capture of the intermediate carbocation by nucleophilic attack of the end product flavan-3-ol to form dimers, which in turn form trimers and so on (Fig. 2.2). There is no self condensation of the carbocation or any oxidative polymerisation of the flavan-3-ol precursors (Haslam, 1989). The hydrolysable tannins are polyesters based on gallic acid and/or hexahydroxydiphenic acid and their derivatives (Haslam, 1989). Recently, gallate esters of proanthocyanidins have been found in green tea and rhubarb, implying a third class, with structural features common to the two above-mentioned classes.



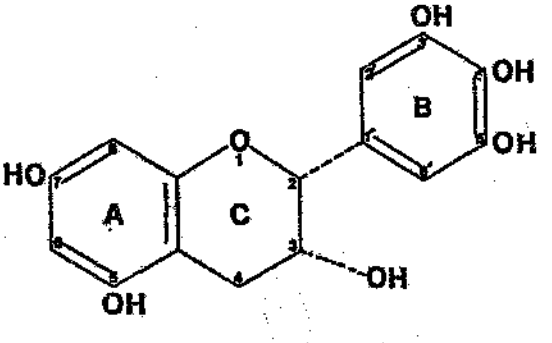
(A)



(B)



(C)



(D)

Fig 2.1 Structures of: (A) (+)-Catechin, (B) (-)-Epicatechin, (C) (+)-Gallocatechin and (D) (-)- Epigallocatechin (Haslam, 1989).

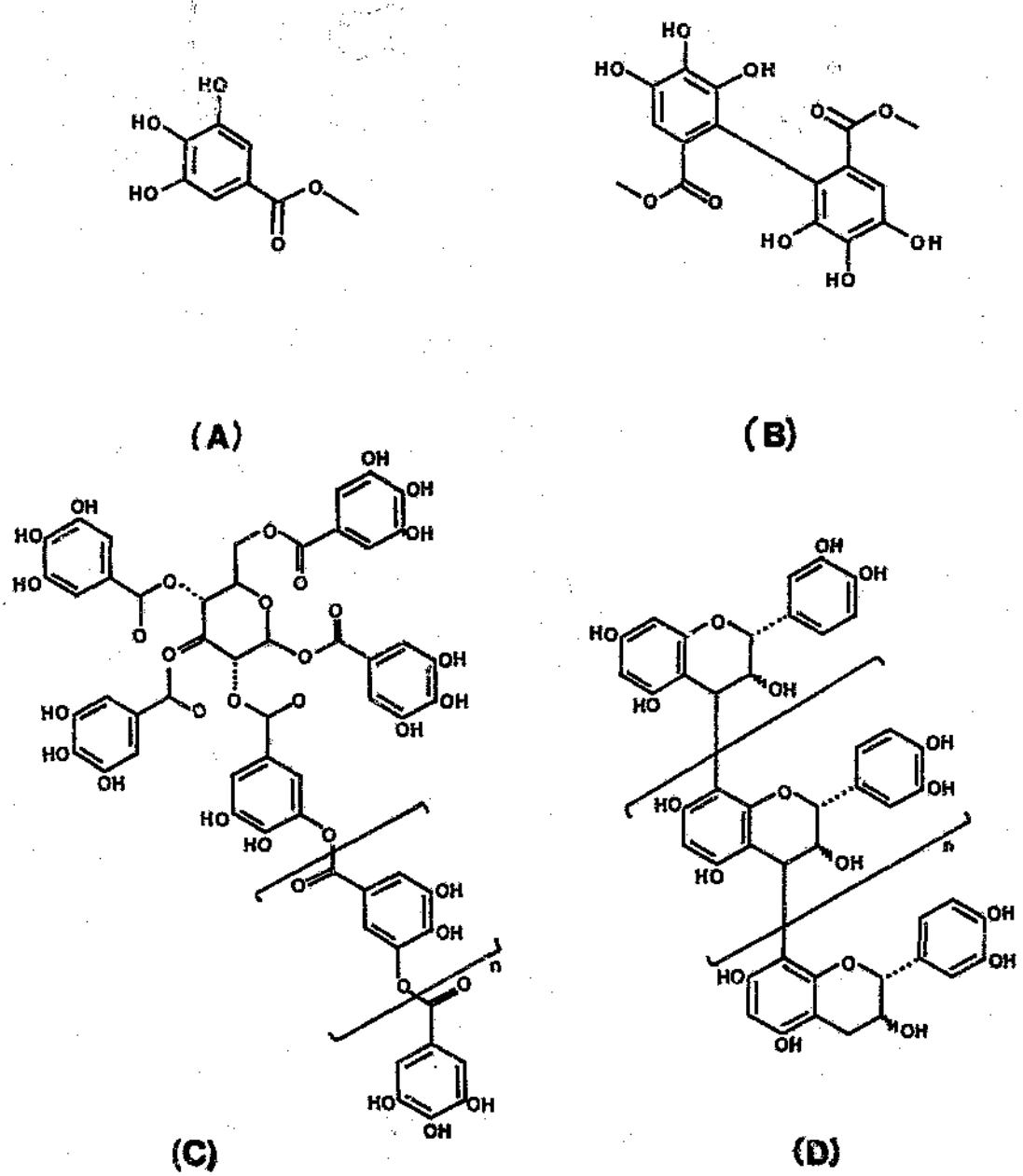


Fig. 2.1 Polyphenols can be divided into two groups - the polyesters (C) of gallic (A) and/or hexahydroxydiphenic acid (B) and their derivatives and the proanthocyanidins (D) (Haslam, 1989).

The multiplicity of phenolic groups common in the polyphenols makes simultaneous binding to several points on and cross-linking of proteins, with subsequent precipitation very favourable. However, astringency and precipitation are not restricted to the polymers since simple phenolic acids (≤ 200 M.W.) also have significant protein binding properties. Polyphenol complexation with polysaccharides has been shown to depend on the molecular size, conformational mobility, shape and water solubility of the polyphenol (Ya *et al.*, 1989). The differing affinity of polyphenols for polysaccharides is the result of a balance between these various factors. Generally, affinity is directly related to the molecular size and conformational flexibility of the polyphenol, and inversely related to its water solubility.

Proanthocyanidins (*syn.* leucoanthocyanidins) will upon acid hydrolysis yield anthocyanidins, thought to have a role in plant pigmentation e.g. the petal pigments cyanidin and delphinidin. The flavan units are released as carbocations or quinone methide species, which are then converted by oxidation to anthocyanidins. The leucoanthocyanidins were divided by Sir Robert and Lady Robinson into three broad classes (Haslam, 1989). There are those that are water insoluble and not soluble in the usual organic solvents, generally yielding colloidal solutions (Class 1). The other two classes are both water soluble, with the distinction that one group is not extractable from aqueous solution by ethyl acetate (Class 2), whereas the other group is (Class 3). The soluble oligomeric

proanthocyanidins fall into the third class, and are less prevalent when compared to the polymeric forms found in the other two classes (Haslam, 1989). Proanthocyanidins that are covalently bound to a carbohydrate (or any other polymer) matrix within the plant cell belong in class 1. Freudenberg and Weinges (1960) collectively designated all colourless substances isolated from plants which form anthocyanidins when heated with acid as proanthocyanidins. Thus the term proanthocyanidin is a chemical rather than a biochemical term, implying no biogenetic relationship (Haslam, 1989). Weinges *et al.* (1969a) made a clearer distinction between these two terms. They reserved the term leucoanthocyanidins for monomeric proanthocyanidins e.g. flavan-3,4-diols, and the term condensed proanthocyanidins for various flavan-3-ol dimers and higher oligomers. The oligomeric proanthocyanidins produce the red colour observed in acidic extracts from plants. Only condensed tannins are found in sorghum.

Of special interest to the present investigation was the work described by Mattice (1989) and Haslam (1989). The oligomers of procyandins formed by the linear 4 → 8 addition of further flavan-3-ol units, yields only two helical structures due to inherent constraints about the interflavan bond. The central core of these linear polymers is composed of the A and C rings of the flavan repeat unit (see Fig. 2.2), while the *ortho*-dihydroxyphenyl B-ring projects laterally from this core. Those formed from units related to (-)-epicatechin e.g. procyanidin B1 or B2 are left-handed

helices, whereas the procyanidin B3 or B4 formed by the successive additions of (+)-catechin units are right-handed (Haslam, 1989). This work is supported by Mattice (1989) who described these 4β → 8 linked polymers as random coils with dimensions smaller than those of unperturbed polystyrene chains of similar molecular weight. By using stationary state fluorescence and other techniques, he showed that the dimension of the higher polymers (both 4β → 6 and 4β → 8 linkages) were very sensitive to the presence of the two rotational isomers viz. (+)-catechin and (-)-epicatechin at the interflavan bond. The handedness of the helix was dependant on the isomer present. When there were only 4β → 8 linkages, a right-handed helix resulted with 3.0 residues per turn, whereas 4β → 6 linkages produced a left-handed helix with 4.1 residues per turn. A combination of these linkages, yielded a helix of different dimensions. The overall shape and intramolecular spatial arrangement between the phenolic groups showed that these linear procyanidin oligomers were of the right size to fit into the major groove of the DNA helix, while at the same time allowing for H-bonding of the B-ring phenolic hydroxyls with the phosphoryl oxygen of the DNA backbone (Haslam, 1989). The oligomeric structure of the procyanidins could allow for several points of contact and hence cross-linking of the DNA. This could have severe consequences on nucleic acid stability. Furthermore, the proanthocyanidin helix itself could resemble DNA, thereby providing an alternative binding site for mutagens or carcinogens, a point which this work sought to investigate.

2.2 SORGHUM POLYPHENOLS

Phenolic compounds found in sorghum grains (*Sorghum bicolor L Mönch*) can be divided into three groups:

1. Phenolic acids
2. Flavonoids
3. Condensed tannins

2.2.1 Phenolic Acids

Phenolic acids can be classified either as benzoic or cinnamic acid derivatives which have the structures shown in Fig. 2.3.

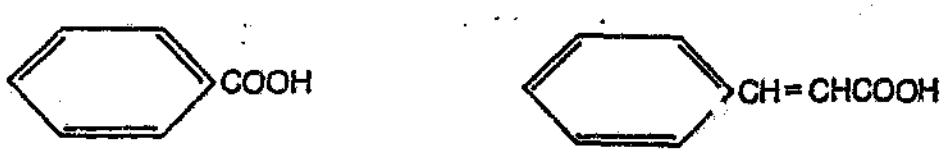


Fig. 2.3. Structures of the benzoic (A) and cinnamic acid (B) derivatives

These derivatives have OH or OCH_3 attached to various sites on the aromatic ring. These phenolic acids can occur either as free acids, soluble or insoluble esters. The free acids and soluble esters are

restricted to the outer layers of the kernel and show great quantitative diversity among the different cultivars, whereas the insoluble, tightly bound phenols that occur in the endosperm are relatively constant from one sorghum grain to another (Hahn, 1984).

2.2.2 Flavonoids

The second group, the flavonoids, are composed of several aromatic rings as depicted in Fig. 2.4.

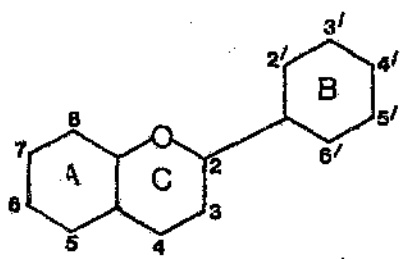


Fig. 2.4 The flavonoid rings, labelled according to the sequence of synthesis.

The A-ring (C_6) is derived from malonyl CoA and the B-ring and part of the C-ring (C_6C_3) from cinnamic acid. The most common A-ring reactions are electrophilic aromatic substitution. Hydroxylation of this ring determines the reactivity and orientation of substitution, partially due to steric effects (McGraw, 1989). The B-ring is

involved in electrophilic additions, metal chelation, oxidation and free radical scavenging (Laks, 1989).

Flavonoids embrace three groups:

(a) flavones

(b) flavonols

(c) flavans (the major group in sorghum)

Within the flavan group, three flavans predominate in sorghum viz. the anthocyanidins (flavan-3-en-3-ols), a major flavan, catechins (flavan-3-ols) or 4-deoxyleucoanthocyandins, and leucoanthocyanidins (flavan-3,4-diols) which are colourless. If a glycoside occurs in position 3 or 7, they are referred to as antho- and leucoanthocyanidins, respectively.

2.2.3 Condensed Tannins

Sorghum cultivars grown in South Africa are particularly rich in tannins, which impart a dark colour to the grain and are thought to be involved in conferring drought, bird and fungal resistance on the grain. Tannins are able to interact with salivary proteins and

glycoproteins giving rise to the characteristic astringent taste of high-tannin grains. It is this ability to bind proteins that inactivates microbial/viral/fungal exoenzymes and hence confers disease resistance on the sorghum grain. Polyphenols can be mobilised in response to wounding and act as toxins at the wound thereby preventing invasion (Haslam, 1979a).

Only condensed tannins (proanthocyanidins) are found in sorghum (Hahn, 1984). Butler (1982) showed that the degree of polymerisation of proanthocyanidins extracted from high-tannin bird resistant sorghum cultivars remained constant and low throughout seed development. However, once the seeds were dried the relative degree of polymerisation increased several fold (also shown by Gupta and Haslam, 1978) suggesting that the bird repellency was due to the relatively short chain oligomeric proanthocyanidins. McGrath and Smith (1990) showed that the sorghum grain used in the present work contained these large chemically reactive polymers. The grain which is consumed in the rural areas of the Transkei where oesophageal cancer is prevalent would be expected to have a high proanthocyandin content since it is stored for extensive periods during which time oxidative polymerisation would be expected to continue.

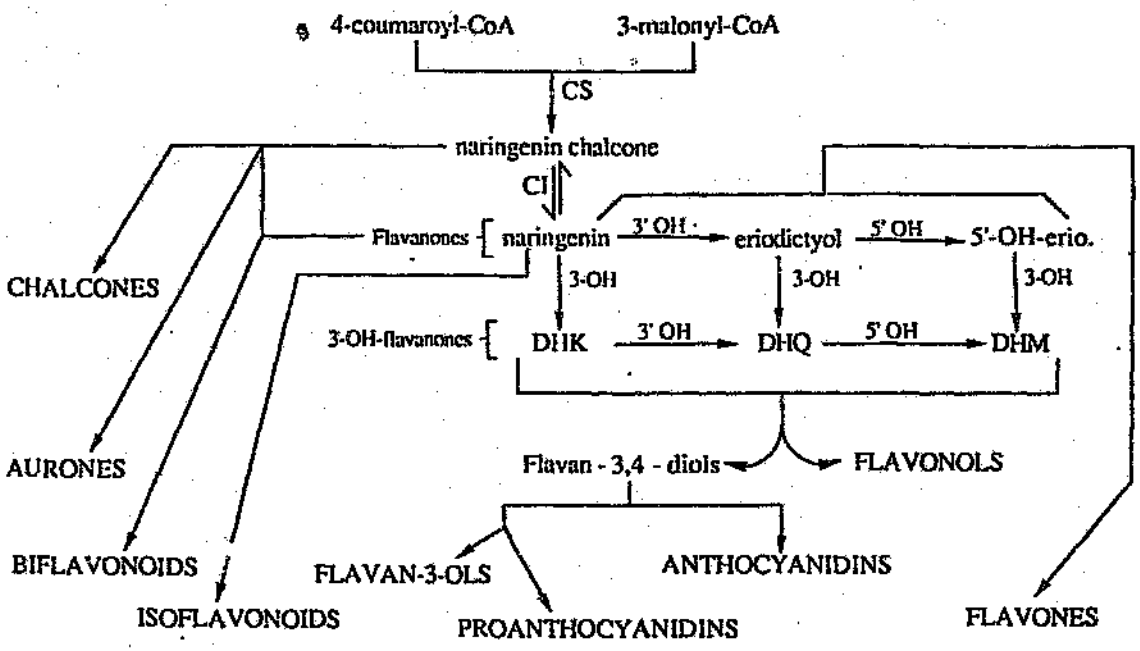


FIGURE 3. Overall pathway to major flavonoid groups with 5,7-hydroxy A-rings (DHK = dihydrokaempferol; DHQ = dihydroquercetin; DHM = dihydromyricetin).

Fig. 2.5 Biosynthetic pathways leading to the variety of flavonoids, some of which are present in sorghum (Stafford, 1990).

2.3 MATERIALS AND METHODS

2.3.1 Sorghum Grain Source

Several cultivars were obtained from Roodeplaat Experimental Farm, Pretoria, South Africa. These included: SSK52, a bird resistant variety, SSK30, SNK3144, PNR8311, NK300 and NK222. Barnard Red, a low polyphenol content variety, was obtained from Tklowve, Potchefstroom, South Africa. The brew packs were received from King Corn, Potchefstroom.

2.3.2 Extraction and Detection of Polyphenols

The polyphenols were extracted from 100g of milled sorghum by allowing the following solvents to percolate through the sorghum which had been placed in a Buchner funnel:

(a) 300 ml chloroform - removes lipids;

(b) 400 ml chloroform:methanol (3:1 v/v) - removes more polar lipids;

(c) 500 ml 80% (v/v) acetone - contains the polyphenols

To quantitate the phenols in the extract and the fractions collected from the column, the method developed by Kaluza *et al.* (1980) was used.

For the assay: 100 µl of sample was made up to 5 ml with H_2O. This was reacted with 1 ml of Folin-Ciocalteu reagent (*Merck*), mixed thoroughly and left for about 1 minute, after which 5 ml of 1 M ethanolamine was added (allows the phenols to reduce the complex). After a further 5 minutes the absorbances were read at 750 nm. The samples should not be left for too long as a precipitate forms, obscuring readings.

Standard Curve: Tannic acid (1 mg/ml stock) was dissolved in methanol, and the concentrations used draw up the curve ranged from 10 µg/ml to 100 µg/ml. The readings for the sorghum polyphenols were read off this curve and expressed as tannic acid equivalents.

2.3.3 Separation of Polyphenols

Fifty grams (wet weight) of Sepharose CL-6B (*Pharmacia*) equilibrated in 10 mM HCl was added to the 80% acetone containing the phenols. The acetone was then removed under vacuum. After all the acetone had been removed (gel must no be dry), the gel was poured into a column (2.5 x 25 cm), which had a 20 mm sepharose plug at the bottom.

Once the column had settled, the following solvents were used as eluants (solutions containing 10mM HCl are referred to as acidic):

(a) 250 ml 10 mM HCl

(b) 250 ml acidic - 80% (v/v) methanol

(c) 250 ml acidic - 80% (v/v) acetone

The flow rate was 0.5 ml/min and 10 ml fractions were collected. The three fractions can be observed as red bands migrating down the column with the eluants.

Three peaks were eluted and designated F_1, F_2 and F_3. The fractions collected were pooled and the volumes of each fraction were reduced under vacuum and then freeze-dried overnight in petri-dishes. The dry weight of each was recorded.

The column can be regenerated by washing with 0.5 M NaOH, followed by several washings with 0.5 M acetic acid to remove any base as the polyphenols oxidize readily under alkaline conditions. A more rigorous cleaning of the Sepharose CL-6B can be obtained by heating the gel in 0.5 M NaOH to 121 ^{0}C in an autoclave for 20 minutes.

2.4 RESULTS AND DISCUSSION

Using the method of Kaluza *et al* (1980) the polyphenols of seven cultivars of sorghum were separated on a Sepharose CL-6B column. All these cultivars were used since it is known that they have different polyphenol compositions, which could possibly account for their varying degrees of susceptibility to fungal attack. Fungal resistance is due not only to the tannin content of the grain but can also be influenced by the presence of benzoic and cinnamic acid phenolic derivatives (Hahn *et al.*, 1983). Hahn *et al.* (1983) observed that the high tannin sorghum grains were found to be more resistant to fungi than the low tannin varieties. Furthermore among the low tannin grains, there were considerable differences in fungal resistance which Hahn *et al.* (1983) postulated must be due to polyphenolic compounds other than tannins, since there was no significant difference in tannin content within this group. These workers found that no simple relationship existed between phenolic acid content and fungal resistance and concluded that resistance must be due both to the polyphenol content and physical properties of the seed. In the present study Barnard Red was included in the series of sorghum cultivars as a non-bird resistant, low-tannin containing cultivar. If the different cultivars affect, to varying degrees, the ability of fungi to grow on them, then it was reasoned that there could be a difference in biological activity in all these varieties, a point which this work sought to investigate.

Most of the sorghum varieties viz. NK222, SSK52, SSK30 and NK300 showed a similar F_3 peak. Of all the varieties, NK222 and Barnard Red showed the largest amount of F_1 (Figs. 2.7 and 2.9). This was expected as these are low-tannin grains. NK222 and SNK3144 had very

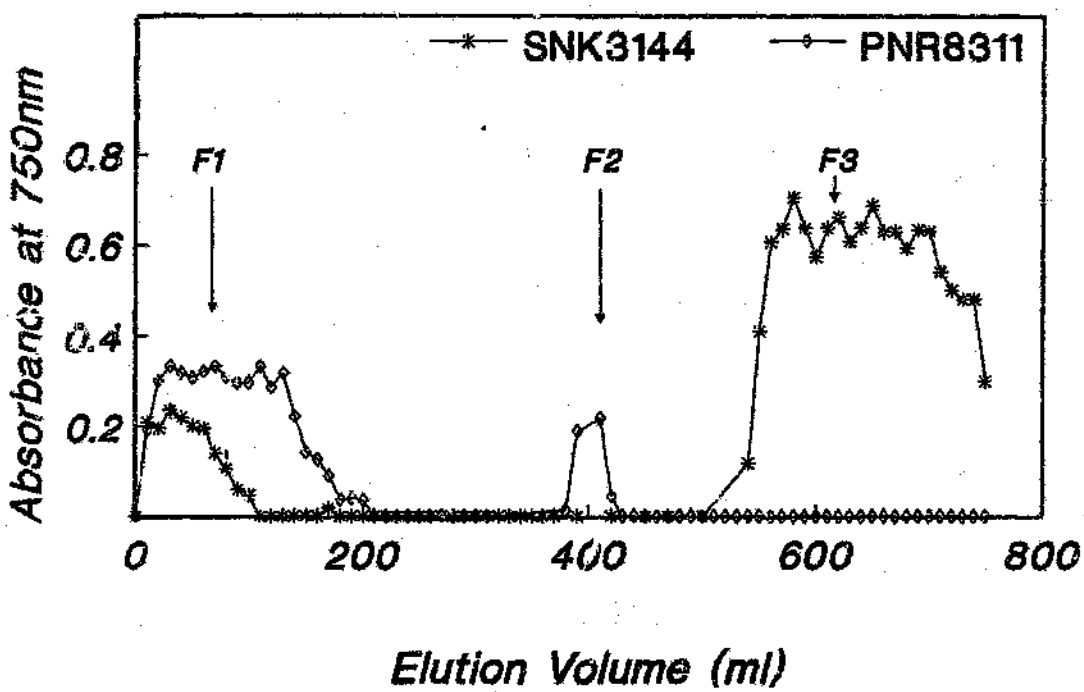


Fig. 2.6 Separation of 80% acetone extracts from sorghum grain cultivars SNK3144 and PNR8311. The three fractions (F_1, F_2 and F_3) were separated on a Sepharose CL-6B column. The absorbance at 750nm was obtained after reacting the phenols with Folin-Ciocalteu and 1 N ethanolamine (see Materials and Methods - Section 2.3).

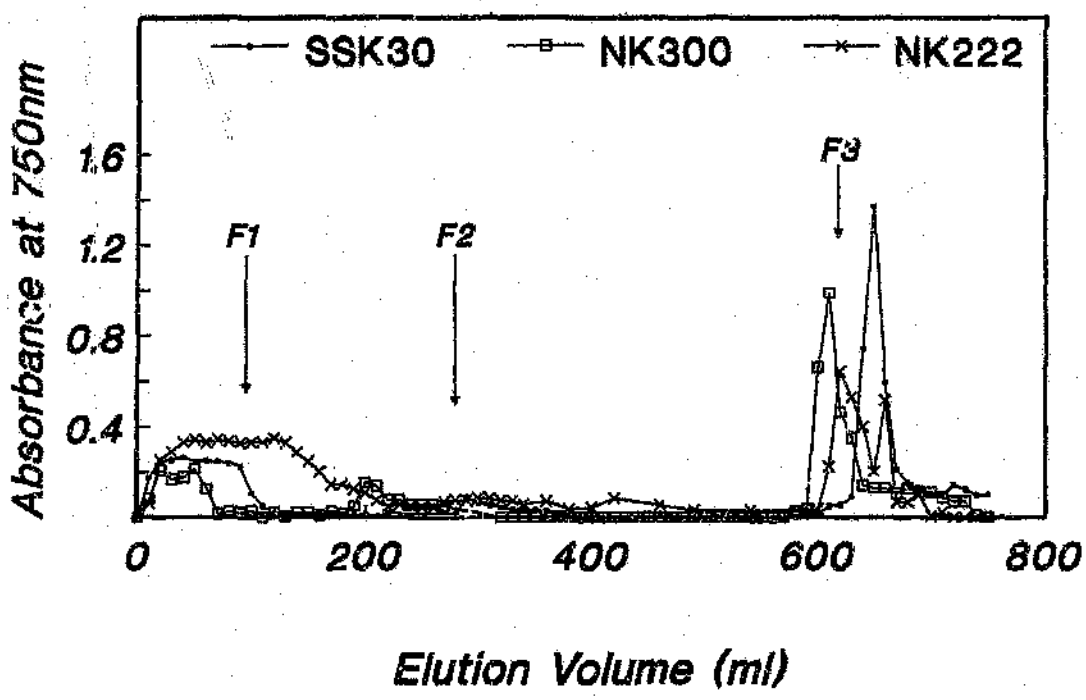


Fig. 2.7 Separation of 80% acetone extracts from sorghum grain cultivars SSK30, NK300 and NK222. The three fractions (F_1, F_2 and F_3) were separated on a Sepharose CL-6B column. The absorbance at 750nm was obtained by reacting the phenols with Folin-Ciocalteu and 1 M ethanolamine (see Materials and Methods - Section 2.3).

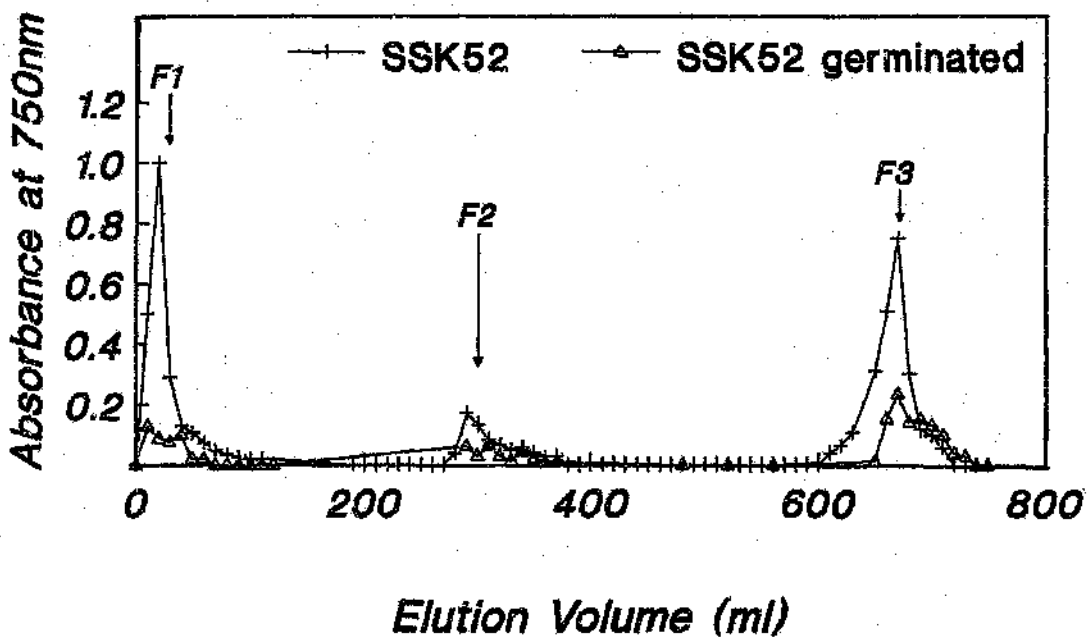


Fig. 2.8 *Separation of 80% acetone extracts from sorghum grain cultivar SSK52. The harvested seeds were also germinated in the lab and then separated into three fractions (F_1, F_2 and F_3) using a Sepharose CL-6B column. The absorbance at 750nm was obtained by reacting the phenols with Folin-Ciocalteu and 1 N ethanolamine (see Materials and Methods - Section 2.3).*

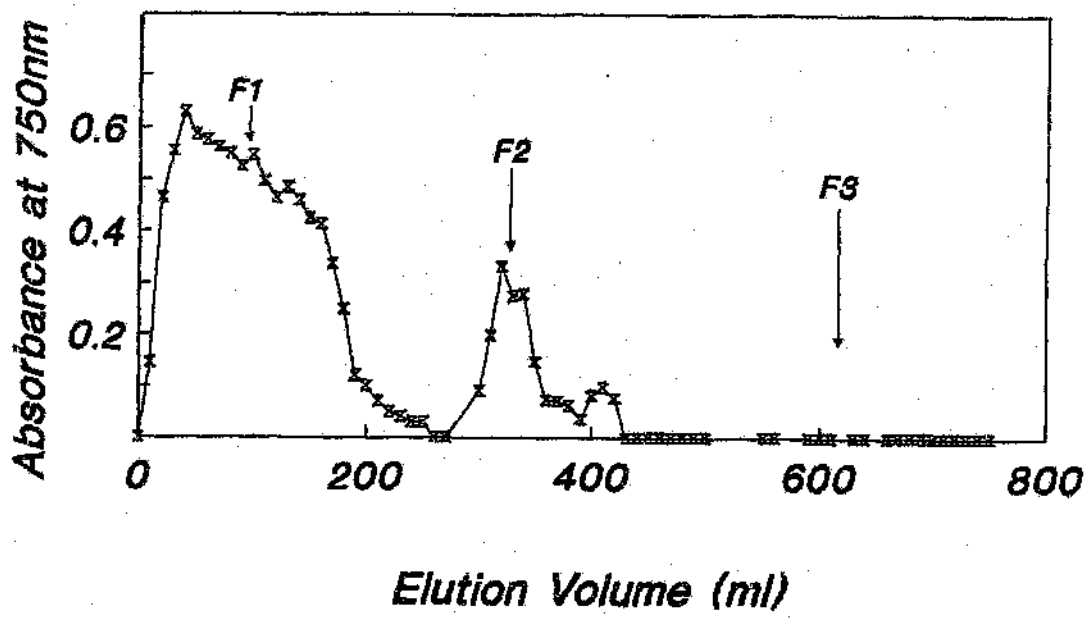


Fig. 2.9 Separation of the 80% acetone extract from Harnard Red. The three fractions (F_1, F_2 and F_3) were separated a Sepharose CL-6B column. The absorbance at 750nm was obtained by reacting the phenols with Folin-Ciocalteu and 1 M ethanolamine (see Materials and Methods - Section 2.3).

low levels of F_2, although the F_3 peak of SNK3144 was quite different from that of the other grains (Figs. 2.6 and 2.7). The F_2 peak of SSK30 and NK300 was very similar, yet they displayed different F_1 peaks. Generally, there appeared to be an abundance of F_1 or F_3 or both, with F_2 peak being quite small by comparison. It was noted by McGrath *et al.* (1982) that germination of the seeds altered the composition and the binding affinities of the polyphenols to column supports. Acetone extracts of harvested and germinated (in this lab) SSK52 were applied to Sepharose CL-6B columns (Fig. 2.8). Almost 40% of the germinated polyphenols could not be recovered from

the column when compared to the 95% recovery from the harvested extract. It has been suggested that germination stimulates the activity of proteinases and carbohydrases (McGrath *et al.*, 1982) which by cleaving the sugars and proteins unmasks more phenolic hydroxyls thereby increasing the binding of the phenolics to the column. It was noted that the column was still coloured by comparison to the one through which the ungerminated extract had been run, indicative of polyphenols still attached to the column. The elution profiles of the polyphenols (Fig 2.8) are similar although the F_1 and F_2 peaks for the germinated seed were much smaller when compared to the ungerminated SSK52 seed. Germination had altered the polyphenols in such a way as to make their recovery from the column impossible, hence they could not be tested biologically. The only form of germinated polyphenols that were tested were those of a sorghum brew mixture which when administered to Drosophila in SMART showed that these were not mutagenic.

Barnard Red sorghum was analyzed and found to contain a large amount of F_1 and some F_2 and no detectable F_3 (Fig 2.9) as expected from a low tannin cultivar. The peculiarity of separation was the turbidity of the 80% extract, which is normally a clear dark red/orange colour. This could not have been a polyphenol-protein precipitate because 80% acetone is used to prevent the coextraction of protein from the grain (Kaluza *et al.*, 1980). Maybe the haze was due to a high starch content. This caused problems in running the column. Instead of adding all of the extract to the gel (as is standard procedure), the volume was first reduced by half (by rotary

evaporation) and then added to the gel and rotary evaporated further, which allowed the column to run well.

Numerous methods have been developed for the separation of phenols and tannins. However, many of these techniques bind the polyphenols so tightly, e.g. polyamide columns, that even after exhaustive washing, there are substantial losses; as much as 70% can be lost (Kaluza *et al.*, 1980). The method devised by Kaluza *et al.* (1980) using Sepharose CL-6B was found to be more suitable for subdividing the crude 80% (v/v) acetone extract from sorghum grain into three complex fractions, F_1, F_2 and F_3, which were used in this work. Sepharose CL-6B, an epichlorohydrin crosslinked polygalactose duplex, contains 6% agarose, and possesses substantial thermal and chemical stability which was particularly useful in the present work where solvents such as acetone and methanol were required to subfractionate the crude extract.

Fraction F_1 contains a complex mixture of small molecular weight phenolics that are water soluble, F_2 comprises the higher molecular weight flavonoids and F_3 the larger proanthocyanidins. The general order of binding on the Sepharose CL 6B column was of the order $F_3 > F_2 > F_1$. Such binding could be explained by hydrogen-bond formation between the phenolic hydroxyls and that of the support, and since the column support is constant, this would imply a difference in the number hydroxyls present in the three fractions; there could be more hydroxyls per molecule or the molecules could be

polymeric. Kaluza *et al* (1980) showed by using a Sephacryl S-200 gel permeation column that the size of the fractions was of the order $F_3 > F_2 > F_1$, although there was a overlap between the fractions. The fact that these fractions exhibit vastly different binding constants on the Sepharose CL 6B column despite overlapping molecular masses, means that size, number and spatial orientation of hydroxyls are important. Elution, however, could not be explained by H-bonding alone (water must always be present to effect elution) since there is an analogous increase in apolarity with size. The fractions are readily removed by aqueous solvents of methanol and acetone i.e. by solvents of decreasing dielectric constant.

The findings of Roux and Evelyn (1958) are in agreement with the observations of Kaluza *et al*. (1980); the high molecular weight tannins showed low mobility in the butanol:acetic acid:water partitioning system. Mobility decreased with an increase in the number of hydroxyls, implying enhanced binding, which would be similar to the pattern observed by Kaluza *et al*. (1980). Roux and Evelyn (1958) observed that the mobility in water of various aglycones of flavonols, flavones and anthocyanidins was low and that modification of the planar C_{15} units by the attachment of sugars e.g. glycosides of flavonols and anthocyanidins, or removal of the double bond in the heterocyclic ring increased water mobility. Variously substituted flavonols showed a decrease in water solubility, whereas addition of an aliphatic hydroxyl in position 4 in place of the carbonyl group increased water solubility. Generally an increase in

phenolic hydroxylation decreased the R_f of the C_{15} compounds in water. Conversely, the R_f value increased with an increase in aliphatic hydroxylation (Roux and Evelyn, 1958). Thus it would appear that separation of the three fractions from sorghum grain cannot be based on the presence of the hydroxyls alone. Hydrophobic factors, possibly due to the heterocyclic aromatic ring of the flavonoids and areas of hydrophobicity in the column need to be taken into account.

Chapter 3. POLYPHENOL BIOLOGY I - MUTAGENICITY

3. POLYPHENOL BIOLOGY I - MUTAGENICITY

3.1 Mutagenicity - An Introduction

To study and test for the possible mutagenic action of the polyphenols present in the various sorghum grain varieties, it was decided to use two well established short-term assays. The two protocols chosen were the Ames test and SMART (somatic mutation and recombination test). These were chosen over the standard animal (*in vivo*) systems for several reasons:

(i) Established carcinogens previously tested in animals have been found to be mutagenic in bacteria. Consequently, one can assume that anything that is mutagenic in bacteria as would be demonstrated in the Ames test can be a potential carcinogen. In fact, it has been predicted that the correlation between carcinogenicity and mutagenicity could be as high as 90% (McCann and Ames, 1976). However, it is not necessarily true to say that all carcinogens are mutagens since not all carcinogens act by causing mutations in the DNA.

(ii) In a survey conducted by Purchase *et al.* (1976) validating several short term tests, the Ames test was found to detect correctly the 120 compounds, which included known carcinogens (91% correct responses) and non-carcinogens (93% correct responses). The Ames assay

was thus able to detect a high percentage of carcinogens accurately without giving many false positives when compared to the other short-term tests surveyed. Furthermore, the bacteria were able to discriminate eight structurally related carcinogen and non-carcinogen pairs used in the study.

(iii) *Salmonella typhimurium*, being prokaryotic, would allow the polyphenols access to the DNA since the number of membrane barriers to be passed would be limited. The sensitivity of the system is greater than that for a eukaryotic assay which means that reasonable and possibly non-toxic levels are sufficient to indicate any biological activity. Any mutation in the DNA could be classified as to the type i.e. base substitution or frame-shift depending on the mutant showing a positive response.

(iv) *Drosophila melanogaster* (used in SMART), on the other hand, is a multicellular, eukaryotic organism and since its 8 (diploid) chromosomes are similar in arrangement to those in man, the results from SMART make extrapolation to man more reasonable. Besides, a comparison of prokaryotic and eukaryotic responses can be made. Dissimilar results could maybe yield some information as to the processing of the compound on

route to the site of action.

(v) Chemical mutagens can be placed into two categories: the directly and the indirectly acting compounds. Direct mutagens are usually electrophiles that are able to interact with negatively charged compounds such as the DNA in the cell and as such can involve covalent binding to the target. Hydrophobic interactions are also possible for intercalative chemicals such as certain antibiotics and chemotherapeutic agents. For the indirectly acting mutagens (promutagens) metabolic activation is required to produce the ultimate carcinogen/mutagen. Such activation is carried out *in vivo* by a group of enzymes known collectively as cytochrome P450s, present predominately in mammalian liver. To mimic such metabolic activation in the Ames test, a crude preparation of cytochrome P450 (in the form of the S9 fraction prepared from rat liver) is incorporated into the mutagenicity assay. An attractive aspect of SMART is that fruitflies, although possessing no liver, do have such a P450 system. Therefore unlike the Ames test, it is not necessary to add the mammalian S9 fraction.

3.2 *The Ames Test - An Introduction*

Once the structure of DNA had been proposed by Watson and Crick in 1953, the field of mutagenesis could be explored. Studies were initially concerned with the types of mutations that occurred and the effects of X-ray and chemically-induced abnormalities. This led scientists to start looking for reliable means of measuring such mutations. Findings of Crick *et al.* (1961) and Whitfield *et al.* (1966) formed the basis of what was to become widely known as the Ames test. Crick had shown that acridine mutagens were able to cause nucleotide additions and deletions in bacteriophages, which led to changes in the reading-frame during the transcription of the DNA. Whitfield *et al.* (1966) were able to demonstrate that ICR (an acridine-like compound) did, indeed, act as frame-shift mutagen in several histidine dependent *Salmonella typhimurium* mutants that had been classified as containing frame-shift mutation sites in the C gene of the histidine operon. ICR was able to cause a change in a base, not necessarily at exactly the same site of the original mutation, such that the correct reading frame was restored in the C gene (Fig. 3.1), allowing for the synthesis of a functional enzyme (coded for by the C gene) involved in the histidine biosynthetic pathway (Ames and Hartman, 1974).

After several years of work, Ames constructed a series of histidine dependent *S. typhimurium* mutants which were classified as TA1535, TA1536, TA1537 and TA1538 (Ames *et al.*, 1973a). These bacteria all

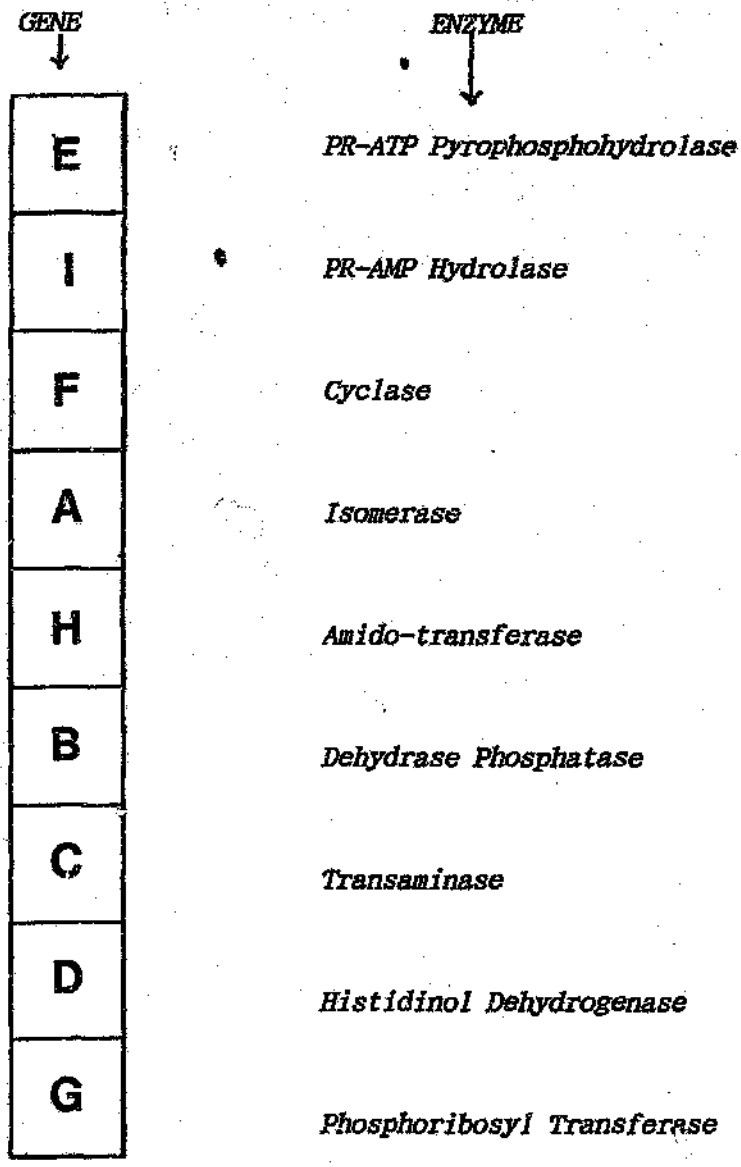


Fig. 3.1 The genes occurring in the histidine operon of Salmonella typhimurium are shown, along with the enzymes (involved in the synthesis of histidine) they encode for (Whitfield et al., 1966). PR = phosphoribosyl.

have the *uvrB* (excision repair) and *rfa* (rough deep mutation affecting membrane permeability) deletions. In addition each strain has strain-specific deletions in the histidine operon. These deletions result in histidine requirement for mutant growth. It is the reversion to histidine independence (his^+) that acts as an indicator of the mutagenicity of a substance. Further work was carried out to enhance the sensitivity of the above series of mutants. This was achieved by the addition of the R-factor (drug resistance plasmid), *pKM101*, giving rise to the mutant strains TA97 (or more recently TA97a which shows better growth qualities), TA98, TA100 and TA102 as indicated in Table 3.1 (McCann *et al.*, 1975 and Levin *et al.*, 1982). This R-factor plasmid carries an ampicillin resistance gene and enhances the effect of the error-prone repair (due to the *uvrB* deletion) system. Strain TA102 also carries a multicopy plasmid, *pAQ1* (which confers tetracycline resistance), which was shown to enhance sensitivity of TA102 to oxidative mutagens (Levin *et al.*, 1982b). The drug resistance conferred on these strains is useful in testing for the presence of the unstable R-factor, which can lost quite readily. The R-factor strains are reverted by mutagens that are weakly or not mutagenic at all in the R-factor deficient parental mutants, the TA1535 series (Maron and Ames, 1983).

3.2.1 Genetic Markers

The following table summarises the genetic features of the bacterial strains used in the Ames test. These features will be discussed in more detail for each bacterial strain in this section.

Table 3.1 The genotype of the bacterial mutants used in the Ames assay is shown. (-) and (+) indicate the absence and presence, respectively, of the rfa and uvrB deletions and the plasmids. Numbers of the histidine mutations denote their position in the genome. In TA102 the histidine mutation is carried on the plasmid, pAQ1.

Deletions		Plasmids		Histidine Mutations			
LPS (*rfa*)	Repair (*uvrB*)	pKM101	pAQ1 (G428)	D6610	D3052	G46	G428
+	+	+	+	97	98	100	
+	-	+	+				102

3.2.1.1 uvrB Deletion

The deletion through the *uvrB* gene includes the deletion of the nitroreductase (*chl*) and the biotin (*bio*) genes. The latter deletion (which cannot be reverted by mutagens) calls for supplementation of the growth with biotin. No excision repair of the DNA is possible with the deletion through the *uvrB* gene. This is useful in detecting DNA damage, e.g. base pair substitution, insertions or deletions caused by mutagens, that would otherwise be repaired and hence the effect of the mutagen not seen.

3.2.1.2 rfa Deletion

When it was observed that di-benzanthracene did not act as a frameshift mutagen, although a closely related carcinogen, benzanthracene epoxide did, it was concluded that maybe the size of the molecule played a role i.e. its entrance was being impeded by the lipopolysaccharide membrane barrier (LPS) (Ames *et al.*, 1973b). Consequently, a *rfa* (rough deep) deletion was made. With this, the LPS was reduced to a ketodeoxyoctonnate lipid core. Thus all four strains contain the *rfa uvrB* deletion, a single deletion through the *gal chl bio uvrB* genes. The deletion results in the partial loss of the LPS, since galactose synthesis stops (because of the *gal* deletion) resulting in a loss of the chain distal to the first galactose unit (Ames *et al.*, 1973b). This loss of the LPS makes the bacteria more permeable and completely non-pathogenic. However,

permeability might still present a problem to large polymers e.g. tannins.

3.2.1.3 pKM101

Plasmid *pKM101* is 35.4 kb long and is derived from plasmid R46 by a single, *in vivo*, deletion of a 14 kb region. Both are naturally occurring plasmids, which, when present in bacteria such as *Escherichia coli* and *Salmonella typhimurium* have been found to make these bacteria more resistant to killing by UV exposure through a process of enhanced (non-lethal) mutagenesis (Mortelmans and Stocker, 1976). The effects in *E. coli* are dependent on a *recA lexA* genotype as is the case in the SOS repair system inducible by DNA damage, and which becomes operational only when the normal repair system becomes non-functional e.g. when overwhelmed by exposure to UV irradiation or a mutagen. The plasmid does not, however, act via SOS induction because it was found the *pKM101* does not cause *recA* induction, the product of which is essential in relieving inhibition imposed in the SOS repair pathway when the normal repair pathway is operative (Lackey *et al.*, 1977; Goze and Devoret, 1979 and Langer *et al.*, 1981). Since one effect of SOS repair is enhanced mutagenesis, one could imagine that the *pKM101* could code for an enzyme involved in an error-prone excision repair as is the case in the SOS response. So far a *muc* gene, which has its analogue in SOS as *umuC*, has been cloned (Perry and Walker, 1982). Thus the plasmid codes for a *muc* gene product inducible by mutagen-induced DNA damage. The

cell would preferentially "correct" the damage by some error-prone system rather than ignore it, thereby preventing cell death. Once the "correction" has been made, replication can continue past the mutagen-induced irregularity, and so becomes fixed in the genome, which in the Ames test would be evident as a reversion to histidine independence.

3.2.1.4 pAQ1

TA102 carries the *pAQ1* plasmid which contains the his G428 mutation and the tetracycline resistance gene. Is has been established that 30 copies of the plasmid are present which implies that 30 copies of the his$^-$ mutation are available for reversion, accounting for the increased sensitivity of this strain to oxidative mutagens (Levin *et al.*, 1982b). All the other strains carry the histidine deletions in the histidine operon region.

3.2.2 Bacterial Strains

3.2.2.1 TA 97/97a

This mutant has extra cytosines added to the run of four cytosines in the wild type, resulting in six consecutive cytosines at the mutated site in the his D gene (Maron and Ames, 1983). TA97 has a second *hot spot* of alternating -GC- base pairs, several codons to

..e 3' side of his D6610 (Levin *et al.*, 1982). *Hot spots* are highly repetitive sequences in the DNA that are prone to spontaneous and induced misrepairing and mismatching, and are the favourite sites of action for frame-shift mutagens (Levin *et al.*, 1982). His D6610 being very similar to the mutational *hot spot* near the his D3052 of TA98, is equally sensitive to fluorene derivatives.

Reversion of the his D6610 by a single base-pair deletion gives rise to a pseudo wild-type histidinol dehydrogenase displaying 60% wild-type activity. However, this activity is sufficient for normal growth in well-defined medium.

3.2.2.2 TA98

In TA98, the his D3052 mutation, containing the -CGCGCG- sequence is reverted by a -CG- deletion. The mutation occurs in the his D gene responsible for histidinol dehydrogenase synthesis (Fig. 3.1). This enzyme catalyses two dehydrogenation steps in the histidine biosynthetic pathway.

The mutant is able to detect frame-shift mutagens that stabilize the shifted pairing that occurs in the highly repetitive DNA sequences. Reversion can be brought about by nitroso-fluorene, aflatoxin B_1, benzo(a)pyrene and daunomycin.

3.2.2.3 TA100

The his G46 mutation in TA100 is in the his G gene. This gene codes for the first enzyme (phosphoribosyl transferase) in histidine synthesis (Ames, 1971; Lehninger, 1975). In the mutated state this sequence substitutes proline (-GGG-) for leucine ( ) in the wild-type (Barnes *et al.*, 1982). Mutagenesis causing base-pair substitution can be detected.

3.2.2.4 TA102

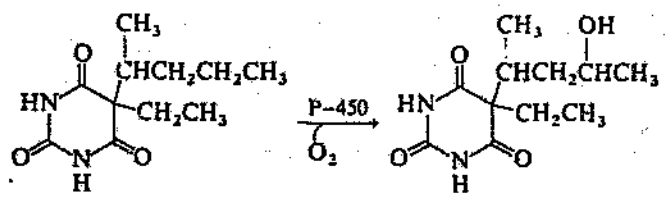
A recent addition to the tester set is TA102. The [illegible] for such a strain arose from the observation that many known oxidative mutagens were weakly detected or not at all by the other strains (Levin *et al.*, 1982b). It differs from the other strains in three aspects. Firstly, an adenine-thymine pair occurs at the mutation site rather than a guanine-cytosine pair. Secondly, a his G428 mutation has been introduced with the multicopy plasmid pAQ1. The mutation site is the -TAA- sequence in the his G gene. Lastly, it has an intact DNA excision-repair system, i.e. no uvrB deletion. It is this intact system that allows for the detection of mitomycin C, a DNA cross-linking agent. If no repair facilities were present, the genome could not replicate itself, leading to cell death. It is for this reason that this mutagen is lethal in the other three strains used in the Ames test (see Table 3.2). The error-prone uvrB excision repair converts the potentially lethal mutation caused by

mitomycin C into a second non-lethal mutation, evident as a reversion. Mitomycin C is included as a positive control in the mutagenesis assay for TA102. Other chemicals detected by TA102 include formaldehyde, glyoxal, various hydroperoxides, bleomycin, quinones (some requiring metabolic activation) and phenylhydrazine (Levin *et al.*, 1982b).

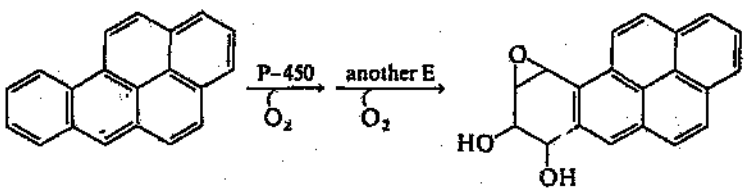
3.2.3 Metabolic Activation

To expand the mutagen (putative carcinogens) detecting capacity of the Ames assay, a metabolic activating system (extracted from liver in the form of the S9 fraction) can be incorporated. Potentially inactive compounds (promutagens) can be converted by this cytochrome P450 (monooxygenase enzymes) system to reactive electrophiles able to interact with proteins, RNA or DNA. Several workers have studied the components present and how these interact to activate various substrates (Guengerich 1977, 1979 & 1991; Guengerich *et al.*, 1982; King and Wiseman, 1987). There are basically three components: NADPH cytochrome P450 reductase, phosphatidylcholine and cytochrome P450, the terminal oxygenase and determinant of substrate specificity (Wright, 1980). The reactions catalysed by the monooxygenases include aromatic and aliphatic hydroxylation, arene and alkene oxide formation, oxidative N-, O-, and S-dealkylation, sulphoxidation, oxidative deamination, desulphuration and dehalogenation (Wright, 1980)and are indicated on the next page.

hydroxylation



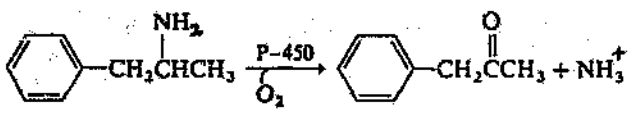
pentobarbital
(sedative; general anesthetic)



benzo[a]pyrene

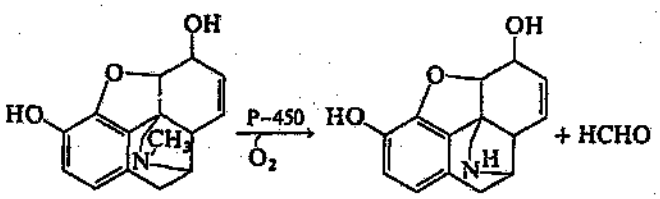
diol epoxide
(DNA mutagen and
protein modifier)

oxidative de: .nation



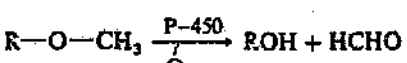
amphetamine

N-dealkylation

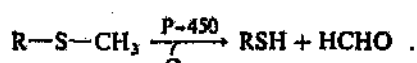


morphine

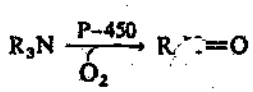
O-dealkylation



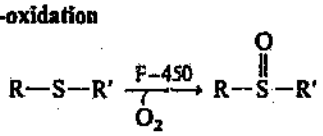
S-dealkylation



N-oxidation



S-oxidation



(reprinted from Bohinski, 1987)

The cytochrome P450 isozymes (each arises from a different gene) are a family of hemoproteins displaying a characteristic peak at 450nm. When liver microsomes are treated with sodium dithionite and then with carbon monoxide, a very well defined peak appears at 450nm, and because the extinction coefficient is known, it is possible to determine the concentration of cytochrome P450 by absorbance spectrophotometry (Omura and Sato, 1964). Some P450 enzymes display a wide substrate specificity whereas others are more selective, and in some cases overlapping substrate specificities have been shown (Guengerich, 1979). There are marked tissue and species difference in the activities and distribution of the monooxygenases, which can vary with age of the animal and with inducer pretreatment. Substrates acted on can be of both endogenous and exogenous (xenobiotic) origin. The endogenous compounds include fatty acids and steroids whereas the xenobiotics embrace drugs, carcinogens and mutagens. Generally, the reaction can be summarised as follows:

$$SH + NADPH + H^{+} + O_2 \rightarrow SOH + NADP^{+} + H_2O$$

where SH = substrate and SOH = hydroxylated substrate. Molecular oxygen is made more reactive so that it can attack inert and poorly soluble (hydrophobic) substrates, converting these into hydrophilic species that can be excreted from the body (King and Wiseman, 1987). Under certain circumstances the activity of these enzymes will convert inactive compounds into highly reactive species which are not excreted. Many lipophilic compounds such as aromatic amines

undergo oxidation reactions via the monooxygenases despite alternative routes for hydrophilic conversion. The addition of oxygen to the amino-N atom to form a N-hydroxy derivative, is the first step in the mutagenic activation of aromatic amines. Such activation can be sterically hindered by the presence of two protons ortho to the amino group, which accounts for the different activities of this group of chemicals in tumour induction. Once such activation has occurred, the functional groups could conjugate via transferase action to endogenous conjugating agents such as glucuronic acid (Wright, 1980). Such conjugation would limit further biotransformations and lead to rapid excretion. However, exceptions were found in studies of 2-aminofluorene activation (Weisburger and Weisburger, 1973). Despite glucuronidation or sulphation of N-hydroxyamides, these can still interact with the DNA. Although the sulphuric acid ester is the major ultimate carcinogenic metabolite, it was found that the deacetylated derivative of N-hydroxy-2acetyl-aminofluorene viz. N-hydroxy-2-aminofluorene was ultimately responsible for the mutagenicity of 2-acetylaminofluorene (Sakai *et al.*, 1978 and Johnson *et al.*, 1980). Cytochrome P450 thus provides other enzymes with substrates to act on. The reverse occurs as demonstrated by the metabolism of the promutagen benzo(a)pyrene (B(a)p), a standard used in the Ames test, where epoxide hydratase provides a substrate for P450.

B(a)p is metabolised (Fig. 3.2) by the combined action of the P450 system and epoxide hydrase (Darnell, 1986 and Wiebel *et al.*, 1975).

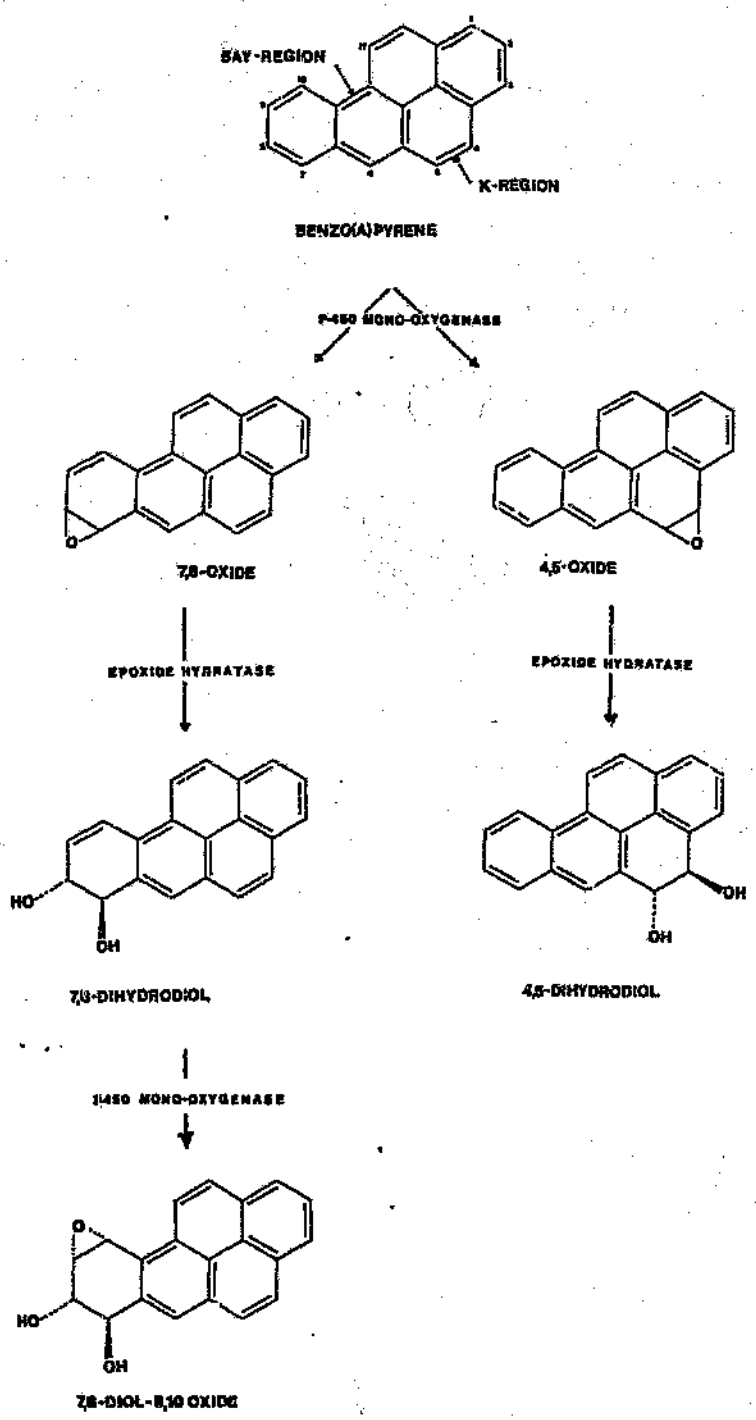


Fig. 3.1 Benzo(a)pyrene metabolism by various cytochrome P450 isozymes, which can lead to two end products: 7,8 diol-9,10 oxide is an electrophilic carcinogen, whereas 4,5-dihydrodiol is not reactive towards DNA (Harvell, 1986).

It was observed that two products resulted, either 4,5 dihydrodiol (non-reactive) or 7,8 diol-9,10-oxide (the ultimate carcinogen) depending on which P450 isozyme (dependant on the inducer administered) is present. When phenobarbital was used to induce rat liver, $P450_{LM2}$ (numbered according to electrophoretic mobility, the letters referring to source, here liver microsomes) was isolated and shown to be involved in the formation of b(a)p-4,5-diol. β-Naphthoflavone (5,6 benzoflavone) induced $P450_{LM4}$. The 3-methylcholanthrene inducible P450, $P450_{LM4}$, preferentially catalyses 9,10-epoxidation of the substrate, leading to the formation of the ultimate carcinogen (Robertson *et al.*, 1983; Hr.gen and Coon, 1976). Normally hydration by epoxide hydratase is a deactivation step resulting in 7,8-oxi) which cannot bind to macromolecules in the cell. But in the presence of the appropriate epoxide hydratase, this metabolite can act as a monooxygenase substrate (Kapitulnik *et al.*, 1978).

In the preparation of S9 to be used in the Ames test, Aroclor 1254 (a polychlorinated biphenyl) is recommended as the most efficient inducer because it produces the largest array and amounts of P450 isozymes, allowing for the widest possible detection of promutagens (Maron and Ames, 1983). Yoshikawa *et al.* (1982) found that a combination of phenobarbital and β-naphthoflavone produced an S9 preparation effective in activating a wide range of substrates, including b(a)p (Eisen, 1986; Haung *et al.*, 1981; Lu and West, 1980 and Suwa *et al.*, 1985) and was used in the present work.

Generally, S-glutathione transferases, present mainly in the cytosol (although membrane forms have been found) and epoxide hydratases are involved in deactivation pathways (Wright, 1980). Both these enzymes are present in S9 fractions. Epoxide hydratase has no cofactor requirement and as such should be fully functional. On the other hand, due to dilution and oxidation during S9 preparation the transferase is inactivated (Dean *et al.*, 1978). Pretreatment of rats with inducers leads to an increase in certain enzymes, with the increase in P450 enzymes being the greatest. Thus the balance between the activation and deactivation pathways is inclined towards the former pathway in pretreated animals. It can, therefore, be argued that susceptibilities to cancer induction in the human population depend to a large extent on the type of cytochrome P450 present in the individual (which can be influenced by dietary and genetic factors) and the interaction of other enzymes with these cytochromes and their end-products.

Mammalian cytochrome P450 enzymes are most abundant in liver, but are also distributed in other organs in the body. P450 occurs in both prokaryotes and eukaryotes. In eukaryotes most of the P450 is located in the endoplasmic reticulum (ER). When the enzymes are isolated, homogenisation of the ER causes small vesicles to form known as microsomes, hence the name microsomal S9. S9 enzymes are present in the nuclear envelope and cell membrane, where they could play a role in the metabolism of carcinogens on route to the genome.

The electron transport chain for the microsomal cytochrome P450 comprises two proteins: cytochrome P450 and cytochrome P450 reductase. Cytochrome P450 (a hemoprotein) contains a protoporphyrin IX group. The reductase is a flavoprotein containing one FAD^{+} and one FMN^{+} nucieotide, the prosthetic groups involved in the oxidation-reduction reactions. In 1980, White and Coon proposed a model for the structure of the active site of cytochrome P450 (Fig. 3.3).

The Active Site and Catalytic Cycle of Cytochrome P450

The protoporphyrin IX ring is located in a hydrophobic cleft and is bound by hydrophobic and electrostatic forces. A sulphur from a cysteine residue in the protein is ligated to the Fe^{3+} in the plane of the heme ring. The iron atom contains five electrons in the outer d-orbital and depending on the spin-pairing can exist in either a low or high spin state (King and Wiseman, 1987). In the low spin state the iron exists as a 6 coordinated heme iron and in high spin as a 5 coordinated atom. Intact microsomes contain an equal mixture of these two spin states. Before the substrate binds, the system is in a low spin state and at a negative redox potential. Upon binding, the substrate induces a spin-state change, resulting in the high spin form. Because the high spin state is at a less negative redox potential, the electrons can flow more readily to the cytochrome P450. It was observed that NADPH cytochrome P450

reductase and cytochrome b_5 can modify the cytochrome P450 spin state. The latter observation proved to be interesting since liver microsomes contain two electron transfer systems that could potentially interact with each other; the aforementioned NADPH P450 reductase:cytochrome P450 and a NADH cytochrome b_5 reductase : cytochrome P450, components of the stearyl CoA desaturase system (Bosterling and Trudell, 1982 and Bosterling *et al.*, 1982).

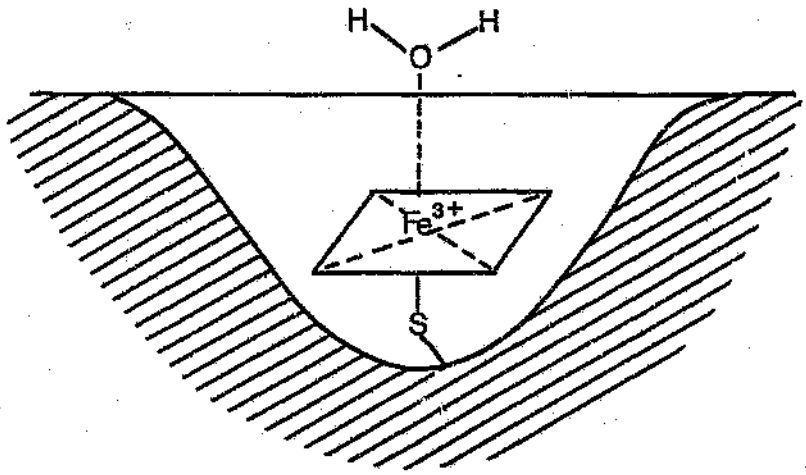


Fig. 3.3 The proposed active site structure of cytochrome P450, showing the iron bound to the sulphur from a cysteine residue in the P450 protein (White and Coon, 1980).

Many researchers have shown that cytochrome b_5 does interact with P450 and depending on the substrate used, this interaction could either be inhibitory or stimulatory, and in some cases no effect was observed (Chiang, 1981; Hoeven and Coon, 1974; Ingelman-Sundberg, 1986 and Prkrovsky *et al.*, 1977 and Yang *et al.*, 1978). The multiple forms of cytochrome P450 could also explain the difference in response to the presence of cytochrome b_5. There could be those requiring cytochrome b_5 mediated transfer of the second electron for oxygenation, whereas other P450's wo. . receive their electrons from NADPH cytochrome reductase (Fig. 3.4).

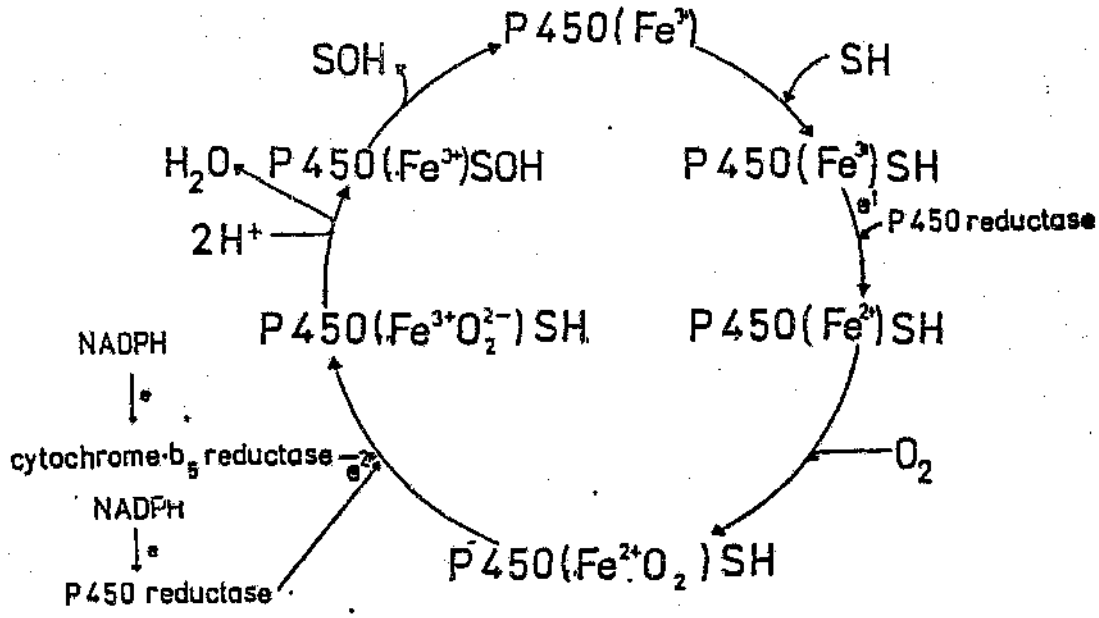


Fig. 3.4 The cytochrome P450 catalytic cycle in which SH is the substrate and SOH the hydroxylated substrate. The first electron (e^1) is donated via the NADPH:P450 reductase, whilst the second electron (e^2) can be received as e^1 or via the NADPH cytochrome b_5 reductase (King and Viseman, 1987).

3.3 THE AMES TEST - MATERIALS AND METHODS

3.3.1 Solutions

All solutions were autoclaved at 121 ^{0}C for 20 minutes and stored at 4 ^{0}C until required.

BROTH:

	per litre
Lab-Lemco Powder (Oxoid 2)	10 g
Peptone (Oxoid)	10 g
NaCl	5 g

This broth was used to culture the bacteria (See Section 3.3.3). For nutrient plates, 15g/litre of agar was added (See section 3.3.5.2 - 3.3.5.5).

MINIMAL GLUCOSE PLATES:

	per litre
Agar	15 g
Distilled H_2O	930 ml
50X VB salts	20 ml
40% (w/v) glucose	50 ml
0.26 mM L-histidine	10 ml

Used for mutagenicity assays (See Section 3.3.6 and 3.3.8).

VOGEL-BONNER SALTS (50VB):

	per litre
Distilled H_2O (at 45 ^{0}C)	670 ml
$MgSO_4.7H_2O$	10 g
Citric acid, monohydrate	100 g
K_2HPO_4	500 g
$NaNH_4HPO_4.4H_2O$	175 g

All ingredients were added individually, allowing each salt to dissolve before adding the next, and the solution made up to 1 litre. This solution was used to make up the minimal glucose plates.

TOP AGAR:

	per litre
Agar	6 g
NaCl	5 g
Distilled H_2O	1000 ml

The top agar was best prepared in 50 ml aliquots. For mutagenicity assays, 5 ml of a solution containing 0.5 mM histidine and 0.5 mM biotin was added to 50 ml of melted top agar, which was kept at 45^{0}C until required. Two ml of agar

was used per plate.

$MgCl_2$ - KCl:

	per litre
$MgCl_2$. 6 H_2O	81.4 g
KCl	123 g
Distilled H_2O	up to 1 litre

This solution was used to prepare the S9 mix.

S9 MIX:

	per 50 ml
Sterile distilled H_2O	19.75 ml
0.2 M potassium phosphate buffer, pH 7.4	25.00 ml
0.1 M $NADP^+$	2.00 ml
1 M glucose-6-phosphate	0.25 ml
$MgCl_2$-KCl	1.00 ml
Liver microsomes (S9)	2.00 ml

The ingredients were added in the order indicated so that the S9 was buffered. This mix was kept on ice till required. Left over S9 was discarded. The volume of liver microsomes

added depended on the protein concentration in the liver extract, here assumed to be 40mg/ml.

AMPICILLIN SOLUTION:

	per 100 ml
Ampicillin trihydrate	0.8 g
0.02 N NaOH	100 ml

This antibiotic was used to test for the presence of the R-factor plasmid, pKM101 (See Section 3.3.5.4).

TETRACYCLINE SOLUTION:

	per 100 ml
Tetracycline	800 mg
0.02 N HCl	100 ml

This antibiotic was used to test for the presence of the pAQ1 present only in strain TA102 (See Section 3.3.5.5).

CRYSTAL VIOLET SOLUTION:

	per 100 ml
Crystal violet	0.1 g
Distilled H_2O	100 ml

This solution was covered with foil to protect it from the light and used to test for the presence of the rough deep mutation of the bacteria (See Section 3.3.5.3).

HISTIDINE AND BIOTIN PLATES:

	per litre
Agar	15 g
Distilled H_2O	914 ml
50X VB salts	20 ml
40% (w/v) glucose	50 ml
Sterile 0.26 mM histidine	10 ml
Sterile 0.5 mM biotin	6 ml

These plates were used to test for histidine requirement (See Section 3.3.5.1).

AMPICILLIN/TETRACYCLINE PLATES:

	per litre
Agar	15 g
Distilled H_2O	910 ml
50X VB salts	20 ml
40% (w/v) glucose	50 ml
Sterile 0.26 mM biotin	10 ml

Sterile 0.5 mM histidine	6 ml
Ampicillin solution	3.15 ml
*Tetracycline solution	0.25 ml

The salt and glucose solutions were added after the agar had been autoclaved. These plates were used to test for ampicillin (*tetracycline - for TA 102 only) resistance. (See Section 3.3.5.4 and 3.3.5.5.).

3.3.2 Bacterial Strains - Source

TA97, TA97a, TA98, TA100 and TA102 were kindly supplied upon request by Professor Bruce Ames, Berkeley, California.

3.3.3 Culturing Bacteria

Bacteria were cultured in nutrient broth and were allowed to grow to a density of 10^9 cells/ml on a rotary shaker kept at 37 ^{0}C. To score the total number of colonies and any toxicity effects of the polyphenols, the bacteria were serially diluted (with 0.9% NaCl) and plated onto nutrient agar plates, then incubated at 37 ^{0}C for 48 hours before the colonies were counted.

For the inoculation of cultures for the Ames test, frozen 0.5 ml

aliquots of bacteria were used. These aliquots were stored at -70 ^{0}C which obviates the use of master plates. Regular testing of the genotype and mutagen response of the bacteria was carried out (See Sections 3.3.5 and 3.3.6).

3.3.4 Preparation of Frozen Cultures for Inoculation

Bacteria were grown up overnight to a density of 10^{9} cells/ml in the presence of antibiotics - ampicillin for all strains, and additionally tetracycline for TA102. Dimethylsulphoxide (DMSO) was added to the cultures as a cryoprotective agent at 0.09ml DMSO/ 1 ml of culture. These were then dispensed into sterile eppendorf tubes and quickly frozen on a bed of crushed dry ice. Once frozen the tubes were transferred to the -70 ^{0}C freezer for storage. Strains stored this way were found to be stable (according to the tests performed in Sections 3.3.5 and 3.3.6) for at least one year.

3.3.5 Genotype Testing

3.3.5.1 Histidine Requirement

The cultures were plated out on selective minimal glucose plates containing biotin (final plate concentration - 3μM, because the biotin gene deletion does not revert), with and without histidine

(25µg/ml). There was no growth on the histidine free plates.

3.3.5.2 uvrB Mutation

This mutation makes the bacteria susceptible to the lethal effects of UV light. Strains were plated onto the nutrient plates in parallel streaks. Several strains could be tested on one plate. Half of the plate was covered with foil so that half of each bacterial streak was covered and the other half open to irradiation with a 15W germicidal UV lamp at a distance of 33 cm for 60 seconds. The irradiated plates were incubated for 24 hours at 37^{0}C. Since TA102 has an intact UV repair system it should grow on both halves, whereas the other strains viz. TA97, TA98 and TA100 should only grow on the unirradiated side. This was observed for all strains.

3.3.5.3 rfa Mutation

Because crystal violet is a large molecule, it will only be able to penetrate the membrane of *rfa* mutants. Strains with the *rfa* character will be killed by crystal violet.

One hundred microlitres of a tester strain was added to 2 ml of top agar at 45 ^{0}C containing 0.5mM histidine and 0.5mM biotin, vortexed briefly and then spread evenly across a nutrient plate. After allowing the top agar to harden, a sterile filter paper disc (approximately 0.5 cm in diameter) containing 10µl of a 1 mg/ml

crystal violet solution, was placed firmly on the seeded plate. The plate was inverted and incubated for 12 hours at 37 ^{0}C. A clear zone of inhibition around the disc indicated the presence of *rfa* mutants.

3.3.5.4 pKM101

TA97, TA98, TA100 and TA102 all contain the R-factor, carrying the ampicillin resistance gene. As the plasmid is unstable and readily lost, the strains were routinely screened for the presence of the plasmid by their sensitivity to ampicillin.

The bacteria were streaked across ampicillin plates (25 µg/ml final (plate) ampicillin concentration) and their growth compared to plates containing no ampicillin. Alternatively, a method similar to that used to test for the *rfa* mutation is applicable, in which ampicillin containing discs were placed on seeded plates. Absence of zones of inhibited growth around the discs indicated ampicillin resistance.

3.3.5.5 pAQ1

TA102 is also tested for tetracycline resistance (*pAQ1* carries the tetracycline resistance gene). The procedure is the same as for ampicillin testing.

3.3.6 Spontaneous and Standard Control Values

A trace of histidine was added to minimal glucose plates during mutagenicity testing to allow a background lawn of auxotrophs to develop. The absence of this lawn was also indicative of toxicity of the test compound under investigation. Characteristic of each tester strain is the number of spontaneous revertants evident against this lawn. The final number of auxotrophs influences this reversion frequency, but since the histidine content is kept constant, the number of auxotrophs will be consistent. Spontaneous reversion is independent of the initial inoculum size within the range 10^5 - 10^8 cells (Green and Muriel, 1976). The following ranges have been observed in several labs (Ames *et al.*, 1975 and Maron and Ames, 1983): revertants/plate (no S9); TA97 (90-180), TA98 (30-50), TA100 (120-200) and TA102 (240-300). Values in this work fell within the ranges given, and more importantly were constant from experiment to experiment. If there was any significant increase or decrease in these values, the strains were re-isolated from the frozen master copies. The addition of S9 increased the frequency slightly.

Standard Mutagens

To check the response of the various strains in this work, several standard mutagens, some requiring S9, were included in every experiment (as shown in Table 3.2 - Spontaneous revertant numbers have been subtracted). Mitomycin C is lethal in all strains with the *uvrB* deletion because it causes DNA cross-linking, a lethal

mutation, which requires excision repair (*uvrB* gene) to convert the mutation to a second, non-lethal mutation in TA102. Values are those reported by Maron and Ames (1983).

Table 3.2 Several of the standard mutagens included in each experiment are shown, some requiring S9 (+) and others not (-). The number of revertants (per plate) induced by each of the mutagens in the four strains is indicated.

			Strains			
Mutagen	µg/100µl*	S9	TA97	TA98	TA100	TA102
Sodium azide	*1.5*	-	*76*	*3*	*3000*	*188*
Daunomycin	*6.0*	-	*124*	*3123*	*47*	*592*
Mitomycin C	*0.5*	-	*0*	*0*	*0*	*2772*
2 Aminofluorene	*10.0*	+	*1724*	*6194*	*3026*	*261*
Benzo(a)pyrene	*1.0*	+	*337*	*143*	*937*	*255*

* *Added to top*

3.3.7 Induction and Excision of Rat Liver Enzymes

Since Aroclor 1254 (the recommended inducer) is a very stable carcinogen and expensive to obtain here, it was decided to use an alternative inducer. Matsushima *et al.* (1976) found that a combination of ß-napthoflavone and phenobarbital produced induction similar to Aroclor both with respect to the amount of protein induced and the ability to catalyse a wide array of substrates in the Ames test. The ß-napthoflavone at a concentration of 8 mg/100g rat body weight was dissolved in warm olive oil and then injected intraperiotineally. Phenobarbital was administered daily in the drinking water at 1 g/litre. The rats were killed by anaesthetising with ether, and the livers removed aseptically.

Preparation of the S9 Fraction:

All the steps were carried out using sterile glassware and cold (on ice) sterile solutions. After the livers were excised, they were weighed in tared beakers, to which 0.15 M KCl was added at 1 ml of KCl per gram of wet liver. The livers were then washed several times in 0.15 M KCl so as to remove any haemoglobin that could inhibit cytochrome P450 (present in this S9 extract) activity. Then the livers were added to a beaker containing 3 vol of 0.15 M KCl/g wet liver and minced with sterile scissors. After this the livers were homogenised and the homogenate centrifuged at 9000g. The supernatant was poured off and saved in 2 ml aliquots in small plastic tubes that are able to withstand very low temperatures.

These aliquots were frozen quickly on a bed of crushed dry ice and stored either in liquid nitrogen, when available, or at -70 ^{0}C. To prepare the S9 mix, the required aliquots were allowed to thawed at room temperature and then kept on ice. To check the sterility of the S9 preparation, 0.5 ml was added to top agar containing histidine and biotin (see top agar) and then poured onto minimal glucose plates. The protein concentration was determined by the Lowry method (Lowry *et al.*, 1952) and was found to be 30 mg/ml. The standard curve for protein determination was done using bovine serum albumin as standa d.

3.3.8 Ames Mutagenicity Assay

For each assay, a fresh overnight culture was used, and the method of Maron and Ames (1983) followed. All four strains were used to test the polyphenols from the different sorghum varieties, which had been separated into three crude fractions (See Chapter 1). The highest concentration used represented the maximum solubility of these polyphenols. They were dissolved in 80% (v/v) methanol for the assay, as a 80% methanol blank showed that this did not affect bacterial viability. With each assay, well defined histidine enriched plates (containing diluted bacteria and test compound) were included to test for toxicity, as examination of the background lawn is quite subjective. Standard mutagens were included in each run to check the response of the strains.

The 2 ml of top agar containing histidine and biotin (see top agar) were dispensed into sterile capped test tubes and held at 45 ^{0}C in a water bath. To this 100µl of mutagen/polyphenols at several concentrations, 100µl of bacteria (and where required 500µl of S9-mix) were added, vortexed at low speed and poured onto the minimal glucose plates. These were left to set on a level board and within an hour, inverted and placed in a 37 ^{0}C incubator. After 48 hours the plates were scored. For plates with few colonies, all the colonies were counted and when the number exceeded 200, several 1 cm^2 squares were counted, averaged and then multiplied by the surface area of the petri-dish (radius = 4.25 cm) to obtain the final number of revertants/plate.

3.3.9 Statistical Treatment of Data

Students t-test was carried out for the Ames test with the different levels of polyphenols to determine if the increase in revertants above the controls was significant (Bernstein *et al.*, 1982; Chu *et al.*, 1981; Weinstein and Lewinson, 1978).

3.4 THE AMES TEST – RESULTS AND DISCUSSION

After having confirmed the presence of the markers (viz. *rfa*, *uvrB*, *his*⁻, *pKM101* and *pAQ1*) engineered into the four strains, and their responses (Fig 3.5 and 3.6) to the standard mutagens sodium azide, daunomycin, mitomycin C and 2-aminofluorene (the latter requires S9 activation), the polyphenol fractions (see Chapter 1) from several of the sorghum cultivars were tested in the four strains, both in the presence and absence of S9. The results of this are depicted in Figs 3.7 - 3.10 and Tables 8.1 - 8.5 (see Appendix - it is required that workers append actual revertant counts for the Ames test).

The different strains of *S. typhimurium* were used to check for the possible action of the phenolics as cross-linking (TA102), base substitution/deletion (TA100 and TA97) or frameshift agents (TA98).

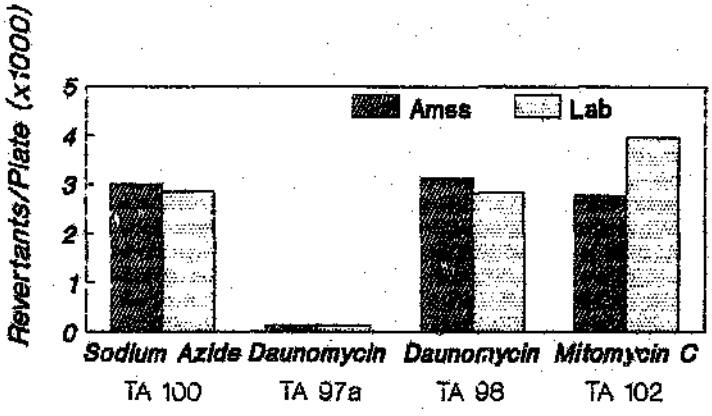


Fig. 3.5 *Comparison of the responses of the four S. typhimurium strains TA97a, TA98 TA100 and TA102 to the standard mutagens (sodium azide, daunomycin and mitomycin C) obtained in the present work (Lab) to those reported by Maron and Ames (1983) (Ames).*

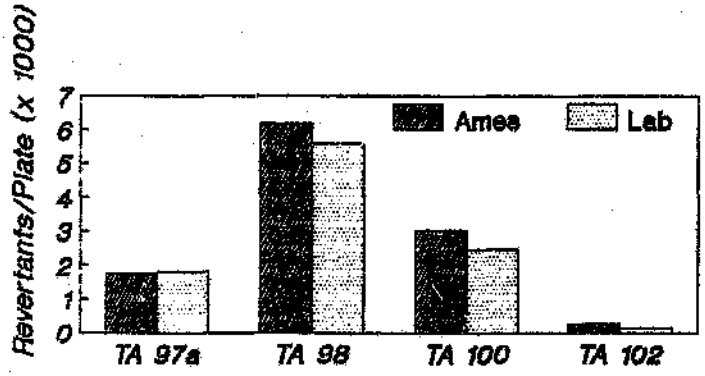


Fig. 3.6 *Comparative responses of the four strains to 2 aminofluorene (requiring S9) obtained in this work (Lab) to the values reported by Maron and Ames (1983) (Ames).*

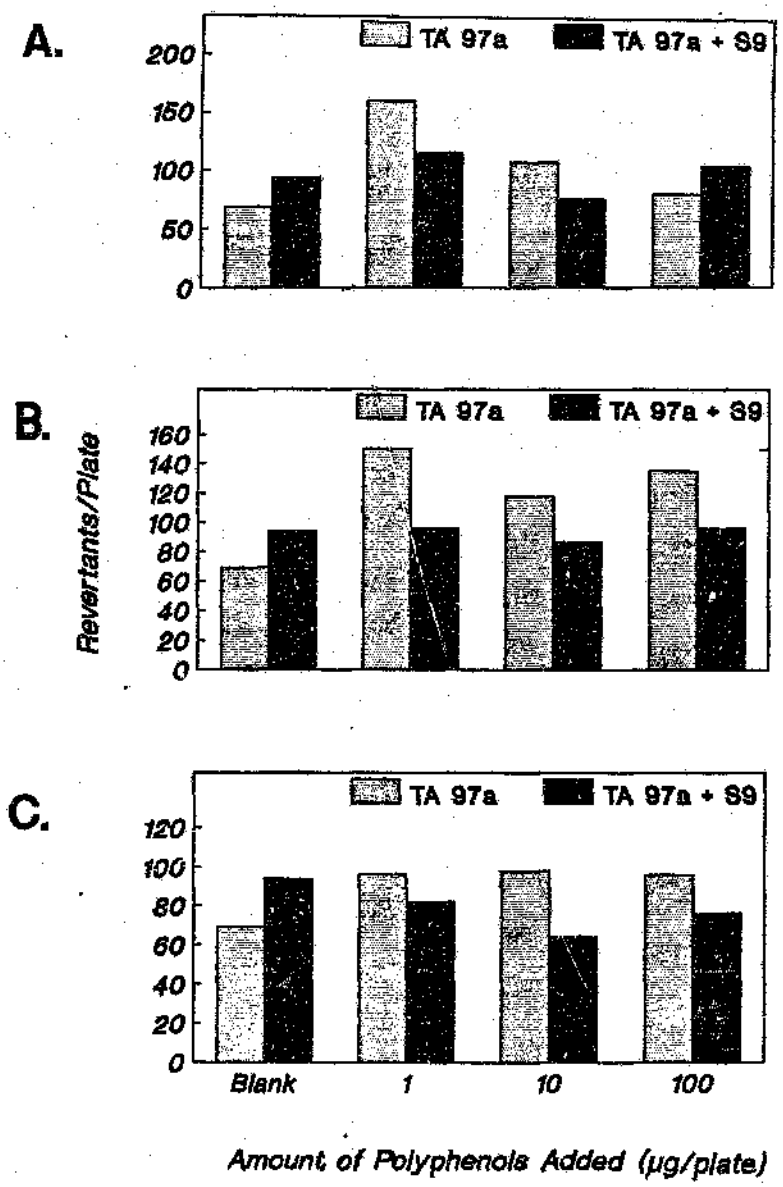


Fig. 3.7 *Incubation of TA97a with (A) F_1, (B) F_2 and (C) F_3 from SSK52, in the absence and presence of S9 enzymes. The blank consists of TA97a, solvent, without and with S9 for the corresponding experimental series.*

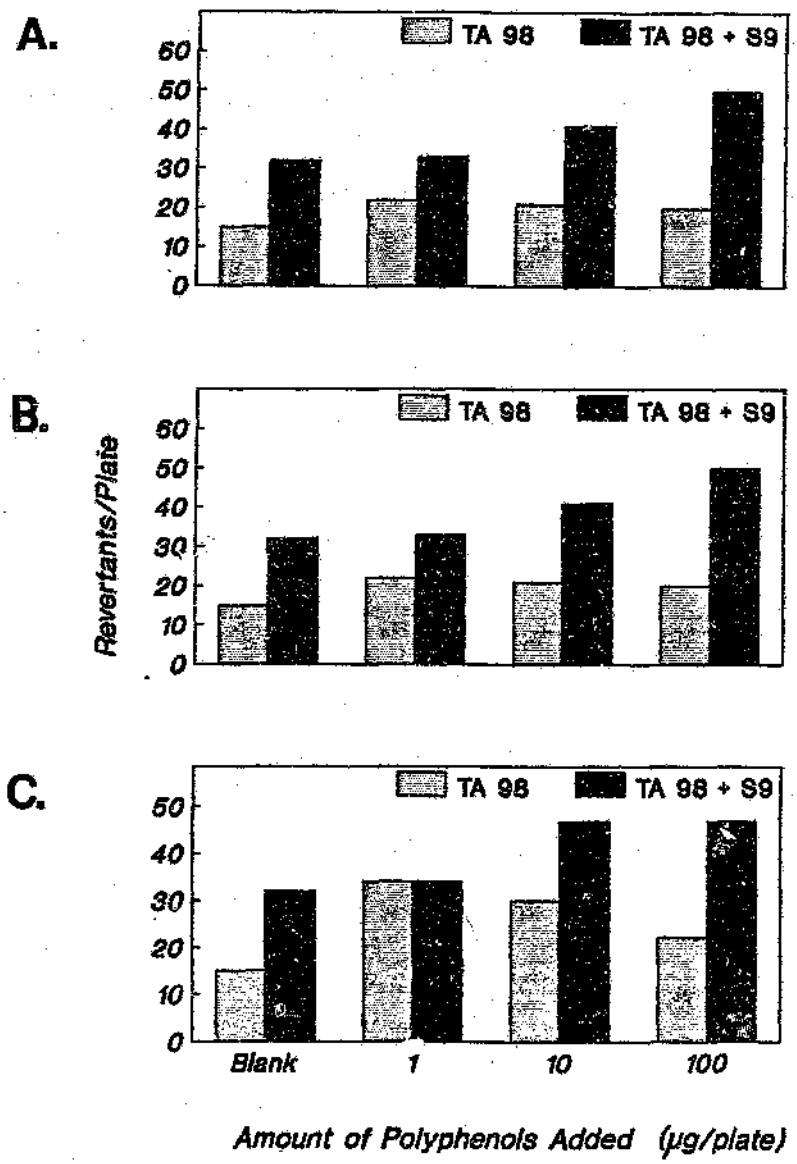


Fig. 3.8 Incubation of TA98 with (A) F_1, (B) F_2 and (C) F_3 from SSES2, in the absence and presence of S9 enzymes. The blank consists of TA98, solvent, without and with S9 for the corresponding experimental series.

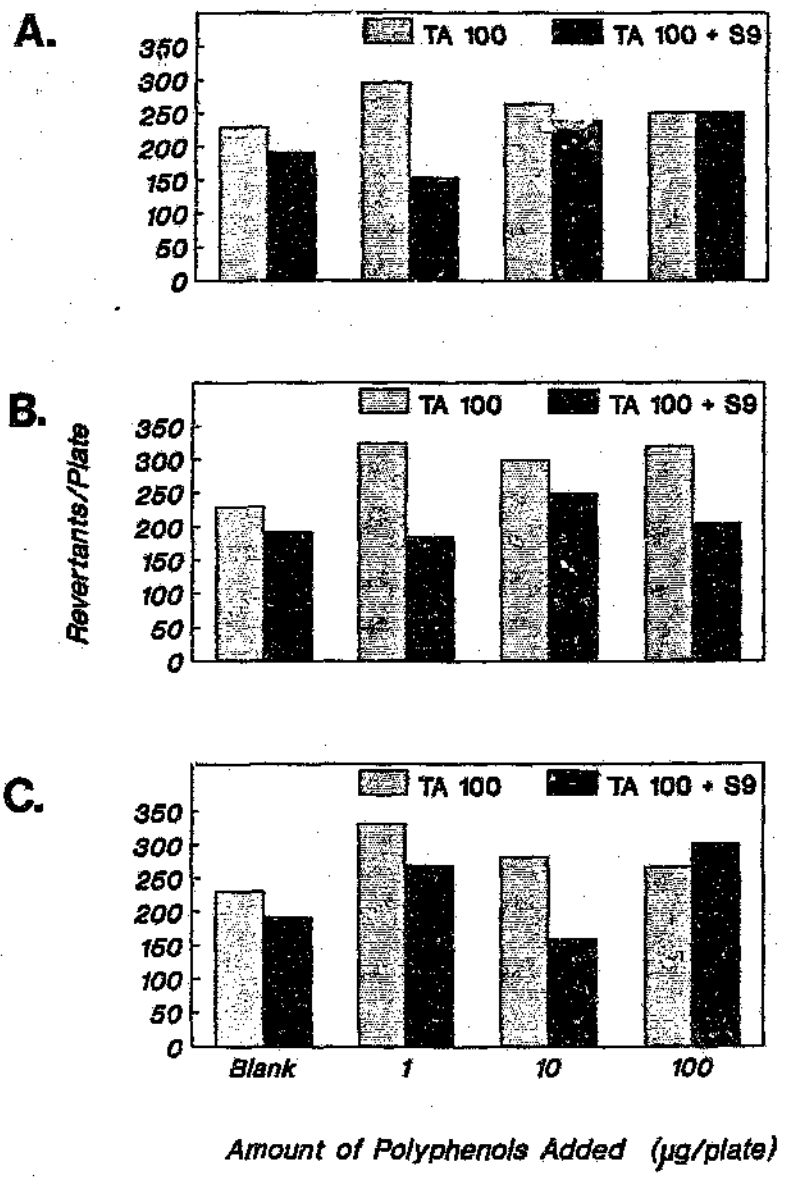


Fig. 3.9 *Incubation of TA100 with (A) F_1, (B) F_2 and (C) F_3 from SSK52, in the absence and presence of S9 enzymes. The blank consists of TA100, solvent, without and with S9 for the corresponding experimental series.*

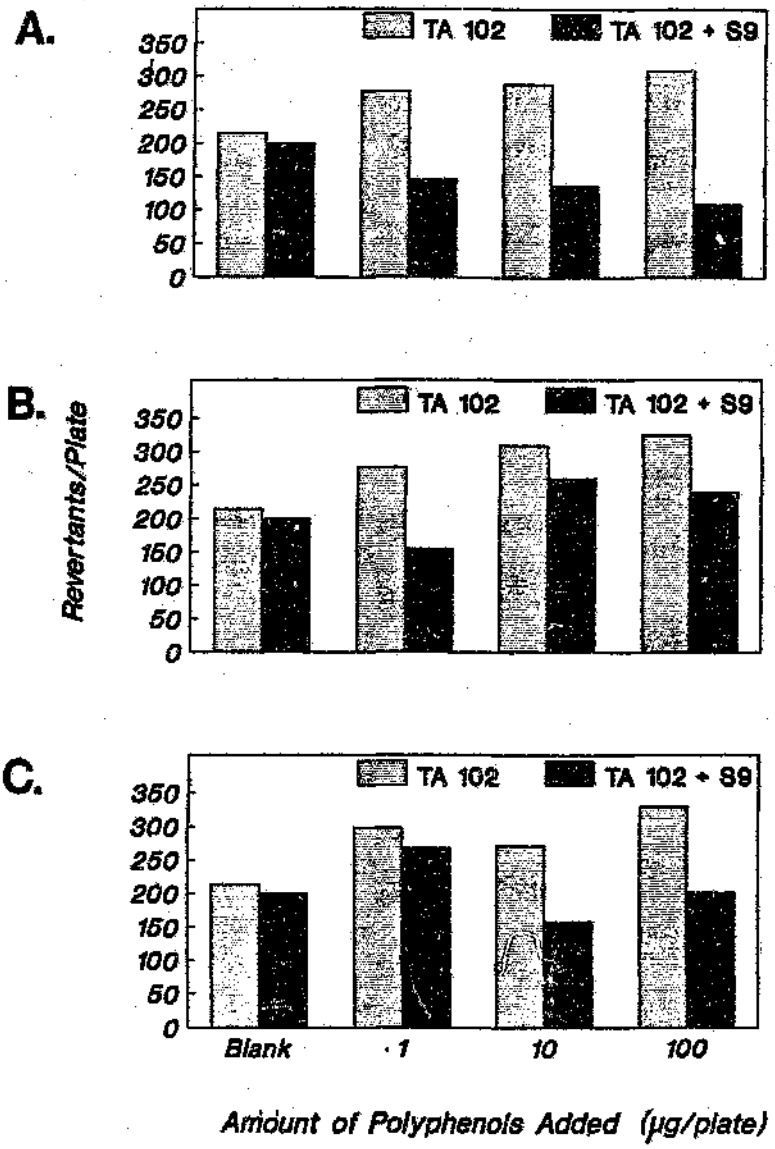


Fig. 3.10 *Incubation of TA102 with (A) F_1, (B) F_2 and (C) F_3 from SSE52, in the absence and presence of S9 enzymes. The blank consists of TA102, solvent, without and with S9 for the corresponding experimental series.*

From the Figs 3.7 - 3.10 and Tables 8.1 to 8.5 it can be seen that there was no significant (by Students t-test) increase in the reversion frequency when compared to the blanks (spontaneous reversion frequency). In some cases, subtracting the number of spontaneous revertants yielded lower values i.e. an actual decrease in reversion (See Appendix - Tables 8.1 to 8.5). This was not due to toxicity (results not shown) since in addition to checking for the presence of bacterial lawn, serial dilutions (c 2000) of the exposed bacteria showed no decline in the number of viable bacteria. Furthermore, the results did not fulfil the other criteria established for determining mutagenicity, the two-fold increase law and a dose-response over at least three log doses (Maron and Ames, 1983; Zimmermann and Taylor-Mayer, 1985). The two-fold rule states that a dose level causing a doubling of the spontaneous mutation rate, is considered to be a positive indicator of mutagenicity. Testing in a narrower range around this level should yield a dose response curve. No dose response or two-fold increases were observed for all three fraction regardless of the cultivar used, both without and with S9 activation. In all cases, the addition of S9 caused a reduction in the number of revertants, except in the case of TA98 and SSK52 polyphenols (Fig. 3.8). The increase in the number of revertants, though not significant, was unexpected in view of all the other findings and appears to be particular to the polyphenols of this sorghum cultivar, and not necessarily to this strain. The reason for this increase is not known. To substantiate our observations from the Ames assay, a further short term assay was

used to test the polyphenols. This test employs the fruitfly, *Drosophila melanogaster*, and is known as the somatic mutation and recombination test (SMART) and forms the basis of the next chapter.

3.5 SOMATIC MUTATION AND RECOMBINATION TEST

3.5.1 Drosophila as a Test Organism – An Introduction

When using short term *in vitro* assays as opposed to animals in detecting mutagenicity, it is essential to use more than one test system, measuring a different genetic endpoint. For the purpose of this study, the fruitfly, *Drosophila melanogaster*, was chosen. The genetics of *Drosophila* is well known making the system ideally suited for genetic manipulation and tailoring. One is dealing with a eukaryote, containing 8 chromosomes. It is possible to treat either the adult flies or the larvae or both depending on the test system selected. Most importantly, although fruitflies do not have livers, they do have the cytochrome P450 enzymes present in fat body cells (Baars *et al.*, 1977). Of the two most used assays, viz. sex-linked recessive test (SLRT) and SMART, the preference was for the latter. Results from this test are evident in the first generation (F_1) as opposed to the F_3 generation in the SLRT, thereby cutting down on the time, 10 days versus a month, required per assay. In the SLRT, the parent(s) are treated as adult flies, and any effects in the sex chromosome are passed on during meiosis to the following generation. SMART involves the crossing of male and female flies and then subjecting the resulting larvae to the mutagen. Mutations are, however, only evident after mitosis has occurred in the larvae and cannot be transmitted in the germ line. The SLRT detects the action of mutagens in the germ cells, whereas SMART measures

mutation in somatic cells.

Limitations of genetic tests is evident from work carried out by Vogel *et al.* (1983). The SLRT and other germ-line assays failed to respond positively to promutagenic aromatic amines and polycyclic hydrocarbons, even when several inducers were administered to enhance S9 activity. Despite the seven-fold increase in the level of S9, the relative inducibility appeared to differ in various parts of the body, with the lowest activity detected in the gonadal tissue. It was concluded from this and other findings that attention should be directed at studying the differences in metabolism in somatic and gonadal tissue, as the former could be more effective. Although this was not demonstrated by Mollet and Würgler (1974), their work did highlight the utility of a somatic test. Based on the work of Becker (1969), they engineered and evaluated the use of the somatic eye mutation and recombination test after feeding mutagens to *Drosophila* larvae. To determine the ease of evaluating the results, students in the laboratory were given flies to analyse.

To make the effects of mutations visible in the eyes, distinct genetic eye markers (carried on the X chromosome) were used. Females homozygous (i.e. both X chromosomes carry identical alleles) for coral coloured eyes were crossed with males (hemizygous since they only have one X chromosome) carrying a white eye marker. The larvae of this cross were subjected to treatment.

If the larvae are not treated, the emerging male adult flies would have coral eyes and the female eyes would be intermediate between coral (recessive marker) and white, viz. light coral. Applying a mutagen to these larvae can cause mutations (on the X chromosome) and somatic recombinations (between the X chromosomes). Both these effects can be seen in the females; males will only show mutations due to the presence of the Y chromosome. Twin spots resulting from recombination in the females are evident as adjacent patterns of coral and white, and single spots will show up as different coloured regions against a light coral (in females) or a coral (in males) background. Female flies are used to determine the frequency of induced recombination and mutation, and the males will give the induced mutation frequency only.

The two compounds used to evaluate the technique were a fungicide with no known mutagenic effect and methylmethanesulfonate, a potent mutagen and carcinogen. This test gave the correct responses and the analysis by the inexperienced students were in good agreement with those of Mollet and Würgler's group. The assay was rapid and provided a minimum of 10 cells/eye for exposure and analysis, and in addition could detect somatic mutation and recombination. A disadvantage of the assay, being somatic, is that the eye spots cannot be analyzed further genetically.

3.5.2 Somatic Mutation and Recombination Test (SMART)

Graf *et al.* (1983 & 1984) engineered SMART but instead of using eyes as Mollet and Würgler (1974) had done, they used wings. The wings can be mounted and results verified long after treatment and because flies can be kept in 70% ethanol, mounting can be carried out at leisure. By using two recessive markers, ***mwh*** (multiple wing hairs - coded by the allele on chromosome 3, position 0,0) and *flr* (flare located on chromosome 3, position 38,8) on the left arm of chromosome 3, genetic mutations and somatic cross-over and recombination between non-sister chromatids could be demonstrated. Both these markers affect the final differentiation of the trichome (hair) formed by a single wing cell, of which there are 10^8 per adult wing. When compared to the single slender trichome that forms per cell, the ***mwh*** marker gives rise to multiple wing hairs in each individual cell, and *flr* results in short malformed hairs as is depicted below.

mwh:

flr:

normal:

Female flies homozygous for the ***mwh*** marker are crossed with males hemizygous for the *flr* marker. *Flr* is lethal in the homozygous state (see Section 3.6.1 for more technical detail). The resulting

larvae carrying the *mwh* and *flr* in the trans-heterozygous state (one chromosome carries *mwh* and the other *flr*) are of interest, as several mutations and recombinations between these markers can occur during mitosis, each resulting in definite phenotypically marked clones on the wing. During mitosis, each chromosome of a homologous pair gives rise to a daughter chromatid, which consists of two sister chromatids. Recombination occurs between the two non-sister chromatids (of the two daughter chromatids).

Multiple wing hair spots are expected to occur more frequently than flare or twin spots and this is confirmed experimentally. Single spots predominate because they can arise from many different types of chromosomal damage:

(i) deletions and point mutations uncovering the recessive markers

(ii) recombination between *mwh* and *flr* loci on 2 non-sister chromatids whereas a similar cross-over but between the *flr* locus and the centromere results in a twin spot.

(iii) disjunction in which the *mwh*$^{+}$ is expressed and *flr* expression suppressed. Such aneuploid (containing (2n+1) or (2n-1) chromosomes) can also arise from large deletions in the chromosome, giving rise to smaller spots as aneuploidy reduces cell viability.

Although point mutations of the *flr* locus to *flr+*, giving rise to flare single spots is theoretically possible, these have not been observed. When the spots are analyzed, the size distribution and frequency is noted. Spot size indicates at what stage the mutagen acted, resultant size decreasing with larval age. This data might help to classify chemicals according to their time of action within the cell.

Several chemicals were tested in SMART. Procarcinogens, including N-methyl-N'-nitro-N-nitrosoguanidine, cyclophosphamide and diethylnitrosamine produced large single spots, whereas aflatoxin B_1 yielded twin spots. Volatile compounds are detectable as was borne out by the wide range of spots produced by 1,2-dibromoethane. An intercalating agent such as 5-aminoacridine, and poly- and monofunctional alkylating agents, mitomycin C and ethylmethanesulphonate, respectively, were positive in SMART. Vinblastine, a spindle poison, expected to have similar effects as non-disjunction produced the expected single spots.

The limitations of SMART need to be determined especially as many weak mutagens, whose effect may be accumulative, cannot be detected by this method. Overall, SMART seems to have some of the features necessary for a quick screening test; large numbers of cells are treated (10^8), it takes only 10 days to perform, has a wide detection range and, in particular, can respond to procarcinogens as the larvae possess P450 enzymes.

3.6 SMART – MATERIALS AND METHODS

3.6.1 *Drosophila* Stocks

Female flies homozygous for *mwh* and wild-type for *flr* were crossed with males heterozygous for *flr* and wild-type for *mwh*. Of the several *flr* alleles available, use was made of flr^3. In the diagrams that follow, this allele will be denoted as *flr*. Phenotypically, the females have *mwh* on their wings. Since the male flies carry the dominant *Ser* marker, their wings are serrate at the lower edges.

To maintain the mutant *flr* marker in the stock, use was made of a balancer chromosome. *Flr* cannot be carried in a homozygous state in the germ cells as it is lethal or closely associated with an allele affecting viability. Inversions present on the balancer chromosome are useful in maintaining such a recessive lethal mutation, which would otherwise be lost from the stocks (Roberts, 1986).

Inversions "suppress" cross-over between normal and inverted regions. Cross-overs are not actually suppressed, the products of cross-over in an inversion leads to the formation of two abnormal gametes that are lethal. Thus only those cells (in the stock) in which no cross-overs occur will survive, ensuring that the genes are kept in the order and position into which they were engineered. If an inverted sequence is long, double cross-overs are frequent and viable, this would separate linked genes. A balancer chromosome is

used to overcome this problem. Balancer chromosomes have been constructed containing many short inversions, (i.e. inversions within inversions), marked by dominant mutations that can be followed (Roberts, 1986). An example of such a balancer is *In(3LR) TM3 Ser* y^+, suppressing cross-over in the left arm of chromosome 3. Each inversion is labelled to indicate the chromosome number and the arm involved viz. *In(3LR)* indicates inversion in the left arm of chromosome 3 with inversion breakpoints in the left and right arms. The balancer carries a dominant serrate wing marker (*Ser*) and a recessive wild-type body colour (y^+). The genotype of the males is thus *mwh*$^+$ *flr*^{3} *Ser*$^+$/*mwh*$^+$ *flr*$^{3+}$ *In(3LR) TM3 Ser* y^+ and that of the females *mwh flr*$^{3+}$/*mwh flr*$^{3+}$.

A more simple representation:

$$\frac{mwh\ +}{mwh\ +} \quad \text{X} \quad \frac{+\ flr}{+\ +}$$

(female) (male)

The crossing of these adult flies results in the following gametes after meiosis in the F_1 generation:

$$\frac{mwh\ +}{+\ flr} \qquad \frac{mwh\ +}{+\ +}$$

The F_1 larvae of the *mwh* +/+ + genotype carry the balancer

chromosome and hence the serrate marker. This is useful in distinguishing the treated larvae once they are adults as they cannot be differentiated during the larval (and exposure to mutagen) stage. These are disregarded since the chances of mutations occurring is very low and hence would give false negative values. The larvae of interest carry the *mwh +/+ flr* genotype. During mitosis several mutations can take place.

Mitosis

mwh + *+ flr*	Duplication of the chromosomes, giving rise to 2 daughter chromatids
mwh + *mwh +*	Each daughter chromatid has two chromatids. Cross-over between the non-sister chromatids can occur.
and	
+ flr *+ flr*	Mutations take place at this stage.

3.6.2 Rearing and Treatment of Flies

Day	Procedure
1	The *mwh* and *flr*^{3} cultures were started
10	Virgin ♀ and ♂ were collected
12	Virgin *mwh* ♀ x *flr* ♂ were crossed
13-14	Egg laying. Adult flies removed.
16-17	Larvae were flushed out of culture

flasks with 20% NaCl and poured into a separating funnel (with 4 - 6 mm outlet). The larvae float in the NaCl and stick to the walls of the funnel. They were washed with NaCl, and the outlet opened, allowing the salt solution out. The tap was closed, and the larvae washed with H_2O, and the funnel opened, and the larvae collected onto a nylon gauze. With a spatula, a match head sized quantity of larvae was dropped into vials containing instant medium, 5 ml of H_2O and mutagen. The vials were stoppered with cotton wool and incubated at 20^0C.

11-23 The adult flies were harvested and preserved in 70% ethanol.

3.6.3 Scoring Mutations

Serrate wings were not mounted for reasons discussed in section 3.6.1. To mount the wings, the flies were washed with water and then transferred singly to a slide containing some Faure's solution. The chloral hydrate was dissolved in water. the glycerol added and mixed. The gum was suspended in a small gauze bag in the above

solution, and allowed to dissolve completely.

Faure's Solution:	
Distilled H_2O	50 ml
Gum arabic	30 g
Glycerol	20 ml
Chloral hydrate	50 g

The wings were removed under a dissecting microscope, and then mounted onto a slide. Twenty wings were mounted, 10 female and 10 male paired wings. These slides were allowed to dry in closed petri-dishes. After about 24 hours, some Faure's solution was applied to a coverslip, inverted and placed over the wings. Carefully ± 150 g weights were placed on the slide and allowed to dry. Once these had dried, they were sealed along the edges with nail varnish. The slides were then ready to be viewed under 400x magnification.

The upper and lower surface of the wings were scanned by adjusting the focus. Different mutations were scored and the number, size and frequency of the spots recorded.

3.6.4 Statistical Treatment of Data

The Poisson null-test hypothesis was used for SMART spot frequency analysis (Graf *et al.*, 1984).

3.7 SMART – RESULTS AND DISCUSSION

To confirm or refute the Ames test results, the fractions from SSK52 (as it is the most extensively used grain in South Africa) were tested in *Drosophila melanogaster* (in SMART) and again the phenols yielded no dose-response over the dose levels tested (Table 3.3). Only single *mwh* spots were observed and by the Poisson null-test hypothesis, the increase in single spots was not significant at the 5% level. Powder from a home-brew beer pack was feed to the fruitflies to check for possible mutagenicity in one of the products derived from sorghum (sorghum beer). There was no significant response to the 20 and 50 mg/ml applications.

The outcome of polyphenol mutagenicity testing was unexpected especially in view of the positive correlation found by Morton (1970) between tannins and carcinogenicity and work by others showing mutagenicity of several plant flavonoids (Stich and Powrie, 1979 and Brown, 1980). It might be argued that the application of a mixture of polyphenols extracted from sorghum might not be detected effectively as the concentration levels of potentially mutagenic components in the extract would be too low or that the level of the other polyphenols could have a masking effect. However, since we wanted to determine if the consumption of sorghum *per se* was a health hazard, the crude extracts were used without further purification. Had any mutagenic activity been detected, then further separation would have been warranted. Besides,

polyphenol mixes have been tested successfully in the Ames assay (Gray and Dugan, 1975; Groenen, 1977 and Kawabata, 1979). Furthermore, the results from the Ames test were confirmed by SMART. Having stated this though further work would need to be done to confirm the effects that metabolism (e.g deglycosylation) could have on these polyphenols (Brown and Dietrich, 1979), before sorghum grain polyphenols can unambiguously be declared non-mutagenic.

Table 3.3 Results for SMART using F_1, F_2 and F_3 from SSK52 and powder from a brew-pack for sorghum beer.

Sample	Amount/ml	#wings analyzed	*mwh* spots/wing
H_2O	-	38	0.58
F_1	10 mg	36	0.50
	1 mg	40	0.33
	100 µg	38	0.55
F_2	10 mg	38	0.47
	1 mg	38	0.76
F_3	10 mg	40	0.60
	1 mg	38	0.63
Brew-pack	50 mg	36	0.44
	20 mg	38	0.45

The work carried out in the following chapter was prompted by the observations that the polyphenols reduced the yield of mutants in the Ames test (See Appendix - Tables 8.1 - 8.5). It was decided to pursue antimutagenic studies using the Ames assay as the method of detection rather than SMART for reasons more detailed in the following chapter.

Chapter 4. POLYPHENOL BIOLOGY II - ANTIMUTAGENICITY

4. POLYPHENOL BIOLOGY II - ANTIMUTAGENICITY

4.1 ANTIMUTAGENICITY - INTRODUCTION

Since the development of the Ames assay in the early 1970's (Ames *et al.*, 1975), immense efforts have been directed at identifying mutagens in the environment, in the hope of reducing human exposure to these compounds. Attention is also being directed at identifying naturally occurring antimutagens as a means of reducing the cancer incidence. Most of the work on antimutagenesis has been studied using the conventional mutagen detecting assays. It would appear from these studies that just as mutagens can bring about their effects via direct DNA interaction, mutagen altered base analogues in DNA replication or repair, and errors in transcription and translation, antimutagens also have many possible modes of action. Clarke and Shankel (1975) defined a true antimutagen as any agent or effect that specifically or preferentially reduced the yield of mutants in short-term assays. It is important that the effects observed should not be the result of toxicity of the chemical under investigation - cell death would automatically reduce the yield of mutants.

Initial studies in the field of antimutagenesis focused on the effect that chemicals had on the genetics, and in particular on repair encoding genes, of various microorganisms (Clarke and Shankel, 1975). It has been subsequently shown that antimutagens

need not necessarily act at the level of genome but can bring about their effects without alterations occurring in the activity of the cell's DNA repair and processing proteins. Kada *et al.* (1982) coined two terms to distinguish these modes of action of antimutagens, namely, bioantimutagens and desmutagens. Bioantimutagens interfere with the DNA replication and/or repair steps (fixation processes). Conversely, desmutagens act by direct interaction with the mutagen thereby preventing its possible binding to and subsequent alteration of the DNA. Evidence for these two types of antimutagens can be distinguished quite readily by using bacterial mutants that carry specific repair or recombinational genes. Desmutagens could be distinguished from the biomutagens by monitoring whether any of these fixation processes are altered; desmutagens would be expected to have no effect .

4.1.1 Desmutagenesis

Kada *et al.* (1987) showed that peroxidase isolated from cabbage was effective in inactivating mutagenic tryptophan pyrolysates. Fibres isolated from different vegetables showed an ability to adsorb pyrolysate mutagens more effectively than cellulose. They postulated that these fibres could resemble DNA, thereby providing an alternative binding site for the mutagens. Polyphenol polymers have been postulated to take on a helical structure similar to DNA (Haslam, 1989) and as such could provide an alternative binding site for the mutagens used in the present work. The question still

remains whether these fibres are really effective *in vivo* because this adsorption could be reversible. Certain NADPH oxidases in vegetables where shown to be effective in preventing the activation of tryptophan derivatives to mutagens by directly inhibiting the S9 metabolic pathway. Polyphenols might well act in a similar way in the presence of S9 and 2 aminofluorene in the Ames test since it is known that these bind strongly to proteins. This could have implications in the body where the cytochrome P450 in the liver not only activates but is involved in detoxification as well, which is essentially an irreversible process. Blocking this pathway could lead to mutagen accumulation *in vivo*. Polyphenols might in addition to their mutagen and protein binding capacities, affect the induction of cytochrome P450 isozymes (Shankel *et al.*, 1987; Grimmer and McGrath, 1991 and Parhboo, 1993). In the Ames assay, β-naphthoflavone is used as one of the inducers in the preparation of cytochrome P450 rich extracts from rat livers. Another pathway of mutagen inactivation could be via conjugation to glucuronic acid, glutathione or other substances in the body, a process which may not be that effective due to the reversibility of the reaction (Wattenberg, 1985).

4.1.2 Bioantimutagenesis

The action of cobaltous chloride as a bioantimutagen in the presence of MNNG was demonstrated in *Bacillus substilis* mutants which have a modified DNA polymerase III (Kada *et al.*, 1987). At concentrations

of the metal which did not affect the viability and hence the growth of these bacteria, the mutation rate in these bacteria was reduced. Subsequently, it has been postulated that cobaltous chloride could have a modifying effect on the *rec A* protein involved in the SOS response. By genetic analysis, Ohta *et al.* (1983) showed that the *rec A* protein was required to mediate the observed antimutagenic effect in the presence of vanillin and cinnamaldehyde. The bioantimutagen could directly activate or even modulate the expression of such repair enzymes. Tannic acid was found to be an effective bioantimutagen in UV irradiated *E. coli* and as such has been thought to enhance the UV excision repair process involving the uvr family of genes (Kada *et al.*, 1985 and Shimoi *at al.*, 1985).

In attempting to determine how the polyphenol rich fractions could be bringing about antimutagenicity, it was decided to use the Ames assay rather than SMART. A decrease in reversion response of the bacteria to known mutagens would be easier to monitor. Historically such tests have been shown to be reliable measures of such testing. Besides, the use of SMART to detect antimutagens has not been investigated extensively nor have the statistics been worked out to allow for meaningful interpretation of the resulting data.

4.2 AMES ANTIMUTAGENICITY TESTING – MATERIALS & METHODS

4.2.1 Antimutagenicity Assays

To check for possible antimutagenic activity, the polyphenols were coincubated with standard mutagens known to produce an established number of revertants in a specific strain (see Table 4.1) in a typical Ames test (see Section 3.3.8 - Ames Mutagenicity Assay). The three fractions from SSK52 were coincubated with TA98 and daunomycin (6µg/100µl) and the number of revertants scored. The antimutagenic activity was also tested against sodium azide (1.5µg/100µl) in TA100 and 2 aminofluorene (10 µg/100µl) requiring S9 activation in TA98 and TA97a. Quercetin was only coincubated with 2 aminofluorene, S9 and TA98 since it was shown to also require S9 activation for mutagenicity and we wanted to see if such a mutagenic flavonoid could be effective in reducing the mutagenicity of the standard mutagen.

4.2.2 Statistical Treatment of Data

Analysis of variance (ANOVA) for antimutagenesis tests were performed with the data derived from these experiments.

Table 4.1 Standard mutagens and the different bacterial strains used to measure the antimutagenic potency of polyphenol-rich fractions from sorghum grain SSK53 in the Ames assay. Values are those reported by Maron and Ames (1983).

Mutagen	µg/100µl	S9	TA97	TA98	TA100
Sodium Azide	1.5	-			3000
Daunomycin	6.0	-		3123	
2 Aminofluorene	10.0	+	1724	6194	

4.3 AMES ANTIMUTAGENICITY TESTING - RESULTS

When it was observed that the sorghum phenolics produced no mutagenic, and in some cases negative, responses, it was decided to investigate the possibility that they could be antimutagenic, by testing the effect of these phenols on the ability of the standard mutagens to produce reversion in the Ames test. The mutagens used included daunomycin, sodium azide and 2-aminofluorene (requiring S9) and were used at those concentrations which produced maximal responses in the specific strains (Table 4.1). In using the diverse mutagens, it was hoped to see some pattern emerge that would indicate a plausible mechanism. Phenolics have been shown to act as antimutagens (Fukuhara *et al.*, 1981; Kada *et al.*, 1985; Stich *et al.*, 1982; Shimoi *et al.*, 1985 and Wood *et al.*, 1982).

The work by van Rensburg *et al.* (1985) on the effect of different grains on a subcutaneously administered oesophageal carcinogen, N-nitrosomethylbenzylamine, provided further impetus to look at the antimutagenicity of sorghum phenolics. The percentage of rats with oesophageal tumours was not affected by a diet of wheat, commercial bird-resistant sorghum (i.e. high tannin content) and polished rice (i.e. low in fibre). However, rats on a millet, red sorghum or brown rice diet had a significantly lower incidence of tumours.

To see whether this effect could be achieved in the Ames test, the phenols were extracted from Barnard Red sorghum, yielding a very

large F_1 fraction, a negligible quantity of F_2 and no F_3 (Fig. 2.9). Several strains were incubated with those standard mutagens that produced the highest reversion frequency in the particular strain, and various levels of F_1 from the red sorghum. The standards used were sodium azide (15 µg/ml) for TA100, daunomycin (60 µg/ml) for TA98 and 2-aminofluorene (100 µg/ml), requiring S9 activation, for both TA98 and TA97a. By using these levels, it would be possible to see any significant reduction in their mutagenic potency. Levels of F_1 varied from 10 mg/ml to 0.1 µg/ml. The results of this are depicted in Fig. 4.1.

4.3.1 Sodium Azide and TA100

The decrease in revertants in the presence of sodium azide was significant (by ANOVA), with a 49% diminution at the 10 mg/ml level, and 40% at the 0.1 µg/ml dose. By increasing the amounts of F_1 incubated there appeared to be an increased suppression although the effect levelled off (Fig. 4.1).

At pH 7.4 (conditions of the Ames assay), sodium azide exhibits its highest potency, although Kleinhof *et al.* (1978) showed that mutagenicity could be enhanced under acidic conditions, when the predominant species would be expected to be the hydrazoic acid form, N_3H ($pK_a = 4.8$) In this neutral form it could penetrate the membrane and once inside be converted into the reactive N_3^- form. With the pH of 7.4, there would be large amounts of N_3^- present which could

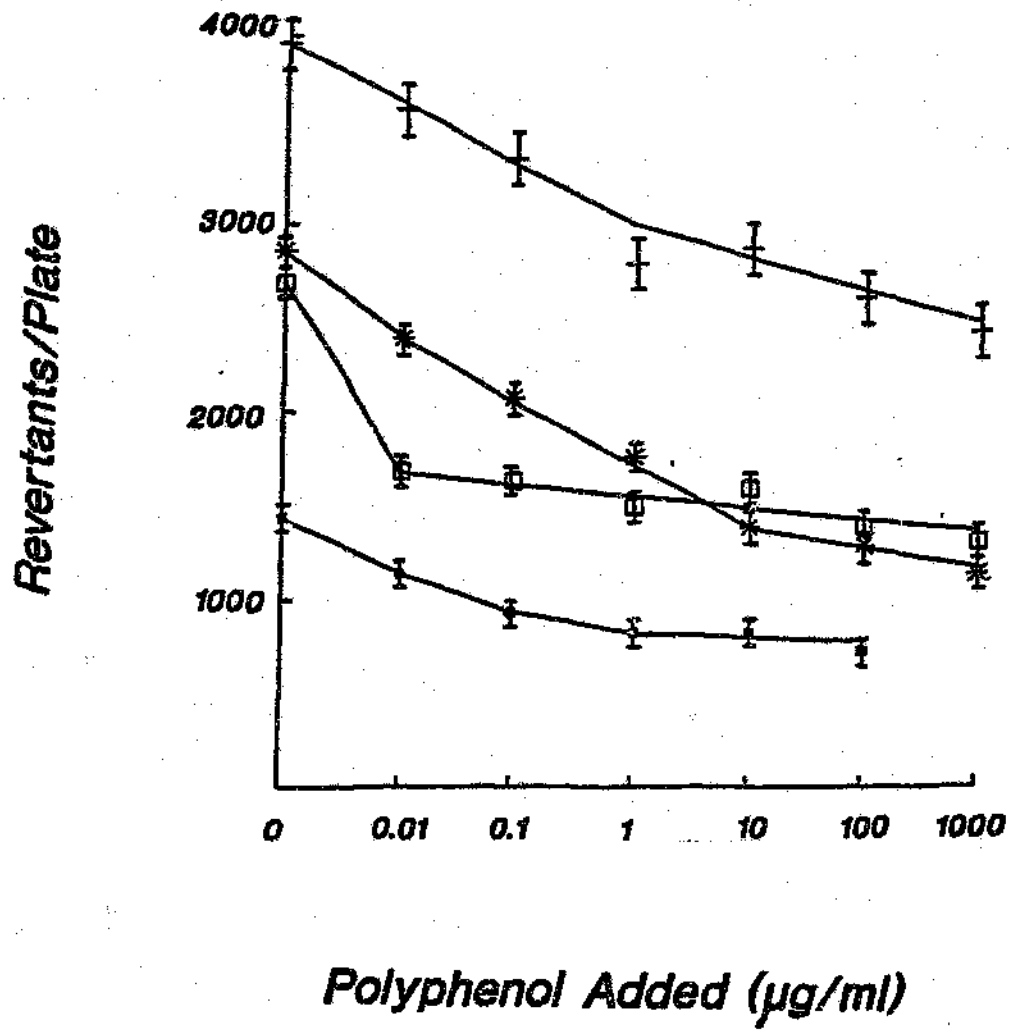


Fig. 4.1 The antimutagenic effect of F_1 on standard mutagen induced reversion frequency of several strains used in the Ames test: TA 100 and sodium azide (□); TA98 and daunomycin (•); TA98, 2-aminofluorene and S9 (+); TA97a, 2-aminofluorene and S9 (▣). The mean of three experiments is shown, with the number of spontaneous revertants having being subtracted. Spontaneous revertants for various strains are: 120 ± 2 for TA100; 27 ± 2 for TA98; 41 ± 5 for TA98 (with S9) and 137 ± 16 for TA97a (with S9). The standard error bars represent the pooled standard error for each experiment.

interact ionically with the phenols.

4.3.2 Daunomycin and TA98

Coincubation of daunomycin with F_1 produced a significant reduction in revertants, with 10 mg/ml suppressing mutagenicity by as much as 60%. There is a dose related decrease in repression, with 0.1 µg/ml causing a 15% decrease (Fig. 4.1).

4.3.3 2 Aminofluorene (2 AF), S9 and TA98/TA97a

2-AF is a more potent mutagen in TA98 than in TA97a with the number of revertants scored for each being approximately 6000 and 1500, respectively. TA97a showed a 20% reduction at 0.1 µg/ml and a very large drop of 92% with 10 mg/ml. No toxic effects were seen. In TA98 there was a similar decrease in mutagenicity. The decrease ranged from 9% at 1 µg/ml to 39% at 10 mg/ml (Fig. 4.1).

This ability to effectively counter the effect of the mutagens is not restricted to the polyphenols from Barnard Red; similar reductions were noted using fractions from the other sorghum grain cultivars. It was decided to focus attention on the cultivar SSK52 since it is a grain presently in use in South Africa.

4.3.4 *SSK52 polyphenols, Daunomycin and TA98*

All three fractions were effective in reducing the mutagenic potency of daunomycin in TA98, though there was a difference between the fractions with the order of efficiency increasing with molecular size viz. $F_1 < F_2 < F_3$ (Fig. 4.2). Based on these results, it was decided to investigate the mechanism of antimutagenesis (see Chapter 5 - Binding Studies) using these polyphenol fractions. We selected daunomycin as the mutagen of interest because it can be obtained commercially as a radioactively labelled molecule, making studies of the antimutagenic behaviour feasible.

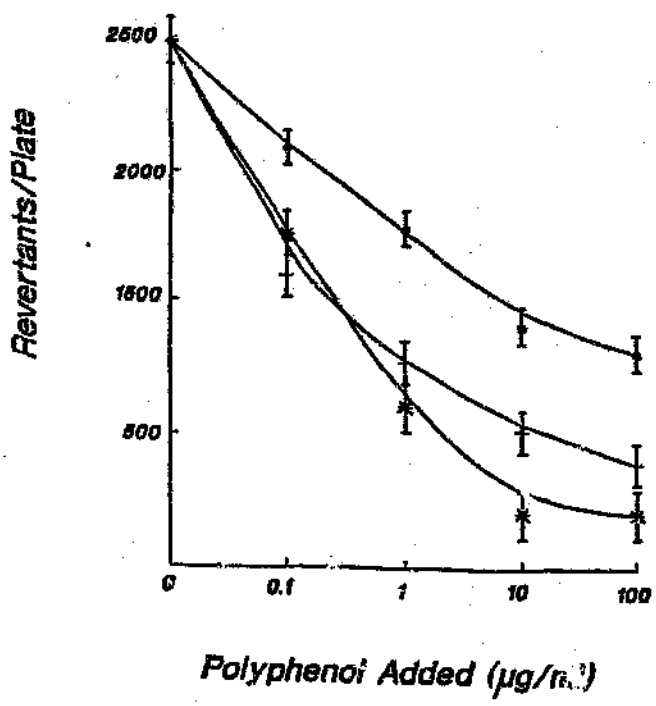


Fig. 4.2 The effect of F_1 (•), F_2 (+) and F_3 (*) on the mutagenic action of daunomycin (6µg/plate) in TA98. The mean of three experiments is shown (with pooled standard error) and spontaneous revertants (30 ± 3) have been subtracted.

4.3.5 Quercetin, TA98 and 2-Aminofluorene (+S9)

Quercetin has been reported to be mutagenic, as was confirmed in this work (see Table 4.2), showing enhanced activity in the presence of S9 (Ogawa *et al.*, 1985). When quercetin was coincubated with a mutagen also requiring S9 activation such as 2-AF in TA98, a marked reduction in reversion frequency in this strain was evident (Table 4.2). Thus a mutagenic polyphenol could act as an antimutagen and hence could have a protective effect, a result also reported by Kurzer (1993) and Kinsella *et al.* (1993).

Table 4.2 The mutagenicity and antimutagenicity of quercetin in strain TA98, both in the absence and presence of S9 activation. The amount of 2-aminofluorene (2-AF) used was 10 µg/plate.

Quercetin	Salmonella strain		
(µg/plate)	TA98	TA98 + S9	TA98, S9 + 2-AF
	Revertants/plate		
0	34 ± 1	50 ± 1	5011 ± 444
0.1	28 ± 4	41 ± 6	
1	36 ± 9	60 ± 10	3590 ± 270
10	80 ± 16	175 ± 31	3045 ± 147
100	313 ± 51	593 ± 26	2839 ± 325
1000	577 ± 162	839 ± 42	2043 ± 788

4.4 AMES ANTIMUTAGENICITY TESTING – DISCUSSION

There appears to be a general reduction in the mutagenicity of the standard mutagens sodium azide, daunomycin and 2AF (+S9).

4.4.1 Sodium Azide and Polyphenol

From the work of Wyss *et al.* (1948) and Berger *et al.* (1953), it was thought that sodium azide caused the accumulation of peroxide by the inhibition of catalase and peroxide. Thus sodium azide mutagenesis was brought about indirectly by peroxide accumulation. However, this was shown not to be the mechanism by Owais *et al.* (1979 and 1988) and Kleinhofs *et al.* (1978). By testing other catalase inhibitors such as potassium and sodium cyanide and sodium arsenate in the TA1530, a repair deficient strain used in the Ames test, no mutagenic response was observed (Kleinhofs *et al.*, 1978). By using cell free extracts from this strain, it was shown that the active mutagen could only be produced in the presence of O-acetylserine. Heat treatment abolished the ability of the bacteria to produce the metabolite (Owais *et al.*, 1981). Owais provided evidence that azide could substitute for sulphide, the natural substrate of O-acetyl-serine(thiol)-lyase (EC 4.2.99.8) due to the low specificity of the enzyme for sulphide, thereby producing β-azidoalanine. These results were supported by infra-red spectrophotometry which provided proof of an organic azide moiety and the presence of an amino group. Evidence for the carboxyl group was only confirmed latter (Owais and

Kleinhofs, 1988) by ^{14}C-labelling. By chemically synthesizing the L- and D-forms of ß-azidoalanine, the stereoselective mechanism of mutagenesis was illustrated. The L-form was the most effective, although it is thought that this is still not the ultimate mutagen. The metabolite is only active in repair deficient bacteria, hence it was postulated that a lesion recognizable by the bacterial excision repair enzymes must be formed. Mutagenesis must therefore take place by a direct misrepairing or misreplication route, similar to that of ethyl methanesulfonate and N-methyl-N'-nitro-N-nitrosoguanidine. In whole animal tests, azide was not biologically active (Owais and Kleinhofs, 1988) because animal cells synthesize L-cysteine from L-methionine rather than from inorganic sulphide. Consequently animals do not possess O-acetylserine (thiol)-lyase.

Polyphenols could interact directly with sodium azide or its metabolite(s), or even interfere with the above-mentioned activation processes. Inorganic sodium azide is very reactive and can react with both electrophiles and nucleophiles, or by nitrogen evolution, can become an electron deficient species (Kleinhofs *et al.*, 1978). All these features make sodium azide ideally suited for interaction with the polyphenols. Our observation of a large initial drop in sodium azide induced mutagenicity (Fig. 4.1) could either be due to a chemical sequestration of the azide, or it could be due to the inhibition of the lyase. The polyphenols could effectively have interacted with all the azide, making further titration ineffectual.

4.4.2 *Daunomycin and Polyphenol*

Daunomycin (Fig. 4.3) is a DNA intercalator and acts as a frame-shift mutagen in TA98, in its -CG- mutational hot-spot. The drug becomes inserted between adjacent base pairs, but this binding was found to be stronger than could be accounted for by intercalation alone. Subsequently, it has been established that N-acetylation decreases DNA affinity, and it appears that the NH_2 of the sugar moiety is involved in stabilising daunomycin. The binding increases with the -CG- content of the DNA which accounts for the specificity towards TA98. All three fractions decreased the mutagenic efficiency of the drug in the order $F_1 < F_2 < F_3$, viz. in order of increasing size. It could be postulated that with the larger molecular mass fraction, the polyphenols take on a helical structure and as such would resemble DNA, providing an alternative binding

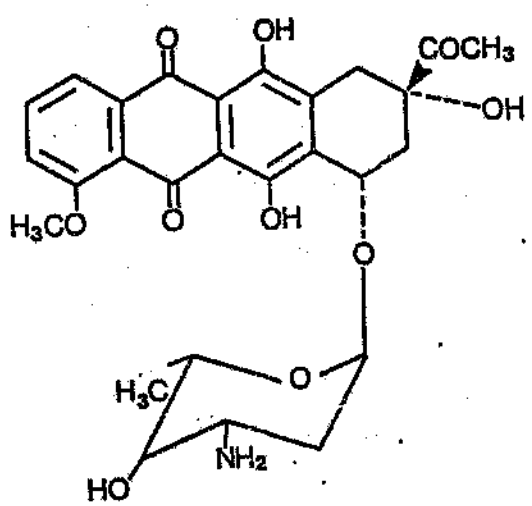


Fig. 4.3 The structure of the mutagen daunomycin, a DNA intercalator.

site for daunomycin. The fact that all three showed antimutagenic behaviour could be explained by the fact each fraction has been found to contain large polyphenols of overlapping molecular mass, though the F_1 fraction has by far the largest sizes (McGrath *et al.*, 1982).

4.4.3 2-Aminofluorene and Polyphenol

When 2AF and polyphenols were coincubated in TA97a and TA98, there appeared to be a more effective reduction in strain TA98 (Fig. 4.1). Similarly, when the same strain and mutagen were titrated with quercetin, the same level of reduction was seen ≈ 50% in both cases, this despite the fact that F_1 was shown to be non-mutagenic and quercetin mutagenic in the presence of S9 (Table 4.2). Monteith (1990) found that coincubation of catechin (identical to quercetin except for a double bonded oxygen at position 4 in the C-ring) at 0.5 mM with 2-acetylaminofluorene (2-AAF) was mutagenic in the Ames test, yet as the concentration increased up to 5 mM, the mutagenicity of 2-AAF was abolished.

Steele *et al* (1985) showed that rats fed catechin yielded P450 with a reduced ability to activate 2-AF to an active mutagen. No such effect was seen for the direct acting mutagens (requiring no P450 activation). . Catechin could also possibly effect metabolite formation. 2-AAF is N-hydroxylated, a rate limiting step in the whole activation process. The hydroxylated intermediate can become

linked to sulphate via the action of sulfotransferase to form a sulphate ester, a direct acting mutagen (DeBrain *et al.*, 1970 ; Sakai *et al.*, 1978 and Weeks *et al.*, 1978). Alternatively, deacetylation follows hydroxylation; in this form it can be acted on by glucuronyl transferase to form a glucosiduronic acid, which has been shown to react quite readily with DNA or RNA (Weisburger and Weisburger, 1975). For 2AF, the route to mutagen formation is via N-hydroxylation. Polyphenols could interact with the activating enzymes or could scavenge the intermediates before they can interact with the C-8 of the guanidine residue in the DNA. Interestingly, 2-AAF was found to produce the SOS response in bacteria whereas no such response was seen for 2-AF administration (Bichara and Fuchs, 1985), implying that the former mutagen causes major structural changes to the DNA.

It would appear that the polyphenols could bring about antimutagenesis either by binding to the enzymes involved in mutagen activation or by scavenging the mutagens and their metabolites. A study on the mechanism of antimutagenesis using radioactively labelled daunomycin and the polyphenols from SSK52 forms the basis of the work to be discussed in the following chapter.

Chapter 5. POLYPHENOL-[illegible]ING STUDIES

5 POLYPHENOL-BINDING STUDIES

5.1 BINDING STUDIES - AN INTRODUCTION

In view of the findings (Grimmer *et al.*, 1992) that polyphenol rich fractions from sorghum grain are effective in reducing the mutagenic potency of several mutagens such as daunomycin, sodium azide and 2-aminofluorene (requiring S9 activation), it was decided to determine how polyphenols could be producing such an effect. To accomplish this the study was restricted to desmutagenesis, and more specifically to the conceivable binding of polyphenols to the mutagen, daunomycin. Binding to the mutagen could occur (i) external to the cell or (ii) within the cell cytoplasm thereby preventing the mutagen from interacting with the DNA. Of the two possibilities the former seems more likely because the polyphenols may be too large to enter the cell.

5.2 THEORETICAL ASPECTS OF BINDING

Much of the work in this area of research has centred around the binding of proteins to nuclei acids because such interactions have important regulatory functions in the cell (Kowalczykowski et al., 1986 and Bujalowski and Lohman, 1987). Halfman and Nishida (1972) and Kronman *et al.* (1981) carried out studies on the interaction of small molecules to proteins. Of specific interest to us was the work by Plumbridge and Brown (1977), Winkle *et al.* (1982) and

Friedman and Manning (1984) on the binding of antibiotics to DNA and will be elaborated on in more detail once the parameters for binding as defined by Scatchard (1949) have been discussed.

No discussion of binding would be complete without mention of the work by Scatchard (1949). This work laid the basis for determining the thermodynamic parameters required to quantitate binding. There are basically three parameters: n, the number of binding sites; K, the intrinsic binding constant and ω, the binding cooperativity parameter. In the discussion that follows it will be seen how different techniques have been employed to arrive at meaningful values for these parameters. Furthermore, the conceptual problems that arise from what appears to be a relatively easy means of measurement will be mentioned. The shortcomings of the various techniques especially when effects like ligand self-aggregation and ionic strength are not taken into account will be disclosed.

When a macromolecule interacts with another molecule (the ligand), one needs to determine the strength of binding, the number of binding sites present and the influence of these sites upon each other. If the various groups on the protein were to act independently, Scatchard (1949) reasoned that one could apply the law of mass action as though each group were on a separate molecule, and that the strength of binding can be expressed as a constant for each group. However, this type of model is ideal and often binding at one site may enhance or inhibit the binding of a subsequent

ligand molecule to the protein. This gives rise to the concepts of positive and negative cooperativity, which can be ascertained from the Scatchard plots. The reaction medium must also be considered and its effect interpreted as an activity coefficient (Scatchard, 1949). Other factors such as electrostatic effects should be kept in mind when interpreting data.

In general the binding (shown below) of a ligand [L] to a macromolecule [M] occurs at well defined sites on the macromolecule, though binding to multiple sites is possible depending on the nature of the macromolecule.

$$M + n\ L \rightleftharpoons ML_n \qquad \text{where } n = 1, 2, 3, \ldots$$

The equilibrium binding (association) constant (K) for this can be written as:

$$K = \frac{[M.L_n]}{[M]\ [L]^n}$$

where n = number of binding sites.

5.3 PRACTICAL MEASUREMENTS OF BINDING

A selected number of methods used in measuring binding will be mentioned. Although each differs in terms of the equipment used, the methods all aim to determine the saturation function (V) of the macromolecule at numerous concentrations of ligand. This saturation function (V) can be defined as the ratio of the concentration of bound ligand $[L]_b$ to concentration of total macromolecule $[M]_t$ (Bohinski, 1987):

$$V = \frac{[L]_b}{[M]_t}$$

When no ligand binds, V will be zero and when $V = n$ at saturating levels of ligand, the macromolecule is fully saturated. The saturation constant (K) can be related to the ligand concentration by the following equation:

$$V = \frac{nK[L]^c}{1 + K[L]}$$

where the value of c is a measure of the interaction between the sites. For values equal to unity, the sites are noncooperative; if $c > 1$ there is positive cooperativity; and if $c < 1$ negative cooperativity exists between the sites.

The methods to be described can either be used to measure the

concentration of the ligand or the macromolecule depending on the method used. Two forms of spectroscopy, absorbance and fluorescence, have been the most widely used especially in measuring aromatic ligands which have very distinctive spectra (Blake and Peacocke, 1969; Halfman and Nishida, 1972; Plumbridge and Brown, 1977; Kronman *et al.*, 1981; Wi[illegible] *et al.*, 1982 and Bujalowski and Lohman, 1987). Equilibrium dialysis (Colowick and Womack, 1969; Revzin, 1990), sedimentation dialysis, partition analysis (Blake and Peacocke, 1969), gel permeation chromatography (Hummel and Dreyer, 1962) and gel electrophoresis (Revzin, 1990) have often provided data which corroborates that obtained by the spectroscopic techniques.

5.3.1 Spectrophotometry

5.3.1.1 Absorbance

The ligands that have been studied have usually been aromatic antibiotics that bind to DNA. When these aromatic compounds bind to macromolecules, the result is usually a bathochromic and a hypochromic (yielding an isobestic point) shift in absorbance spectrophotometry. These features are very characteristic of DNA intercalators (Blake and Peacocke, 1968; Plumbridge and Brown, 1977). It is important that the concentrations used to determine the monomer or unbound spectra must be relatively low because many of these drugs have a tendency to self aggregate in solution. Even in

the bound state this can become a factor. Initially at low concentrations the antibiotics intercalate via their aromatic rings between the successive base pairs in the DNA helix. As the concentration used to titrate and obtain the isobestic point increases, the drug begins to bind via electrostatic interaction mediated by the cationic groups present in the antibiotics to the exterior of the helix by way of the phosphate backbone. A further increase in drug concentration could cause stacking upon the externally bound drug. When these compounds are irradiated with polarized light, the fluorescence polarisation decreases since the rotational motion of the drug will decrease upon binding. To enable one to calculate the concentration of the free and bound ligand certain conditions have to be met. The nucleic acid must be non-absorbing at the λ_{max} of the drug. Both the free and bound ligand must adhere to de Beer's law over the entire concentration range used which implies that the extinction coefficient of the bound (ϵ_b) and the free (ϵ_f) ligand must be constant. By implication the ϵ_b must not vary as the ratio (r) of drug to macromolecule changes. Practically, one needs to work with concentrations of ligand which obey this law. The bound ligand's extinction will vary experimentally in some cases (Blake and Peacocke, 1969). Furthermore, there should only be one species of free, and similarly bound, ligand present in solution. If more than two species are present the relative proportions should not change with r. Clearly once these conditions are met it is possible to determine the isobestic point from which the value of r can be calculated. The

isobestic point may not be achieved if there is significant self aggregation. The extent to which all these conditions are satisfied needs to be determined experimentally for each case under investigation. The degree of deviation from the prerequisite condition will need to be judged and a decision made as to whether this is significant experimentally or whether the deviation can be ignored. Often binding curves generated from such data will show up deviations. Upwards inflections observed in specific areas of the curves for proflavine and DNA interactions indicated a different, maybe a weaker form of binding at specific r values (Blake and Peacocke, 1968). A value of $r \leq 0.3$ appears to represent the true monomer range as determined by the minimal shift seen upon titration. The concentration ranges for ligand are usually in the 10^{-5} range, this depending on the particular ligand under investigation.

5.3.1.2 Fluorescence

The fluorescence peaks of many aromatic dyes are very distinctive and, just as in absorbance spectrophotometry, binding to DNA results in a decrease in the fluorescent signal emanating from the ligand. The intensity of the signal is lower yet much more sensitive to changes in nucleic acid concentrations because much lower levels of the drug can be used, usually in the 10^{-7} M range. Heterogeneity in binding sites are thus more readily determined with this method (Blake and Peacocke, 1969).

Although the above discussion has centred on the signal arising from binding of aromatic ligands to DNA, it has been shown that proteins can also fluoresce when bound to nucleic acids. In studies conducted by Bujalowski and Lohman (1987) on interactions between single-strand binding proteins (the ligand) and single-stranded nucleic acids, the quenching of the intrinsic fluorescence of tryptophan in these proteins was monitored. This property of proteins was exploited by Kronman *et al.* (1981) to monitor not the ligand but the macromolecule, in this case bovine α-lactalbumin, and the binding of divalent metal ions (ligand) to it. Not only did the change in fluorescence or absorbance indicate binding, but here it was shown that removal of the tightly bound calcium ion from the α-lactalbumin could be correlated to a conformational change in the protein.

Thus signal changes of both the ligand and the macromolecule can be monitored and with prior structural knowledge predictions can be made as to the extent of and the effect of this binding on the molecules involved.

5.3.2 Equilibrium and Rate Diffusion Analysis

There are two ways of measuring binding; equilibrium dialysis or rate of dialysis. In the first case, an unbound ligand is allowed to diffuse through a membrane which is not permeable to the complexed ligand. It is relatively easy to measure the

concentration of the free diffused ligand in the appropriate chamber. The apparatus consists of two chambers isolated from each other by a dialysis membrane. Equilibrium dialysis measures the steady state concentrations of this diffusible ligand and as such can be a very lengthy process, sometimes as long as 1-3 days. Such a time span can be a disadvantage in measuring non-specific protein-DNA interactions as these complexes are very labile (Revzin, 1990). This is where measurement of the rate of dialysis is an advantage. The technique was used by Colowick and Womack (1969) for measuring the dissociation constant and the number of binding sites of an enzyme-substrate complex, by monitoring the rate of diffusion of the radioactively labelled substrate. Since the substrate equilibrates with the enzyme, the rate of diffusion into the lower chamber is indicative of the amount of unbound substrate in the upper chamber. When the rates of isotope entering and leaving the lower chamber are equal (viz. steady state conditions), the concentration of unbound ligand can be determined. The method was also used by Ridlington and Butler (1969) to measure the binding of phosphate to ribonuclease A. The advantage was that the rapid equilibrium attained between the phosphate and protein instead of that across the membrane could be measured.

Equilibrium competition experiments can be carried out using selective filters. Studies on the *lac* repressor binding to non-operator DNA were based on the fact that DNA bound to the labelled repressor could not pass through the membrane, whereas free DNA

could (Jones and Berg, 1966; Riggs *et al.*, 1968; Lin and Riggs, 1972). Thus determining the amount of radioactivity on the filter gave an idea of the extent of binding.

Equilibrium dialysis although requiring lengthy periods can cover a wider range of concentrations and hence greater r values can be attained than in spectroscopy. Despite this the disadvantages of long equilibration times and possible binding of the ligand to the membrane, has meant that equilibrium dialysis has found very limited application.

5.3.3 Centrifugation

This technique has found particular application in non-specific protein-DNA interactions (Revzin, 1990). A solution is made up containing both components which are allowed to equilibrate. Once this has been attained the solution is subjected to very high centrifugal forces, usually in an ultracentrifuge. At the start of the spin, both DNA and protein are uniformly distributed throughout the solution. The free protein, being small, will remain distributed throughout the whole tube, whereas the DNA-protein complex will move as a boundary. The distance travelled by this boundary is dictated by the force and time of the centrifugation. The concentration of DNA and protein can be determined by monitoring at the appropriate wavelengths and making minor corrections for radial dilution. The absorption of light above the sedimentation

boundary is due to free ligand only, whereas below the boundary the absorbance will be due to both bound and free ligand (Blake and Peacocke, 1968). Since one knows the total concentration of ligand added, the free ligand concentration can be calculated from the plateau portion of the boundary. Most of these experiments are carried out in either sucrose or glycerol gradients, the one disadvantage being that large quantities of both components are required. In a way, this method is similar to equilibrium dialysis, in which the sedimentation boundary is analogous to the dialysis membrane. The number and position of the sedimentation boundary will depend on the association constant (Gilbert and Jenkins, 1959 and Revzin, 1990). If the DNA and protein interact on a 1:1 basis and have a low association constant (they will move independently), two boundaries will be evident. Conversely, if their affinity for each other is high, then a single boundary will result. Although concern has been expressed about the effect that the high centrifugal forces could have on the complexes, affinity constants established with this method are in good agreement with those found using column chromatography, run at atmospheric pressure (Revzin, 1990). The limitations on the concentration of ligand used will depend, at the lower limit, on the amount of binding, if any, that may occur between the ligand and the centrifuge tube and at the upper limit on the degree of self aggregation.

5.3.4 Partition Analysis

By using this technique it is possible to induce the partitioning of the free from the bound ligand between an aqueous and a non-miscible organic phase whose partition coefficient for the ligand with respect to the water is known (Blake and Peacocke, 1968). Although partitioning allows for a clean separation of the two species of ligand, addition of the organic phase would change the dielectric constant and would thus influence binding. This could explain why this method has not been used extensively. The operative concentration range is the same as for equilibrium dialysis, though the method is more rapid.

5.3.5 Column Chromatography

5.3.5.1 Elution Volume Determination

This method was developed by de Haseth *et al.* (1977) using DNA-cellulose chromatography. In essence, the protein is loaded onto a cellulose column saturated with DNA and eluted with a salt solution at a fixed concentration. The volume of the salt solution required to elute a given amount of protein can be related to the association constant of the protein-DNA complex. The strength of binding is directly related to the elution volume. When a plot of the percentage of total protein remaining on the column was made against

the elution volume a linear relationship was found. By predicting certain models, de Haseth *et al.* (1977) were able to relate the association constant to the above mentioned parameters. The range of affinity constants that could be determined were of the order 10^4 to 10^6 M^{-1}.

5.3.5.2 Gel Exclusion Chromatography

Just as equilibrium dialysis or partition analysis measure the distribution of the ligand across a membrane or in another phase, respectively, gel permeation measures the distribution of the free ligand within a porous gel matrix. The most commonly used supports are polydextrans, polyacrylamide or polyagarose which act as molecular sieves, and are selected to allow only the free ligand and not the complexed form to penetrate the matrix of the gel; the macromolecule is totally excluded. These supports are used since they are relatively inert in buffers of moderate ionic strength so that adsorptive interactions do not interfere with the determinations which can be in the µg/ml range. This technique was established by Hummel and Dreyer (1962) and has been used by Frankel *et al.* (1985).

In essence the Hummel-Dreyer method involves equilibrating a column with a solution containing the ligand at a particular concentration. A protein solution containing a total ligand concentration equal to that of the column saturating solution is

then run through the column. If the protein binds the ligand, the solvent of this sample will become depleted with respect to the ligand. Thus when the column is run, the protein will be separated from the ligand depleted solvent, moving ahead with its bound ligand. The result is an elution profile exhibiting a peak in ligand concentration above the column ligand saturation baseline as indicated in Fig. 5.1.

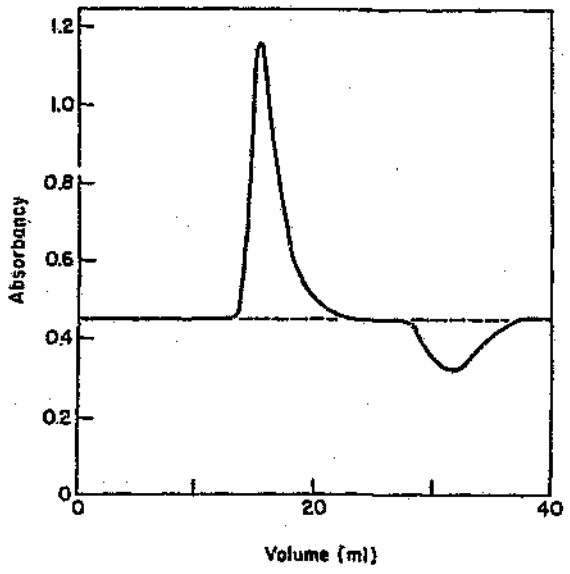


Fig. 5.1 The Hummel-Dreyer method of determining binding constants using the elution profile obtained by gel chromatography. The area of the trough represents the amount of ligand bound to the column and is equal to area of the peak (Hummel and Dreyer, 1962).

This peak would represent excess ligand bound. An analogous trough will follow, this representing the depletion that resulted from the bound ligand which is carried ahead with the protein (Fig. 5.1). Theoretically, the area of the peak and the trough will be equal. The area of the trough is measured since it is more reliable, and knowing the amount of protein applied, the binding ratio is determined at the particular column ligand saturating level.

5.3.6 Gel Electrophoresis

Electrophoresis has been popular as a means of studying specific protein-DNA interactions (Revzin, 1990). The gel support is usually polyacrylamide, agarose or a mix of the two. In theory free DNA will move more rapidly into the gel than the complexed DNA when subjected to an electric current. This approach can be limiting if the complexes dissociate during the run, although the low ionic salt buffers used tend to favour protein-DNA interactions. Problems might arise in DNA-antibiotic interactions where binding might not involve covalent linkages. The application of mobility shift gels to quantitate nonspecific interactions has, however, been limited because the dissociation of these complexes is so rapid upon onset of electrophoresis. Knowledge of the amount of DNA loaded and quantitation of the bands allows for the computation of free DNA.

5.3.7 Other Techniques

The above discussion is by no means a comprehensive treatise of all the methods available in this area of research. Only those which could find application in the particular problem at hand have been elaborated on. However, mention will still be made of a few more modern techniques that have been used in this area of research.

Electron microscopy (EM) is not a true quantitative technique but can nevertheless provide optical information especially in cases of cooperative binding. This method was particularly useful in the elucidation of the nucleosomal structure of chromatin. Delius *et al.* (1972) used EM to visualise complexes of the gene 32 protein of bacteriophage T4 to DNA. Regions of DNA containing the protein appear thicker than those free of the protein.

The last two methodologies to be mentioned are spectroscopic techniques and include electron spin resonance (ESR) and optically detected magnetic resonance (ODMR). Bobst *et al.* (1982) used ESR to study gene 32 protein complexation to single stranded nucleic acid. Using commercially available probes, spin labelled polynucleotides were prepared. When non-specific binding occurs, the ESR spectrum is modified. However, control experiments showed that the protein had a slightly higher affinity for the labelled poly dT; despite this the method can still be used to determine *relative* binding affinities.

The second method, ODMR, was utilised by Casa-Finet *et al.* (1987) to study complexes of single-strand binding (SSB) proteins to poly dT. In the N-terminal of the protein there are three tryptophan residues which were found, by phosphorescence spectra and lifetimes, to participate in stacking interactions with thymine residues in poly dT. ODMR also revealed that there was a second *trp* binding site in this particular protein from *E. coli* which was not present in other SSB proteins studied. Thus a considerable degree of information can be obtained at the molecular level from these techniques which in future could be supplemented by information obtained from site directed mutagenesis in proteins and nucleic acids.

5.4 BINDING STUDIES - MATERIALS AND METHODS

5.4.1 Instrumentation

5.4.1.1 Spectrophotometers

For absorbance measurements the dual beam UV-Visible UV-160A *Shimadzu* and for fluorescence, the *Hitachi* Model 850 (with a 150W Xenon lamp) were used. Both machines were checked on a regular basis for accuracy using filters and standards provided by the manufacturers.

5.4.1.2 Scintillation Counter

All isotope measurements were carried out in the *LKB 1219 Rackbeta (Wallac)* liquid scintillation counter linked to a PC and printer. Standardisation programs for single and dual isotope counting were used to tailor these programs to the experimental conditions. Channel spill-over in dual labelled samples was accounted for during the standardisation procedures.

5.4.2 Source and Preparation of Radioisotopes

For all radioactive isotope counting the following scintillation cocktail was used: Toluene:Triton X100 (2:1 v/v) containing 0.05 g/l of 1,4-bis(5-phenyloxazol-2-yl)benzene (POPOP) and 4 g/l of 2,5 diphenyloxazole (PPO). The solvents used were analytical grade

and POPOP and PPO were obtained from *Sigma*.

The tritiated water (185 MBq/mmol) and [U- ^{14}C] sucrose (20 MBq/mmol) were obtained from *Amersham International*, Buckinghamshire, England. [$^{3}H(G)$] Daunomycin (59.2 GBq/mmol) dissolved in 100% ethanol was purchased from *DuPont*, New England Nuclear Division, Wilmington, Detroit. The chemical purity of daunomycin was reported (by the suppliers) to be 99.0% and was checked using thin layer chromatography Silica Gel. The solvent system in the first direction was chloroform:methanol:water (13:6:1 v/v) followed in the second direction by n-propanol:water:acetic acid:pyridine (8:2:2:1 v/v). From this method the sample was found to be 95% pure and was used without further purification.

5.4.3 Preparation of E. coli for all In Vivo Binding Studies

Initial experiments were carried out using the Salmonella mutants employed in the Ames test. However, the uptake of labelled thymidine by these mutants was very small (2-4% depending on the strain used) when compared to that which could be achieved with *E. coli*. (20%). It is for this reason that all work described in this section was carried out using *E. coli*.

The culturing of *E. coli* (JM101) was carried out with stocks that had been stored in 15% (v/v) glycerol at -70 ^{0}C. The broth used to grow the cultures to log phase contained Bacto-tryptone 10 g/l,

Bacto-yeast extract 5 g/l, both from *Difco*, Detroit, and NaCl at 10 g/l. This solution was adjusted to pH 7.5 where necessary with 1 M NaOH and autoclaved at 121^{0}C for 20 minutes. Protein concentrations were determined with the Lowry method (Lowry *et al.*, 1952) using bovine serum albumin as a standard.

5.4.4 Two In Vivo Binding Assays to Measure the Effect of Polyphenols on the Uptake of ^{3}H-Daunomycin: (1) onto DNA of E. coli and (2) into E. coli cells

To measure the effect of polyphenols on the uptake of daunomycin two methods were selected. The first method sought to measure changes in the amount of daunomycin bound to bacterial DNA. To take into account any possible toxic effects due to the polyphenols and hence any change in growth characteristics, the DNA was labelled with ^{14}C-thymidine. A further advantage of this was that the amount of daunomycin bound to the DNA could be expressed as a ratio of labelled daunomycin to labelled DNA. Changes in this ratio (assuming no toxic effects viz. ^{14}C-thymidine constant) in the presence of polyphenols would be indicative of changes in the amount of daunomycin associated with the DNA. This methodology will be discussed in Section 5.4.4.1.

A second method of measuring polyphenol desmutagenicity was to develop an experimental approach that would measure the amount of

daunomycin within the bacterial cell. This was done by using changes in the internal volume of a cell as used by Rottenberg (1979) to measure membrane potentials in cells. In this work ^{14}C-labelled sucrose, ^{3}H-labelled daunomycin and tritiated water were used in a procedure detailed in Section 5.4.4.2.

5.4.4.1 Use of ^{14}C-Thymidine labelled DNA to Measure Changes in ^{3}H-Daunomycin DNA Targeting in the Presence of Polyphenols

To carry out labelling of bacterial DNA, ^{14}C-thymidine was added to 30 ml of sterilized rich medium (Bacto-tryptone 10 g/l; Bacto yeast extract 5 g/l and NaCl 10 g/l) at a specific activity of 206 µCi/µg thymidine and daunomycin at 0.2 µCi/µg. Both isotopes were made up as one solution, to reduce variability in the ratio of the two, and 30 µl of this solution added to the broth. Radioactive counting was carried out as before (see Section 5.4.1.2 and 5.4.2).

The broth was inoculated with *E. coli* and 30 µl of the daunomycin and thymidine mix, with subsequent addition of varying amounts of the three fractions of sorghum polyphenols from SSK52. A stock solution of polyphenols of 30 mg/ml was made up and different amounts added to the broth so that the final concentrations in 30 ml was 0.6 (maximum solubility), 0.2, 0.1 and 0.01 mg/ml. For F_2 and F_3, a few drops of methanol had to be added first to solubilise the

weighed powder since straight addition of water failed get the polyphenols into solution. The cultures were grown on a rotary shaker with a 5cm orbit (120 rpm) at 37 ^{0}C for 10 hours. The cultures were then subjected to centrifugation at 10 000g for 10 minutes and the resulting pellet resuspended in isotonic saline, washed and centrifuged at the same gravitational force as before. The pellet was then resuspended in 5 ml of water and sonicated for 20 seconds, after which the solutions were centrifuged for 10 000g for a further 20 minutes. A sample was taken from the pellet (consisting of cell debris) and the supernatant (containing material of interest) and the radioactive count determined. The supernatant was concentrated by freeze-drying, after which it was reconstituted in 200 mM phosphate buffer, pH 7.5 and loaded onto a 1.0 X 15 cm Biogel A 5M (Biorad) column equilibrated in and eluted with the same buffer. Biogel A 5M consists of 6% agarose and has a fractionation range from 10 000 to 5 000 000 Daltons. DNA from *E. coli* is reported to have a molecular weight of 3.1 x 10^{9} (Ausubel, 1988) and was thus expected to elute in the excluded volume.

5.4.4.2 Indirect Measure of ^{3}H-Daunomycin-DNA Binding Using Changes in ^{3}H-Daunomycin:^{14}C-Sucrose and Cell Volume, as Indicated by $^{3}H_2O$:^{14}C-Sucrose

The bacteria were grown overnight to an absorbance of 0.6 at 540nm. Once this growth had been achieved, the cultures were centrifuged at

10 000rpm (12 000g) in a *Sorvall* SS34 rotor head for 10 minutes (Padan *et al.*, 1976). The pellet was washed twice in 0.1M phosphate buffer, pH 7.2, and centrifuged at the same gravitational force. The resulting pellet was resuspended in minimal volume of the buffer and the protein content established, and then adjusted so that the protein concentration in the assay would be 2 mg/ml. The assay was carried out in sterile 1.5 ml eppendorf tubes containing 100 µl of buffer and varying amounts of polyphenol, 50 µl of a sucrose and tritiated water mixture without or with daunomycin (made up so that the counts were well within range of a calibration curve established for the scintillation counter) and 100 µl of bacteria. A stock solution of F_1 polyphenol was made up, diluted one hundred fold, and various volumes (amounts of 200 ng to 1000 ng) were added to the incubation mixture (with appropriate adjustments of assay volumes with buffer), yielding concentrations of 0.8 to 4 µg/µl in the final assay volume of 250 µl, compared to 1.2 ng/µl of daunomycin in the mix. Once the assay had been carried out for 20 minutes (the optimum time determined experimentally), the eppendorf tubes were centrifuged at 13 000 rpm in a *Hereaus* biofuge for 10 minutes. The pellet was resuspended in 200 µl of buffer of which 50 µl was added to 4 ml of scintillation fluid and counted.

Bakker *et al.* (1976) used the following equation to calculate the internal volume (a) of the cell as a percentage of the total water content of the pellet by using the tritium and ^{14}C-labelled sucrose in the pellets and supernatants (as indicated in the following

equation).

$$\alpha = \left[1 - \frac{\frac{(^{14}C)}{(^{3}H)}pellet}{\frac{(^{14}C)}{(^{3}H)}supernatant} \right] \times 100\%$$

A more reliable measure of the internal volume (V_i) could be achieved by using the equation (shown below) as derived by Rottenberg (1979).

$$V_i = V_s \cdot \left[\frac{(^{3}H_p)}{(^{3}H_s)} - \frac{(^{14}C_p)}{(^{14}C_s)} \right]$$

where $^{3}H_p$ and $^{3}H_s$ are the tritium counts in the pellet and the supernatant, respectively. Similarly, $^{14}C_p$ and $^{14}C_s$ are the counts due to ^{14}C-sucrose in the pellet and supernatant, respectively. V_s is the volume of the supernatant (which can be measured). The internal volume is usually expressed relative to the protein content of the pellet. The above equation was used in the present work.

5.4.5 *In Vitro Binding Assays: Diffusion Analysis, Spectrophotometry and Column Chromatography*

5.4.5.1 *Equilibrium and Rate of Diffusion Analysis*

Two thick (10mm) perspex plates (100 mm long) had four wells ground out along the entire length of each plate. A Visking dialysis membrane (or alternately millipore filters) was placed between two such plates and clamped together with each plate providing half a well on either side of this membrane. The dialysis membrane (*Medicell* International Ltd., London, Britain) is composed of cellulose with a pore size of approximately 2.4 nm and a molecular weight cut off value of 12 000 - 14 000. Furthermore, because fine holes were drilled into each well from the upper surface of the plate, it was possible to withdraw samples from each well using a microsyringe and to submit these to liquid scintillation counting using the same scintillation cocktail as before (See section 5.4.2). The final volume used in each well was 100 µl. Experiments were carried out in triplet or quadruplet.

The daunomycin (59.2 GBq/mmol) used here was identical to that described in Section 5.4.2 of this chapter. An initial thousand fold dilution was carried out on a sample of this isotope, then an equal volume of this was diluted with non-radioactive daunomycin to give 50 ng/ml, 60 ng/ml and 500 ng/ml for the different experiments. All assays were performed in 0.51 M 2[Tris(hydroxymethyl)methyl-

2-amino-ethanesulfonic acid (TES) (from *Sigma*) and NaOH buffer, pH 7.2. TES has a pK_a of 7.4 at 25 °C.

5.4.5.2 Spectrophotometry

5.4.5.2.1 Absorbance

All assays were carried out at room temperature (20 ^{0}C) in a 10 mM potassium phosphate buffer (pH 7.4) containing 150 mM NaCl and 1 mM ethylenediaminetetracetic acid (EDTA) (Xodo *et al.*, 1988). Daunomycin (concentration range 5 to 50 µM - within the range used by Xodo *et al.* 1988) was titrated with varying amounts of DNA (stock solution 4 µg/µl) and polyphenols. The absorbance at 480nm was used to monitor any binding since at wavelengths in the UV region daunomycin, DNA and the polyphenols have strong and overlapping spectra. All components have minimal absorbance except daunomycin which has an ϵ = 11 500 M^{-1} cm^{-1} at 480nm (Chaires *et al.*, 1982) which was useful in calculating the amount of free and bound drug, using the de Beer-Lamberts law, which states that the absorbance is linearly related to the concentration and extinction coefficient of a substance. To calculate the free and bound drug concentrations, the following calculations were carried out (Xodo *et al.*, 1988).

$$C_B = \left[\frac{A^O - A}{A^O - A_F} \right] \times C_T$$

where A^0, A_F and A are the absorbances of the drug (here daunomycin) in the absence of DNA, in the presence of a large excess of DNA and a mixture of free and bound drug, respectively. C_T is the total concentration of the drug in solution. The concentrations of bound (C_B) and free (C_F) drug needed for the parameters in the Scatchard plot were calculated according to the following:

$$C_F = C_T - C_B$$

5.4.5.2.2 Fluorescence

The same buffer was used as for the absorbance measurements. Daunomycin could be monitored at 1 µM levels with an excitation at 470 nm and an emission at 592.3 nm, wavelengths corresponding to those reported by Xodo *et al.* (1988). To obtain the parameters for the Scatchard plot the following calculations were carried out.

$$C_F = C_T \frac{\left(\frac{F}{F_o} - P\right)}{(1-P)}$$

where F and F_0 are the fluorescence intensities in the presence of DNA and in the absence of DNA, respectiveiy. The ratio of the fluorescence intensities of the free drug and fully saturated drug is used to obtain a value for P. The bound (C_B) drug concentration can be determined from the total (C_T) and free (C_F) as shown below:

$$C_B = C_T - C_F$$

5.4.5.3 Column Chromatography (Hummel-Dreyer Method)

Columns (1cm X 25 cm) of Sephadex G-10 and G-50 were equilibrated in 0.9% (w/v) NaCl solution. A solution of daunomycin (10 mg/ml) was loaded onto the column. The column was washed with increasing concentrations of NaCl up to 1 M. However, when this failed to remove the daunomycin from the column, a methanol:water gradient was applied, and at 20% (v/v) the daunomycin was eluted from the column. Initial trials using plastic end parts for the column were found to bind the daunomycin as it moved out of the column to the fraction collector. These parts were replaced by glass wool which did not bind the daunomycin.

5.5 BINDING STUDIES - RESULTS AND DISCUSSION

Several of the conventional methods used to study binding interactions were employed to provide evidence of the desmutagenic behaviour of polyphenols. The two *in vivo* techniques used included methods (i) to determine the amount of daunomycin associated with the DNA by using column chromatography to isolate DNA from *E. coli* grown in the presence of varying concentrations of F_1 polyphenols and (ii) to monitor the uptake of daunomycin into *E. coli* as a function of cell volume (^3H_2O:^{14}C-sucrose) and ^{3}H-daunomycin:^{14}C-sucrose ratio changes. The *in vitro* procedures included equilibrium and rate of diffusion analysis, absorbance and fluorescence spectrophotometry and column chromatography (Hummel-Dreyer method). Of all the methods utilised, the extraction of the daunomycin:DNA complex from *E. coli* was found to be the best and provided evidence for the desmutagenic action of the polyphenols. The other assays were not suitable for the present study either due to the insolubility of the polyphenols or because of their binding to the equipment used in these studies. All the above mentioned techniques will be detailed in this chapter.

5.5.1 *Polyphenol Effect on ^{3}H-Daunomycin and ^{14}C-Thymidine Ratio as a measure of DNA Targeting in E. coli*

To measure interaction of polyphenols with daunomycin, it was decided to quantitate the binding of daunomycin to DNA in the absence and presence of polyphenols. If polyphenols (maybe F_2 or F_3) bind the daunomycin outside the cell or if polyphenols coat the cell thereby preventing daunomycin entry into the cell, this would effectively decrease the amount of daunomycin that could interact with the DNA. In the case where polyphenols (the small F_1 fraction) bind but can enter the cell, the daunomycin-polyphenol complex could either remain in the cytoplasm or it could still bind to the DNA as a daunomycin-polyphenol complex, in which case no difference in the amount of drug bound would be seen. Thus if a negative result is obtained i.e. no change in the amount of daunomycin bound to DNA, binding cannot be ruled out. It was with this in mind that the other techniques mentioned were used to either provide supporting evidence or to disprove these postulates.

The three compounds involved in the binding assays viz. daunomycin, DNA and polyphenol all have overlapping UV spectra and since both DNA and daunomycin needed to be measured accurately, it was decided to label the DNA with ^{14}C-thymidine. This would allow one to monitor the DNA during purification, as well as to indicate any changes in growth of the bacteria in the presence of the polyphenols (allowing one to monitor any toxic effects due to the polyphenols). Because

the antimutagenicity had been observed using *S. typhimurium* in the Ames test, these bacteria were used as the test model.

FAILURE OF SALMONELLA MUTANTS AS THE TEST ORGANISMS: *E coli* is preferred

The ^{14}C-labelled thymidine was fed to the bacteria at various concentrations to optimise uptake. It was found that when using the Salmonella bacterial mutants, it was not possible to get more than 2 to 4% uptake of the label, depending on the strains used. Even a brief treatment with 1% (v/v) Tween (Billen and Olson, 1978) and 0.25 M sucrose failed to force the uptake of thymidine into the cell by transient permeablisation. This could be due to the genetic deletions that were made in these bacterial strains to increase their sensitivity to mutagens in the Ames test (Maron and Ames, 1983). The uptake of mechanism of thymidine may have been impaired. A change was made from *S. typhimurium* to *E coli.*, whereupon a 20% optimum uptake was recorded. Once this had been achieved, daunomycin was added and the DNA extracted. As was expected, using the conventional DNA extraction procedure which involves sodium dodecyl sulphate and salt treatment, followed by chloroform:isoamyl alcohol and then ethanol extraction, only 1% of the daunomycin remained associated with the DNA. Most of the daunomycin was in the alcohol fractions. An alternative way of breaking open the cell and extracting the DNA, separating free from bound daunomycin, was needed.

Sonication of the bacteria proved to be effective in breaking open the cells. The sonicated cells were centrifuged to remove cell

debris, and the supernatant filtered by centrifugation in a biofuge, using the polysulfone (low drug binding) ultrafiltration units recommended by *Millipore*. It was anticipated that the DNA-daunomycin would be retained by the membrane, whereas the free daunomycin would move through due to the selected pore size of the membrane. However, using a solution of daunomycin only and subjecting this sample to centrifugation, it was found that the daunomycin bound to the membrane. An alternative way of separating the daunomycin-DNA complex from the rest of the cellular contents was column chromatography.

It was found that daunomycin had a strong affinity for Sephadex which is normally used in gel permeation (see Section 5.5.5.). Biogel 5M proved to be a suitable alternative; close on a 100% recovery of the daunomycin loaded onto such a column could be achieved using a 200 mM potassium phosphate buffer (pH 7.5) as the eluent. The next step involved the dual labelling experiment in which *E coli*. was fed ^{14}C-labelled thymidine and ^{3}H-labelled daunomycin and resulting DNA extracted from these cells. The bacterial cells were washed, sonicated and then centrifuged, and the resulting pellet loaded onto a Biogel 5M column. Elution was carried out as before. The results of this experiment are shown in Fig. 5.2.

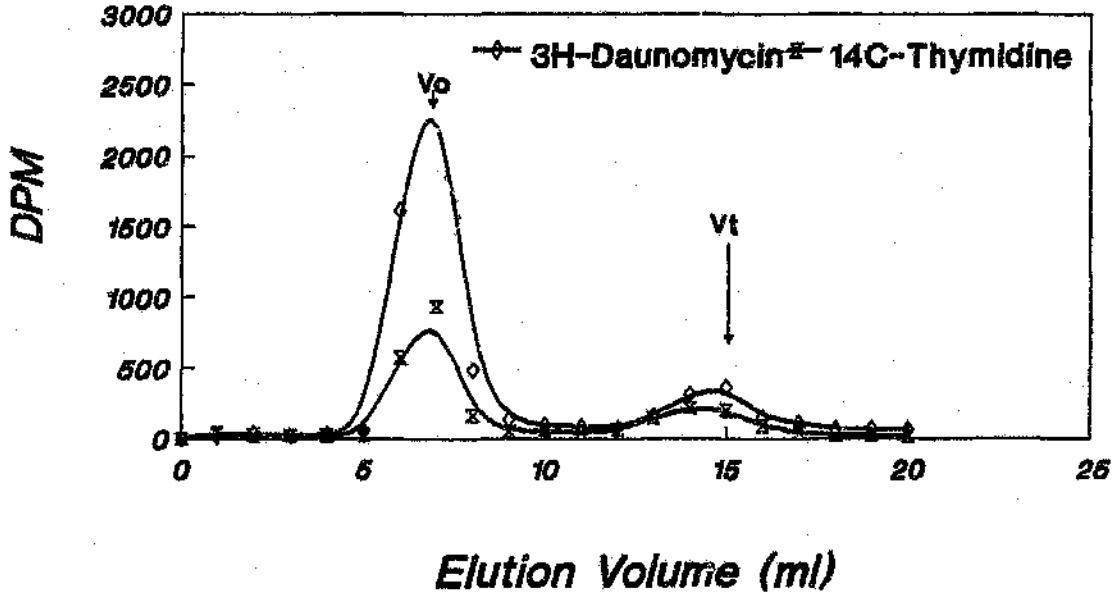


Fig. 5.2 *The cytoplasmic content of E.coli. was loaded onto a BioGel-5M column (1 x 15cm) and eluted with a 200 mM phosphate buffer, pH 7.5. The unincorporated ^{14}C-thymidine and free ^{3}H-daunomycin were separated from the dual labelled (daunomycin:thymidine) DNA complex which eluted in the void volume.*

The results obtained in Fig. 5.2 opened the way to study the effect of polyphenols on daunomycin-DNA binding. *Escherichia coli* was grown in the presence of the three fractions and the results obtained plotted (Fig. 5.3) as the ratio of ^{3}H-daunomycin to ^{14}C-thymidine versus the amount (expressed as mg of polyphenol/ml of growth medium) of phenolics added to the medium. All three fractions from SSK52 were effective in reducing the amount of daunomycin associated with the DNA (thymidine content was constant). There appeared to be no significant difference between the three (except at the lower concentrations) which was expected based on the antimutagenicity observed in the Ames test (Fig. 4.2) where the order of binding efficiency increased with molecular size viz. $F_1 < F_2 < F_3$ in the presence of daunomycin and *S. typhimurium* strain TA98. Thus it has been demonstrated that in the case of daunomycin, the polyphenols act as desmutagens since they reduce the effective concentration of the mutagen associated with the DNA.

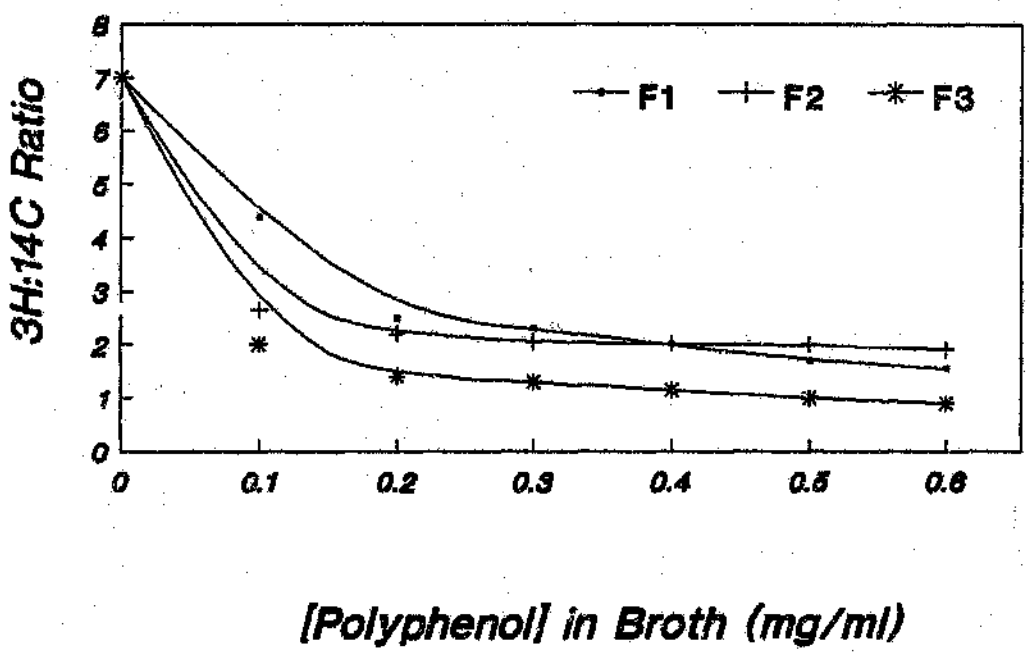


Fig. 5.3 *The decrease in the amount of ^{3}H-daunomycin associated with ^{14}C-thymidine labelled DNA (as measured by the change in ^{3}H:^{14}C ratio) from E coli. grown in the presence of polyphenols extracted from sorghum grain SSK52.*

5.5.2 ^{3}H-Daunomycin Uptake into *E. coli* Cells

To determine the uptake of daunomycin and the effect of polyphenols on this uptake into *E coli.*, a method was required to measure any deviations in cell density, and hence the resulting pellet size containing the daunomycin marker. One way to achieve this was to have one constant isotope (here ^{14}C-sucrose) against which to measure the relative change of the ^{3}H-daunomycin, expressing change as a ratio of these two isotopes. Another way of ensuring consistency in the control, was to measure another parameter, namely that of cell volume. Determinations of cell volume have been used to measure the uptake of compounds (used to calculate internal pH) into *E coli.* and were particularly useful in the development of the chemiosmotic theory (Padan *et al.* 1976; Mitchell, 1968 and Winkler *et al.*, 1966).

In attempting to measure cell volume, two labelled probes were required, one which could diffuse throughout the whole system, and another which would only occupy the area external to the cell wall. The two isotopes selected were tritiated water which measures both interstitial space and internal cell volume, and ^{14}C-sucrose which will only occupy the interstitial space. By calculation (see Section 5.4.4.2), it was possible to get the value for the internal volume, expressed as a volume per amount of protein. A value of 2.68 µl ± 0.25 (SD)/ mg of protein (as determined by the Lowry method (1951)) which compares favourably with a value of 2.7 µl/mg reported by Winkler and Wilson (1966) for *E.coli.*

The next step was to carry out a titration (result shown in Fig. 5.4) of daunomycin to establish (i) if the drug was taken up into the cell and (ii) over what concentration range there was a linear relationship between uptake and the amount of daunomycin added to the medium (with simultaneous cell volume monitoring). Logically the concentration to be selected for titration with the polyphenols (F_1 used) would have to be in the linear region, otherwise small changes in daunomycin uptake would not be registered. As an initial probe into the system, it was decided to select 80ng (a final concentration of 1.6 µg/ml - *see* Fig. 5.4) of daunomycin against which to titrate polyphenols of the following concentrations 0.4, 0.8, 1.6, 3.2 and 4.0 µg/ml. Such low values were necessary to prevent precipitation of the polyphenols, which could distort the isotope ratio in the pellet. Data obtained in this experiment was used to calculate the internal cell volume (according to the method of Rottenberg (1979)) and the ratio of daunomycin to sucrose, the results of which are presented in Table 5.1. Monitoring of the cell volume using tritiated water and sucrose served as a control for any variability in volume from one preparation to another, and in the presence of polyphenols would indicate if these had moved into the cell.

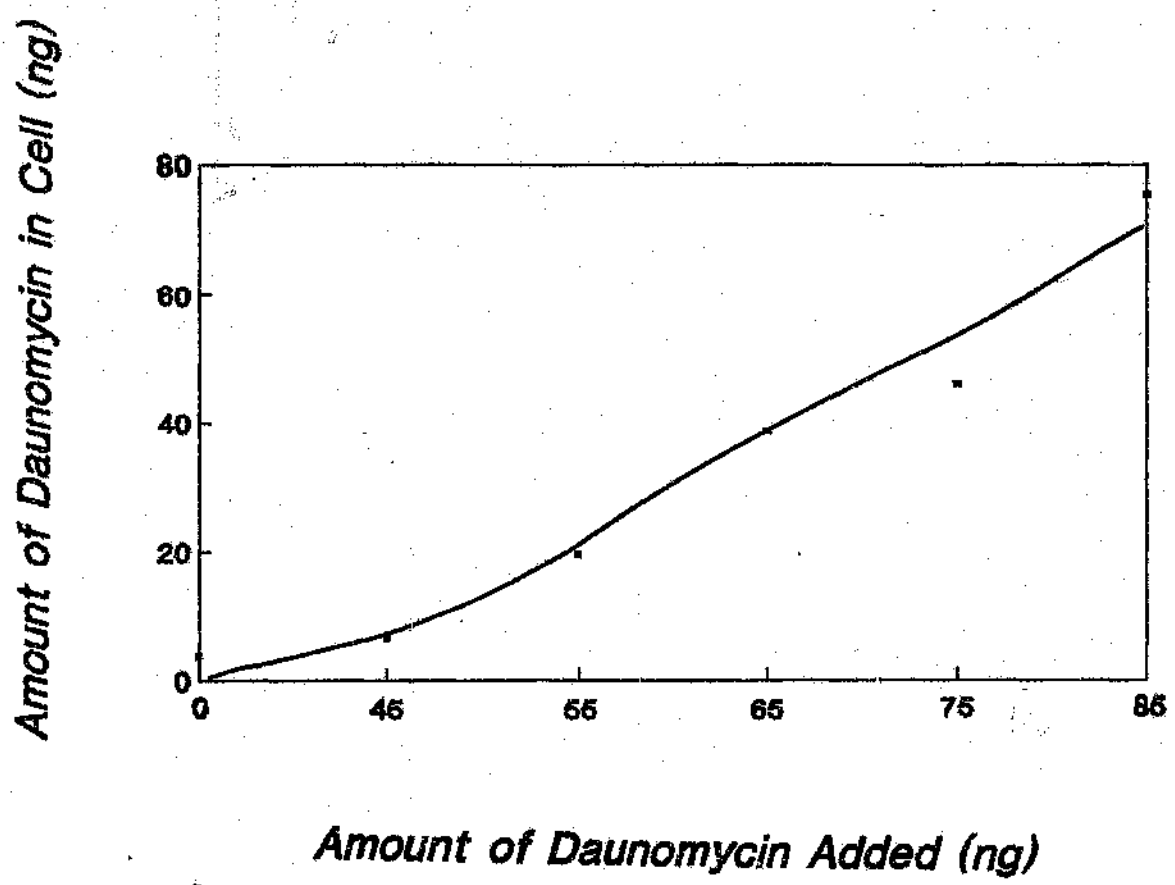


Fig. 5.4 *^{3}H-Daunomycin uptake into E coli, as measured by the method of Rottenberg (1979). E coli cells were incubated with ^{3}H-daunomycin and ^{14}C-sucrose for 20 minutes and then centrifuged. The resulting pellet was resuspended and the amount of each isotope present determined. Using these values it was possible to calculate the amount of daunomycin in the cell or bound to it (see Table 5. 1).*

Table 5.1 The effect of F_1 polyphenols on the uptake of ^{3}H-daunomycin[ǂ] (1.6µg/ml) into E coli. This was determined both as a change (i) in cell volume as measured by ^3H_2O:^{14}C-sucrose and expressed in µl/mg protein and (ii) the ratio of ^{3}H-daunomycin:^{14}C-sucrose. For each level of F_1 tested, the mean of six replicates was taken, and the standard deviation of this mean shown (n=6).

F_1 (µg/ml)	Volume[i] (µl/mg protein)	Daunomycin[ǂ] Uptake (ng)	Daunomycin (ng)[ii] / Sucrose (µg)
0.0	2.68 ± 0.25	61.3 ± 0.44	0.95 ± 0.050
0.4	2.80 ± 0.10	68.5 ± 0.80	1.06 ± 0.001
0.8	2.50 ± 0.20	64.7 ± 0.14	1.00 ± 0.001
1.6	2.87 ± 0.18	59.3 ± 0.21	0.92 ± 0.002
3.2	2.67 ± 0.11	72.4 ± 1.06	1.12 ± 0.001
4.0	2.55 ± 0.22	62.8 ± 0.50	0.98 ± 0.001

As can be seen there was no difference in the amount of daunomycin taken up into the cell in the absence or presence of various concentrations of polyphenols, which is particularly evident in the daunomycin:sucrose ratio. This finding seems to contradict those obtained from the previous experiment (Fig. 5.3) which showed that the polyphenols reduced the amount of daunomycin associated with the DNA viz. the amount of daunomycin taken up by the cell had decreased. The amount of sucrose did not vary significantly over the whole series (64.43 µg ± 7.59) of experiments since it might be argued that the polyphenols could be displacing the sucrose. If this were true and the amount of daunomycin had decreased due to polyphenol binding, a constant ratio of daunomycin to sucrose would have resulted. However, the amount of daunomycin was constant. Furthermore, it can be seen that the volume recorded (calculated using the ^3H_2O:^{14}C-sucrose ratio - see Section 5.4.4.2) was constant

over the entire polyphenol titration range. This would seem to prove that the polyphenols are not able to enter the cell, since none of the water was displaced and hence the formation a polyphenol-daunomycin complex in the cell is ruled out. There appears to be no evidence of binding from this method to support the antimutagenic effect of F_1 on daunomycin as seen in the Ames test (Fig 4.2) and Fig 5.3.

Only F_1 polyphenols were used since it was predicted that they could be carrying out their antimutagenic activity by preventing the entry of daunomycin into the cell. The other two fractions would most likely precipitate in the pellet due to the large amount of bacterial protein present, and hence were not tested. It was decided to seek an alternative method for measuring uptake into the cell to corroborate the findings presented in Table 5.1.

5.5.3 Equilibrium and Rate Diffusion Analysis

To perform equilibrium and rate dialysis, a nitrocellulose dialysis membrane was selected with a pore size of 2.4nm so that it was impermeable to *E coli*. which has a diameter of 2.2 μm and a length of 10 μm (Darnell, 1986).

In the absence of any other components other than daunomycin, it was predicted that an equilibrium would be established across the dialysis membrane. If purified DNA or *E coli*. were placed together

with the daunomycin (it is known that DNA binds the drug; the drug can also diffuse into the bacteria, whereupon it binds to the bacterial DNA), the effective daunomycin concentration that could diffuse across the dialysis membrane would be decreased. Samples taken from the well on the other side of the membrane would show a decrease in the amount of daunomycin that had diffused across the dialysis membrane and since the concentration gradient is less steep, the rate of diffusion would also be affected.

Initial experiments were aimed at determining how long it would take to establish such an equilibrium. From Fig. 5.5, it can be seen that even after 28 hours the ratio had not reached unity even when the concentration was increased from 50µg/ml to 500µg/ml, although the initial (over the first hour) rate of diffusion had increased by about 1.5 times. Upon further analysis, it was found that up to 60% of the daunomycin was lost, presumably bound to the membrane. This was confirmed by removing and counting the membrane. Even on changing the membrane to a specially treated low binding type (as recommended by *Millipore*), binding still occurred. If daunomycin binds to the membranes, it is likely (as has subsequently been found in this laboratory) that the polyphenols would also bind. This would introduce another binding parameter into the whole study which could not be quantitated for the polyphenols. Thus it was decided not to investigate this method any further.

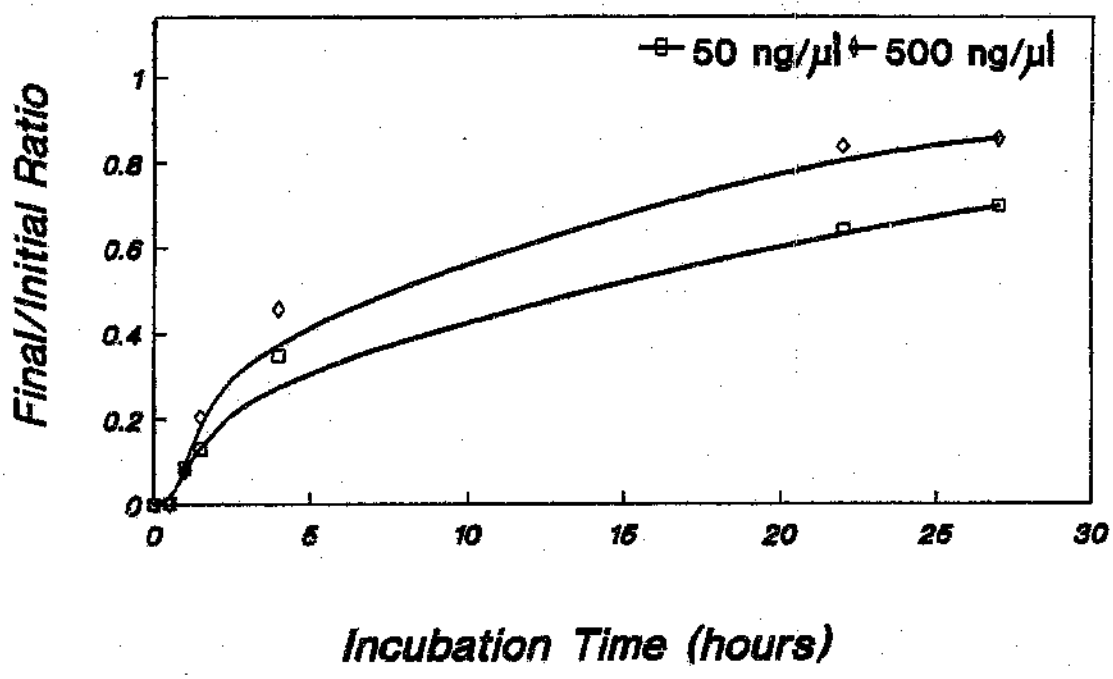


Fig. 5.5 *Measurement of equilibrium and rate of diffusion of ^{3}H-daunomycin across a permeable membrane placed between two chambers (see Section 5.5.2). Daunomycin was added to one chamber (initial) and allowed to diffuse across the membrane into the second chamber (final) over a period of 30 hours. Samples were taken at several time intervals to measure the amount of daunomycin on either side of the membrane, and the equilibrium ratio (^{3}H-daunomycin $_{final}$ / ^{3}H-daunomycin $_{initial}$) determined from this. Daunomycin concentrations used were 50 and 500 ng/µl.*

5.5.4 Spectrophotometry

5.5.4.1 Absorbance

Protein-polyphenol interactions have been monitored using UV spectrophotometry (Takechi and Tanaka, 1987; Bergmann, 1986). The extent of interaction can be quantified by monitoring the UV spectrum of the polyphenol in the 210-280 nm region (Czochanska, 1980). For example the polyphenol ß-penta-O-galloly-D-glucopyranose has absorbance maxima at 215nm and 280nm (Gray, 1978; Warminski, 1989). Proteins obscure readings in the lower region, and all proteins except those rich in tyrosine and tryptophan show minimal absorbance at 280nm; consequently the concentration of the polyphenol in the presence of precipitating proteins can be determined at the higher wavelength. It was observed that the height and position (in some cases) of this peak varied when proteins added to the polyphenol caused precipitation. This peak varied with the different amounts and types of precipitating proteins added. I tried to set up a similar system using the polyphenol and daunomycin spectra. However, as can be seen from the spectra of these two compounds (Fig. 5.6), both have very strong absorbances in this region, making it impossible to monitor the two compounds simultaneously. Besides, if DNA were added, this would have a very strong absorbance in the UV region. As can be seen from Fig 5.6, daunomycin has a very distinctive peak at 480 nm, a wavelength at which both the DNA and polyphenol showed minimal

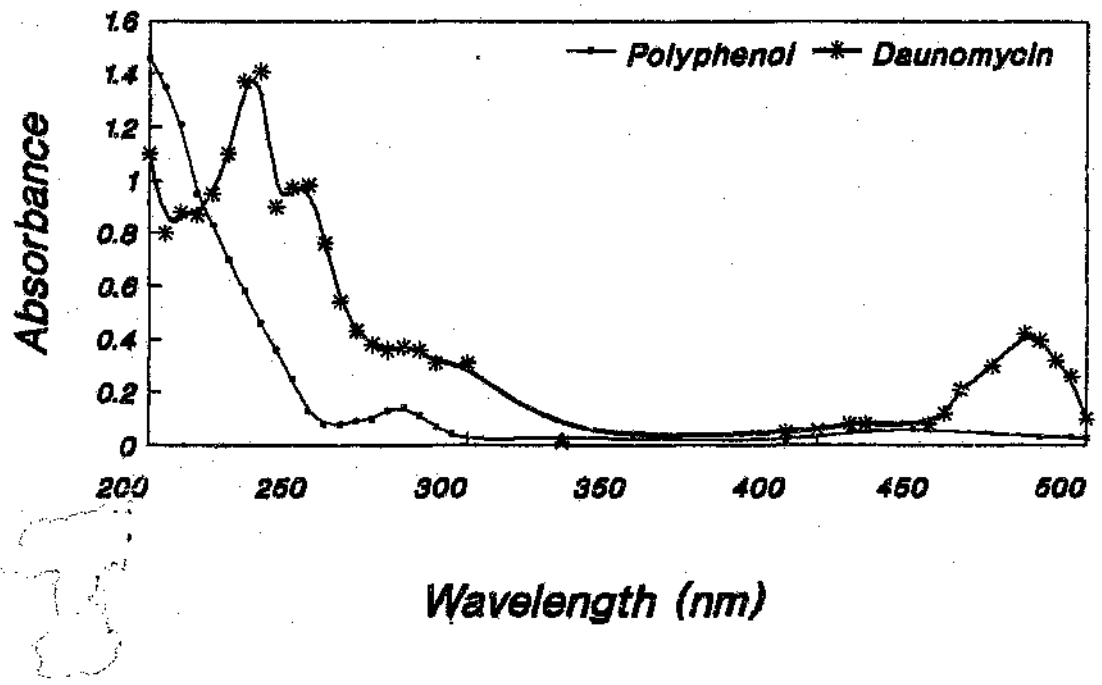


Fig 5.6 Absorbance spectra of daunomycin and polyphenol, showing the significant overlap in the UV region but with a distinctive 480nm peak for daunomycin, which was used to monitor daunomycin binding to DNA.

absorbances, and when DNA was added to daunomycin there was a decrease in the 480 nm peak (Xodo *et al.*, 1988). When daunomycin (6 ng/µl) was titrated with increasing amounts of DNA, it was possible to bind almost 100% of the drug (Fig 5.7). Titration with a more purified F_3 fraction (F_g) using the same concentrations as for DNA, produced no significant decrease in absorbance of the daunomycin at 480nm, suggesting limited interaction between the drug and the polyphenol.

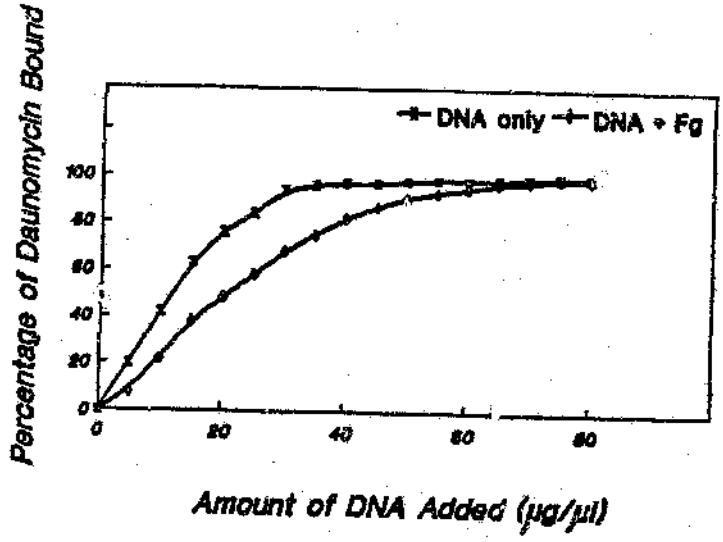


Fig. 5.7 The binding of DNA to daunomycin as monitored by a decrease in daunomycin (6 ng/µl) absorbance at 480nm. A prior addition of 24 ng/µl F_g (a more purified form of F_3) to daunomycin was made, with subsequent titration of DNA.

However, when 24 ng/µl (this was the maximum amount that could be added before precipitation was seen in the assay) of the F_g was added prior to DNA titration, this decreased the amount of daunomycin bound at low concentrations of DNA (Fig. 5.7), though at higher DNA concentrations all the daunomycin was eventually bound. Whether the polyphenols actually bind to the daunomycin needed to be established. Higher concentrations of polyphenols could not be used as it was noted that a fine precipitate formed, which could have reduced the amount of light reaching the photomultiplier thereby giving a false increase in absorbance.

This same apparent non-reactivity of daunomycin was observed when quercetin was used as a potential binding target. It was found that quercetin on its own caused quenching which was traced to the fine precipitate that formed in the assay. Quercetin is only soluble in alcohol and precipitated when added to the aqueous DNA-daunomycin solution. Hence data obtained from absorbance spectrophotometry would not be a reliable means of calculating the amount of free and bound daunomycin (required for Scatchard plotting). An attempt was made to use fluorimetry since much lower concentrations of the drug and hence of the other components would be needed to produce any change in the binding spectra, possibly eliminating the problem of polyphenol precipitation.

5.5.4.2 Fluorescence

Just as absorbance of the drug decreases upon binding, it was found that titration with DNA brought about an analogous decrease in emission at 592.3 nm when daunomycin was irradiated with light at 470nm. The amount of daunomycin was a thousand fold less than required for absorbance spectrophotometry. When the percentage decrease in fluorescence of various concentrations of daunomycin was plotted versus the amount of DNA or polyphenol (Fig. 5.8 and 5.9), a definite decrease in both cases was noted.

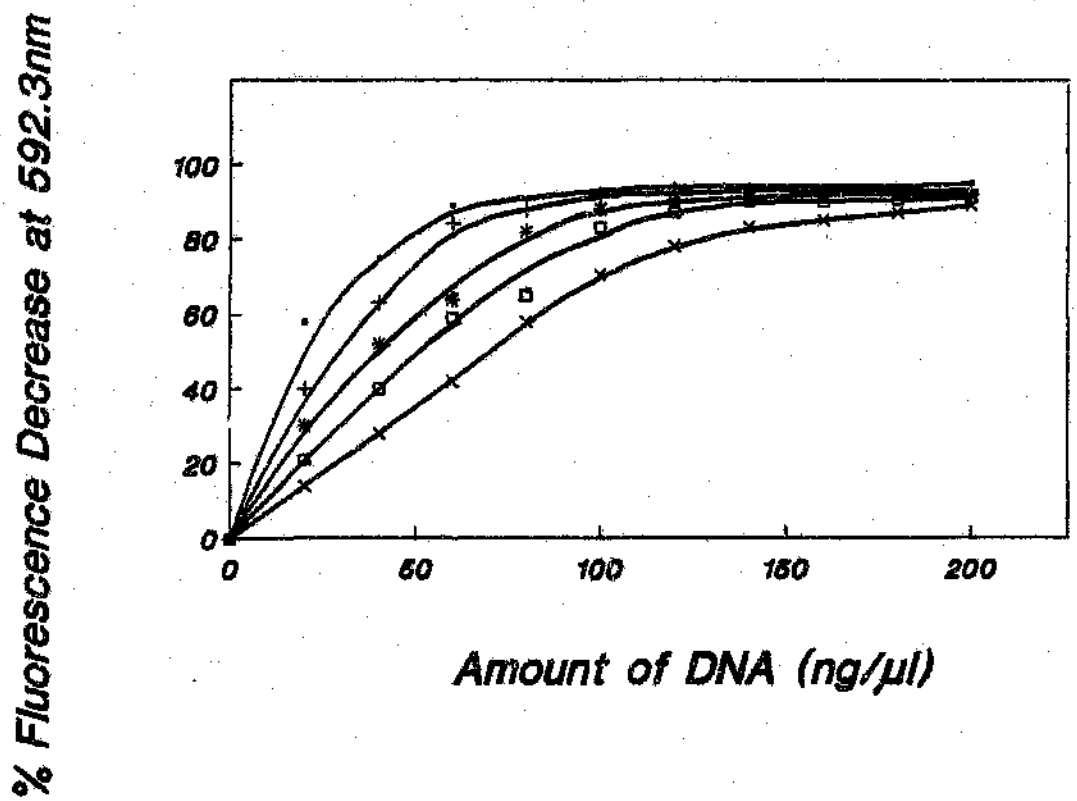


Fig. 5.8 *Daunomycin at varying concentrations (as indicated in the legend below) was titrated against DNA, and the decrease in the amount of free daunomycin was measured as a percentage decline in 592.3 nm fluorescence.*

4.11 pg/µl 8.22 pg/µl 12.3 pg/µl
16.4 pg/µl 20.5 pg/µl

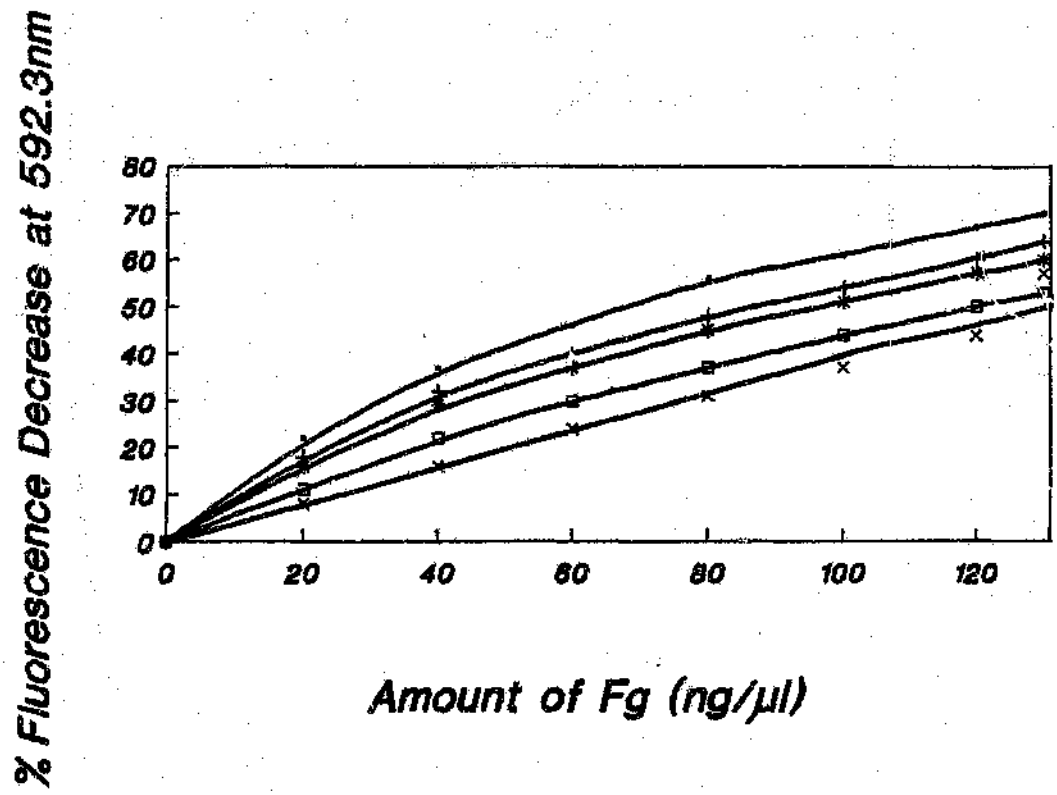


Fig. 5.9 *Daunomycin at the concentrations (indicated in legend below) used in Fig. 5.8, was titrated with F_g and the decrease in free daunomycin monitored by a percentage decrease in 592.3 nm fluorescence.*

4.11 pg/µl 8.22 pg/µl 12.3 pg/µl
16.4 pg/µl 20.5 pg/µl

For a comparative study, the lowest level of daunomycin (4.11 pg/µl - as used in Fig. 5.8 and Fig 5.9) was incubated with either DNA or polyphenols (Fig. 5.10). As can be seen DNA was far more efficient on a weight for weight basis in producing a decrease in fluorescence than the polyphenols. Although the polyphenols brought about a decrease in 592.3 nm emission it has not been proven as is the case for DNA, that this represents binding of the polyphenol to the drug. To prove actual binding to daunomycin, it was decided to use the Hummel-Dreyer gel chromatography technique to determine the binding constant of daunomycin for DNA and polyphenol.

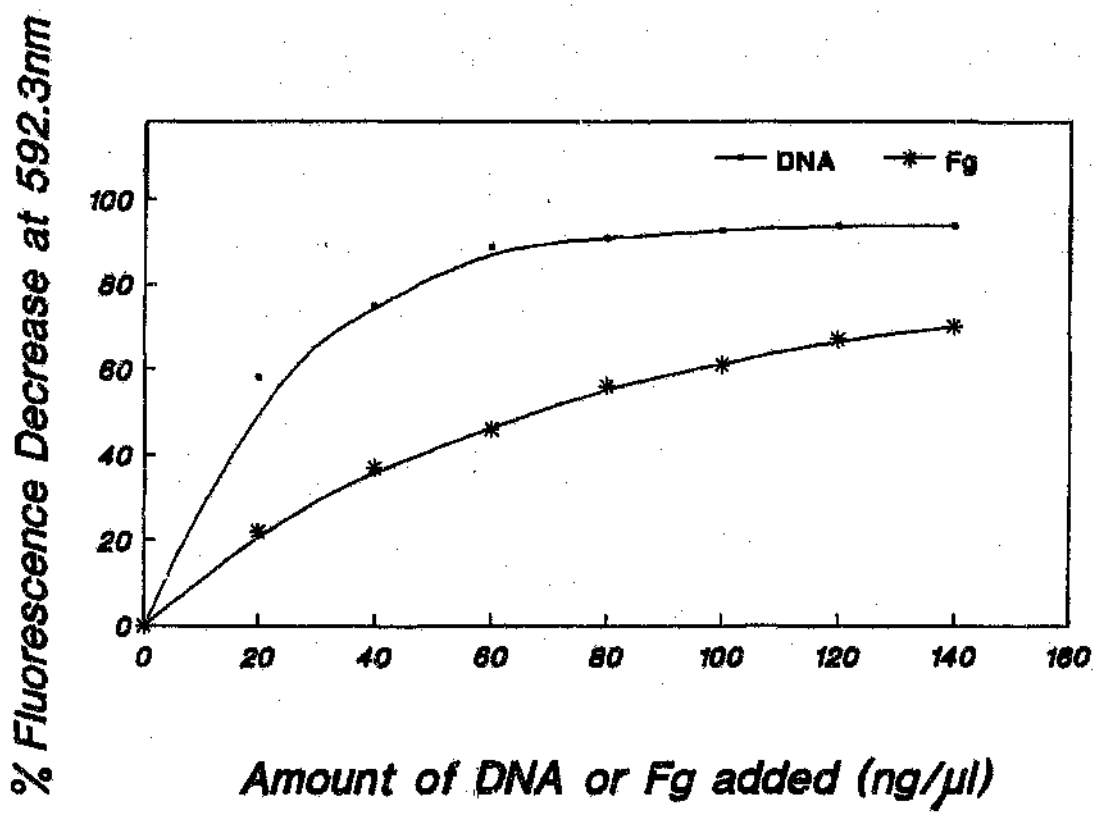


Fig. 5.10 Comparative studies of daunomycin binding to increasing (■) DNA and (*) F_g concentrations. Percentage decrease in fluorescence (at 592.3nm) measures the decrease in free daunomycin (initial concentration was 4.11 µg/µl) in the final assay volume.

5.5.5 *Column Chromatography - Hummel-Dreyer Technique*

This method depends on the fact that daunomycin but not the DNA or polyphenol is able to enter the pores of the gel from which it can be eluted with solutions of mild ionic strength from the column. Sephadex G-10 was selected as the column support because of its small pore size. Daunomycin was loaded onto the column in a potassium phosphate buffer, pH 7.5, containing 15 mM NaCl, and elution was attempted with increasing salt concentrations. Even a 1 M salt solution failed to remove the daunomycin from the column. The pore size was increased by using a Sephadex G-50 since it was thought that daunomycin could be binding in the small pore-sized gel, yet the same effect was observed. A methanol gradient was applied and at 20% (v/v) the daunomycin was eventually eluted. This defeated the object of this method which depends on selective retardation of the ligand (here daunomycin), and since it was shown that polyphenols also show an affinity for the column packing material, this procedure could not be applied to the current binding studies.

Thus of all the methods applied, only fluorescence spectrophotometry and extraction of the daunomycin-DNA complex by column chromatography provided evidence of the desmutagenic activity of the polyphenols.

Chapter 6. SUMMARY

6 SUMMARY

Polyphenols were extracted from various sorghum grain cultivars and were fractionated into the following three major groups on a Sepharose CL-6B column: F_1 containing low molecular weight water soluble phenolics, F_2 with the larger sized aqueous methanol soluble polyphenols and F_3 which possesses polyphenols soluble in aqueous acetone. The F_3 fraction contains polyphenols with molecular masses of up to 50 000.

From the work conducted the following conclusions can be drawn about the biological activity of the polyphenols.

(i) In chapter 3 the polyphenols from these various cultivars were shown not to be mutagenic at the levels tested in both the Ames assay and Somatic Mutation and Recombination Test.

(ii) In chapter 4 the polyphenol fractions F_1, F_2 and F_3 from sorghum grain variety SSK52 were coincubated with the standard mutagens sodium azide and TA100, daunomycin and TA98, 2-aminofluorene (requiring S9 activation) and both TA98 and TA97a in the standard Ames test. These polyphenols were shown to reduce the ability of these standard mutagens to induce reversion in the appropriate strains. They appeared to exhibit antimutagenic activity in the

order $F_3 > F_2 > F_1$. Barnard Red F_1 polyphenols were also found to be effective antimutagens, confirming the work carried out by van Rensburg *et al.* (1985) on the reduction of experimentally induced oesophageal tumours in rats feed a Barnard Red supplemented diet.

(iv) An interesting observation was made in chapter 4 about the biological activity of quercetin. Quercetin is a known mutagenic polyphenol in TA98 (especially in the presence of S9), a finding confirmed by us. However, we have subsequently shown that it can act as an antimutagen in the presence of the mutagen 2-aminofluorene (requiring S9 activation) in TA98. Thus although a compound may be an antimutagen, it does not necessarily follow that it is not mutagenic.

(v) In chapter 5 it was concluded that the polyphenols act as desmutagens because they were able to reduce the effective concentration of the mutagen. This was shown by fluorometric techniques and more directly and convincingly by measuring the amount of radioactive mutagen (^{3}H-daunomycin) actually taken up by *E. coli* and bound to its DNA in the absence and presence of polyphenols. The effectiveness of the polyphenols in reducing the DNA-daunomycin binding was in the same order of efficiency as observed in the biological antimutagenic assays carried out in Chapter 4. During the course of these binding studies,

it was noted that uptake of labelled thymidine by the *Salmonella typhimurium* mutants was very low (2-4%) when compared to that in *Escherichia coli* (20%). The reason for this was not investigated.

FUTURE WORK

Future work would be directed at developing a reliable method of detecting mutagen-polyphenol binding as the techniques used in this study were not satisfactory due to the strong binding (which could not be quantitated accurately) of daunomycin and/or polyphenol to the apparatus used in the binding studies. The polyphenols in the three fractions would need to be deglycosylated and retested in the two mutagen detecting assays to prove beyond doubt that polyphenols in sorghum grain do not present a health hazard. Further separation of the three complex fractions used in the present work could help prove that what was observed is a general, non-specific binding of polyphenols to the mutagens.

A complete change in direction might well look at the possibility of the polyphenols acting as antioxidants and scavengers of free radicals. This ability might well explain the mutagenic / antimutagenic activity of the polyphenols noted on p. 98 because polyphenols may be able to generate free radicals as well as scavenge them.

Chapter 7. REFERENCES

7. REFERENCES

Ackers, G. K. (1973) *Studies of Protein Ligand Binding by Gel Permeation Techniques.* Methods in Enzymology, XXVII, p. 441-455.

Ames, B. N., Durston, W. E., Yamasaki, E. and Lee, F. D. (1973a) *Carcinogens are Mutagens: A Simple Test System Combining Liver Homogenates for Activation and Bacteria for Detection.* Proceedings of the National Academy of Science (USA), 70(8), p. 2281-2285.

Ames, B. N., Lee, F. D. and Durston, W. E. (1973) *An Improved Bacterial Test System for the Detection and Classification of Mutagens and Carcinogens.* Proceedings of the National Academy of Science (USA), 70, p. 782-786.

Ames, B. N. and Whitfield, H. J. Jr. (1966). *Frameshift Mutagenesis in Salmonella.* Cold Spring Harbour Symposium on Quantitative Biology, 31, p. 221-225.

Ames, B. N. and Hartman, P. E. (1974) *The Histidine Operon of Salmonella typhimurium.* In: Handbook of Genetics - Bacteria, Bacteriophages and Fungi, R. C. King (Editor), Plenum Press, New York, p. 223-235.

Ames, B. N., McCann, J. and Yamasaki, E. (1975) *Methods for Detecting Carcinogens and Mutagens with the Salmonella/Mammalian-Microsome Mutagenicity Test.* Mutation Research, 31, p. 347-364.

Ames, B. N. (1971) *The Detection of Chemical Mutagens with Enteric Bacteria.* In: Chemical Mutagens: Principles and Methods for their Detection, A. Hollaender (Editor), Plenum Press, New York, Volume 1, p. 267-282.

Ames, B. and Hooper, K. (1978) *Does Carcinogenic Potency Correlate with Mutagenic Potency in the Ames Assay.* Nature, 274, p. 19-22.

Arimoto, S. Y., Ohara, T., Namba, T., Hayatsu, N. and Hayatsu, H. (1980) *Inhibition of the Mutagenicity of Amino Acid Pyrolysis Products by Hemin and other Biological Pyrrole Pigments.* Biochemical and Biophysical Research Communications 92, p. 662-669.

Ausubel, F. M. (1988) Current Protocols in Molecular Biology, John Wiley and Sons, New York.

Baars, A. J., Zijlstra, J. A., Vogel, E. and Breimer, D. D. (1977) *The Occurrence of Cytochrome P450 and Aryl Hydrocarbon Hydroxylase Activity in Drosophila melanogaster microsomes, and the Importance of the Metabolizing Capacity for the Screening of Carcinogenic and Mutagenic Properties of Foreign Compounds.* Mutation Research, 44, p. 257-268.

Bakker, E. P., Rottenberg, H. and Caplan, S. R. (1976) *An Estimation of the Light-Induced Electrochemical Potential Difference of Protons Across the Membrane of Halobacterium Halobium*. Biochemica et Biophysica Acta, 440, p. 573-586.

Barnes, W., Tuley, E. and Eisenstadt, E. (1982) *Base-sequence Analysis of His$^+$ Revertants of the hisG46 Missense Mutation in Salmonella typhimurium*. Environmental Mutagenesis, 4, p. 297-302.

Berger, H., Haas, F. L., Wyss, O. and Stone, W. S. (1953) *Effect of Sodium Azide on Radiation Damage and Photoreactivation*. Journal of Bacteriology, 65, p. 538-543.

Bergmann, W. R. (1986) *Fluorescence of Proanthocyanidin Polymers*. PhD thesis, Agricultural and Mechanical College, The Louisiana State University, Louisiana, USA., p. 1-294.

Bernstein, L., Kaldor, J., McCann, J. and Pike, M.C. (1982) *An Empirical Approach to the Statistical Analysis of Mutagenesis Data from the Salmonella Test*. Mutation Research, 97, p. 267-281.

Bichara, M. and Fuchs, P. P. (1985) *DNA Binding and Mutation Spectra of the Carcinogen N-2-Aminofluorene in Escherichia coli: A Correlation between the Conformation of the Premutagenic Lesion and the Mutation Specificity*. Journal of Molecular Biology 183, p. 341-351.

Billen, D. and A. C. Olson (1978) *The Use of Tween-80-Permeabilized Mammalian Cells in Studies of Nucleic Acid Metabolism.* Methods in Cell Biology, XX, p. 314-340.

Blake, A. and Peacocke, A. R. (1968) *The Interaction of Aminoacridines with Nucleic Acids.* Biopolymers 6, p. 1225-1253.

Bobst, A. M., Langemeier, P. W., Warwick-Koochaki, D. E., Bobst, E. V. and Ireland, J. C. (1982) *Nucleic Acid Binding Affinity of Bacteriophage T4 Gene 32 Protein in the Cooperative Binding Mode.* Journal of Biological Chemistry, 257, p. 6184-6193

Bohinski, R.C. (1987) *Protein Binding and the Cooperative Effect.* In: Modern Concepts in Biochemistry, Woodstock Publishers, New York, pp. 153-157, 451-452 & 601.

Bösterling, B., Trudell, J. R., Trevor, A. J. and Bendix, M. (1982) *Lipid-Protein Interactions as Determinants of Activation or Inhibition by Cytochrome b_5 of Cytochrome P450 Mediated Oxidations.* Journal of Biological Chemistry, 257(8), p. 4375-4380.

Bösterling, B. and Trudell, J. R. (1982) *Association of Cytochrome b_5 and Cytochrome P450 Reductase with Cytochrome P450 in the Membrane of Reconstituted Vesicles.* Journal of Biological Chemistry, 257(9), p. 4783-4787.

Bradshaw, E. and Harington, J. S. (1986) *The Problem of Oesophageal Cancer Relative to Other Cancers in Southern Africa, 1949–1979.* South African Journal of Science, 82, p. 57–58.

Brown, J. P. and Dietrich, P. S. (1979) *Mutagenicity of Plant Flavonols in the Salmonella/Mammalian Microsome Test: Activation of Flavonol Glycosides by Mixed Glycosidases from Rat Caecal Bacteria and Other Sources.* Mutation Research 66, p. 223–240.

Brown, J. P. (1980) *A Review of the Genetic Effects of Naturally Occurring Flavonoids, Anthraquinones and Related Compounds.* Mutation Research 75, p. 243–277.

Buening, M. K., Chang, R. L., Huang, M., Fortner, J. G., Wood, A. W. and Conney, A. H. (1981) *Activation and Inhibition of Benzo(a)pyrene and Aflatoxin B_1 Metabolism in Human Liver Microsomes by Naturally Occurring Flavonoids.* Cancer Research, 41, p. 67–72.

Bujalowski, W. and Lohman, T. M. (1987) *A General Method of Analysis of Ligand-Macromolecule Equilibria Using a Spectroscopic Signal from the Ligand to Monitor Binding. Application to Escherichia coli Single Strand Binding Protein-Nucleic Acid Interactions.* Biochemistry 26, p. 3099–3106.

Butler, L. G. (1982) *Relative Degree of Polymerisation of Sorghum Tannin During Seed Development.* Journal of Agricultural and Food Chemistry, 30, p. 1090-1097.

Calendi, E., DiMarco, A., Reggiani, M., Scarpinato, B. and Valentini, L. (1965) *On Physio-Chemical Interactions between Daunomycin and Nucleic Acids.* Biochemica et Biophysica Acta, 103, p. 25-49.

Casa-Finet, J. R., Khamis, M. I., Maki, A. H., Ruvolo, P. P. and Chase, J. W. (1987) *Optically Detected Magnetic Resonance of Tryptophan Residues in Escherichia coli SSB Gene Product and E. coli Plasmid-Encoded Single-Stranded DNA Binding-Proteins and their Complexes with Poly(deoxythymidilic)Acid.* Journal of Biological Chemistry, 262, p. 8574-8583.

Chaires, J. B., Dattagupta, N. and Crothers, D. M. (1982) *Studies on Interaction of Anthracycline Antibiotics and Deoxyribonucleic Acid: Equilibrium Binding Studies on Interaction of Daunomycin with Deoxyribonucleic Acid.* Biochemistry, 21, p. 3933-3940.

Chaires, J. B., Fox, K. R. Herrera, J. E., Britt, M. and Waring, M. J. (1987) *Site and Sequence Specificity of the Daunomycin-DNA Interaction.* Biochemistry, 26, p. 8227-8236

Chiang, J. K. L. (1981) *Interaction of Purified Microsomal Cytochrome P450 with Cytochrome b_5.* Archives of Biochemistry and Biophysics, 211, p. 662-673.

Chu, K. C., Patel, K. M., Lin, A. H., Tarone, R. E., Linhart, M. S. and Dunkel, V.C. (1981) *Evaluating Statistical Analysis and Reproducibility of Microbial Mutagenicity Assays.* Mutation Research, 85, p. 119-132.

Clarke, C. H. and Shankel, D. M. (1975) *Antimutagenesis in Microbial Systems.* Bacteriological Review, 39, p. 33-53.

Colowick, S. P. and Womack, F. C. (1969) *Binding of Diffusible Molecules by Macromolecules: Rapid Measurement by Rate of Dialysis.* Journal of Biological Chemistry, 244(3), p. 774-777.

Crespi, M., Muñoz, N., Grassi, A., Aramesh, B., Amiri, G. and Mojtabai, A., Casale, A. (1979) *Oesophageal Lesion in Northern Iran: A Premalignant Condition ?* The Lancet, p. 217-220.

Crick, F. H. C., Barnett, L., Brenner, S. and Watts-Tobin, R. J. (1961) *General Nature of the Genetic Code for Proteins.* Nature, 192, p. 1227-1232.

Czochanska, Z., Foo., L. Y., Newman, R. H. and Porter, L. J. (1980) *Polymeric Proanthocyanidins. Stereochemistry, Structural Units and Molecular Weight.* Journal of the Chemical Society (Perkin I), p. 2278-2286.

Dahlquist F. W. (1978) *The Meaning of Scatchard and Hill Plots.* Methods in Enzymology, XLVIII, p. 270-299.

Dandliker, W. B., Dandliker, J., Levison, S. A., Kelly, R. J., Hicks, A. N. and White, J. U. (1978) *Fluorescence Methods for Measuring Reaction Equilibria and Kinetics.* Methods in Enzymology, XLVII . 380-396.

Darnell, J. (1986) *Macromolecules in Prokaryotic and Eukaryotic Cells.* In: Molecular Cell Biology, J. Darnell, H. Lodish and D. Baltimore (Editors), Scientific American Books, USA, p. 136-137.

De Baun, J. R., Miller E. C. and Miller, J. A. (1970) *N-Hydroxy-2-acetylaminofluorene Sulfotransferase: Its Probable Role in Carcinogenesis and in Protein-(methion-S-yl) Binding in Rat Liver.* Cancer Research, 30, p. 577-595.

Dean, B. J., Huston, D. H., Wright, A. S. and Stevenson, D. E. (1978) *The Protective Action of Glutathione Against Bacterial Mutagenicity of Cis-1,3-Dichloropropene.* The Pharmacologist, 20(3), Abstract No. 50, p. 154.

DeHaseth, P. L., Gross, C. A., Burgess, R. R. and Record, M. T. (Jr). (1977) *Measurement of Binding Constants for Protein-DNA Interactions by DNA-Cellulose Chromatography.* Biochemistry, 16, p. 4777-4779.

Delius, H., Mantell, N. J. and Alberts, B. (1972) *Characterization by Electron Microscopy of the Complex Formed between T4 Bacteriophage Gene 32 Protein and DNA.* Journal of Molecular Biology, 67, p. 341-350.

Eisen, H. J. (1986) *Induction of Hepatic P450 Isozymes.* In: Cytochrome P450 - Structure, Mechanism and Biochemistry, P. P. Oritz de Montellano (Editor), Plenum Press, New York, p. 315-344.

Elliger, C. A., Henika, P. R. and MacGregor, J.T. (1984) *Mutagenicity of Flavones, Chromones and Acetophenones in Salmonella typhimurium.* Mutation Research, 135, p. 77-86.

Fink, G. R., Kloptowski, T. and Ames, B. N. (1967) *Histidine Regulatory Mutants in Salmonella typhimurium. A Positive Selection for Polar Histidine-Requiring Mutants for Histidine Operator Constitutive Mutants.* Journal of Molecular Biology, 30, p. 81-95.

Frankel, A. D., Ackers, G. K. and Smith, H. O. (1985) *Measurement of DNA-Protein Equilibrium using Gel Chromatography: Application to the HinfI Restriction Endonuclease.* Biochemistry, 24, p. 3049-3052.

Freudenberg, K. and Weinges, K. (1960) *Systematik und Nomenklatur der Flavonoide.* Tetrahedron, 8, p. 336-345.

Friedman, R. A. G. and Manning, G. S. (1984) *Polyelectrolyte Effects in Site-Binding Equilibria with Application to the Intercalation of Drugs into DNA.* Biopolymers 23, p. 2671-2714.

Fukuhara, Y., Yoshida, D. and Gotō, F. (1981) *Reduction of Mutagenic Products in the Presence of Polyphenols during Pyrolysis of Protein.* Agricultural and Biological Chemistry, 45(5), p. 1061-1066.

Gilbert, G. A. and Jenkins, R. C. L. (1959) *Sedimentation and Electrophoresis of Interacting Substances. II. Asymptotic Boundary Shape for Two Substances Interacting Reversibly.* Proceedings of the Royal Society of London, Ser. A., 253, p. 420-425.

Glick, Z. and Joslyn, M. A. (1969) *Effect of Tannic Acid and Related Compounds on the Absorption and Utilization of Proteins in the Rat.* Journal of Nutrition, 100, p. 516-520.

Goze, A. and Devoret, R. (1979) *Repair Promoted by Plasmid pKM101 is Different from SOS Repair.* Mutation Research, 61, p. 163-179.

Graf, U., Juon, H., Katz, A. J., Frei, H. J. and Würgler, F. E. (1983) *A Pilot Study on a New Drosophila Spot Test.* Mutation Research, 120, p. 233-239.

Graf, U., Würgler, F. E., Katz, A. J., Frei, H., Juon, H., Hall, C. B. and Kale, P. G. (1984) *Somatic Mutation and Recombination Test in Drosophila melanogaster*. Environmental Mutagenesis, 6, p. 153-188.

Graf, J. C. (1978) *Adsorption of Polyphenols by Polyvinlypyrrolidone and Polystyrene Resins*. Phytochemistry, 17, 495-497.

Gray, J. I. and Dugan, L. R. (1975) *Inhibition of N-Nitrosamine Formation in Model Food Systems*. Journal of Food Science, 40, p. 981-990.

Green, M. H. L. and Muriel, W. J. (1976) *Mutagen Testing using Trp$^+$ Reversion in Escherichia coli*. Mutation Research, 38, p. 3-32.

Grimmer, H. R. and McGrath, R. M. (1991) *Antimutagenicity and Cytochrome-P450 Inducing Effect of Polyphenols from Sorghum Grain*. Presented at the 10th South African Biochemical Congress, University of Natal, Pietermaritzburg, South Africa.

Grimmer, H. R., Parbhoo, V. and McGrath, R. M. (1992) *Antimutagenicity of Polyphenol-Rich Fractions from Sorghum bicolor Grain*. Journal of Science, Food and Agriculture, 59, p. 251-256.

Groenen, P. J. (1977) *A New Type of N-Nitrosation Inhibitor.* In: Proceedings of the 2nd International Symposium on Nitrite in Meat Products, B. J. Tinbergen and B. Krol (Editors), Pudoc, Wageningen, Netherlands, p. 171-179.

Guengerich, F. P. (1977) *Separation and Purification of Multiple Forms of Microsomal Cytochrome P450: Activities of Different Forms of Cytochrome P450 Towards Several Compounds of Environmental Interest.* Journal of Biological Chemistry, 252, p. 3970-3979.

Guengerich, F. P. (1991) *Reactions and Significance of Cytochrome P450 Enzymes.* Journal of Biological Chemistry, 266(16), p. 10019-10022.

Guengerich, F. P., Dannan, G. A., Wright, S.T., Martin, M. V. and Kaminsky, L. S. (1982) *Purification and Characterization of Liver Microsomal Cytochromes P450: Electrophoretic, Spectral, Catalytic and Immunochemical Properties and Inducibility of Eight Isozymes Isolated from Rats Treated with Phenobarbital and β-Napthoflavone.* Biochemistry, 21, p. 6019-6030.

Guengerich, F. P. (1979) *Isolation and Purification of Cytochrome P450 and the Existence of Multiple Forms.* Pharmacology and Therapeutics, 6, p. 99-121.

Gupta, R. K. and Haslam, E. (1978) *Plant Proanthocyanidins. Part 5. Sorghum Polyphenols.* Journal of the Chemical Society (Perkin I), 8, p. 892-895.

Hahn, D. H., Faubion, J. M., and Rooney, L. W. (1983) *Sorghum Phenolic Acids, their High Performance Liquid Chromatography Separation and their Relation to Fungal Resistance.* Cereal Chemistry, 60(4), p. 255-259.

Hahn, D. H., Rooney, L. W. and Earp, C. F. (1984) *Tannins and Phenols of Sorghum.* Cereal Foods World, 29(12), p. 776-779.

Halfman, C. J. and Nishida, T. (1972) *Method for Measuring the Binding of Small Molecules to Proteins from Binding-Induced Alterations of the Physical-Chemical Properties.* Biochemistry, 11, p. 3493-3498.

Hardigree, A. A. and Epler, J. L. (1978) *Comparative Mutagenesis of Plant Flavonoids in Microbial Systems.* Mutation Research, 58, p. 231-239.

Haslam, E., Lilley, T. H., Cai, Y., Martin, R. and Magnolato, D. (1988) *Traditional Herbal Medicines - The Role of Polyphenols.* Planta Medica, 55, p. 1-8.

Haslam, E. and Lilley, T. H. (1988) *Natural Astringency in Foodstuffs - A Molecular Interpretation.* In: CRC Critical Reviews in Food, Science and Nutrition, CRC Press, Inc. Boca Raton, Florida, 27(1), p. 1-40.

Haslam, E. (1989) Plant Polyphenols - Vegetable Tannins Revisited, Cambridge University Press, Cambridge, England, p. 1-81 and p. 155-166.

Haslam, E. (1974) *Polyphenol-Protein Interactions.* Biochemical Journal, 139, p. 285-288.

Haslam, E. (1979a) *Vegetable Tannins.* Recent Advances in Phytochemistry, 12, p. 475-523.

Haslam, E. (1977) *Symmetry and Promiscuity in Procyanidin Biochemistry.* Phytochemistry, 16, p. 1625-1640.

Haslam, E. (1977) *Structure, Conformation and Biosynthesis of Natural Procyanidins.* Proceedings of the 5th Hungarian Bioflavonoid Symposium, Mátrafüred, Hungary, p. 97-110.

Haslam, E. (1982) *The Metabolism of Gallic Acid and Hexahydroxydiphenic Acid in Higher Plants.* Progress in the Chemistry of Organic Natural Products, 41, p. 1-46.

Haugen, D. A. and Coon, M. J. (1976) *Properties of Electrophoretically Homogenous Phenobarbital-Inducible and β-Naphthoflavone-Inducible Forms of Liver Microsomal Cytochrome P450.* Journal of Biological Chemistry, 215, p. 7929-7939.

Hemingway, R. W. (1989) *Reactions at the Interflavanoid Bond of Proanthocyanidins.* In: Chemistry and Significance of Condensed Tannins, R. W. Hemingway, J. J. Karchesy and S. J. Branham (Editors), Plenum Press, New York, p. 265-284.

Huang, M., Chang, R. L., Fortner, J. G., Conney, A. H. (1981) *Studies on the Mechanism of Activation of Microsomal Benzo(a)pyrene Hydroxylation by Flavonoids.* Proceedings of the National Academy of Science (USA), 78, p. 6829-6836.

Huang, M., Johnson, E. F., Müller-Eberhard, U., Koop, D. R., Coon, M. J. and Cooney, A. H. (1981) *Specificity in the Activation and Inhibition of Flavonoids of Benzo(a)pyrene Hydroxylation by Cytochrome P450 Isozymes from Rabbit Liver Microsomes.* Journal of Biological Chemistry, 256(21), p. 10897-10901.

Hummel, J. P. and Dreyer, W. J. (1962) *Measurement of Protein Binding Phenomenon by Gel Filtration.* Biochimica et Biophysica Acta, 63, p. 530-536.

Ingelman-Sundberg, M. (1986) *Cytochrome P450 Organization and Membrane Interactions.* In: Cytochrome P450 - Structure, Mechanism and Biochemistry, P. R. Oritz de Montellano (Editor), Plenum Press, New York, p. 119-151.

Inoue, T., Ohta, Y., Sadaie, Y., and Kada, T. (1981) *Effect of Cobaltous Chloride in Spontaneous Mutation Induction in a Bacillus substilus mutator strain.* Mutation Research, 91, p. 41-45.

Johnson, E.F., Levitt, D. S., Müller-Eberhard, V. and Thorgeirsson, S. S. (1980) *Catalysis of Divergent Pathways of 2 Acetylamino-fluorene Metabolism by Multiple Forms of Cytochrome P450.* Cancer Research, 40, p. 4456-4459.

Jones, O. W. and Berg, P. (1966) *Studies on the Binding of RNA Polymerase to Polynucleotides.* Journal of Molecular Biology, 22, p. 199-209.

Kada, T., Inoue, T., Ohta, T. and Shirashu, Y. (1981) *Environmental Desmutagens and Antimutagens.* In: Environmental Mutagenesis and Plant Biology, E J. Klekowski Jr. (Editor), Prager, New York, p. 134-151.

Kada, T. and Shimoi, K. (1987) *Desmutagens and Bioantimutagens - Their Modes of Action.* BioEssays, 7(3), p. 113-115.

Kada, T., Morita, K. and Inoue, T. (1978) *Antimutagenic Action of Vegetable Factor(s) on the Mutagenic Principle of Tryptophan Pyrolysate.* Mutation Research, 53, p. 351-353.

Kada, T., Kato, M., Aikawa, K. and Kiriyama, S. (1984) *Adsorption of Pyrolysate Mutagens by Vegetable Fibers.* Mutation Research, 141, p. 149-152.

Kada, T., Kaneko, K., Matsuzaki, S., Matsuzaki, T. and Hara, Y. (1985) *Detection and Chemical Identification of Natural Bio-antimutagens.* Mutation Research, 150, p. 127-132.

Kaluza, W. Z., McGrath, R. M., Roberts, T. C. and Schröder, H. H. (1980) *Separation of Phenolics of Sorghum bicolor (L) Moench Grain.* Journal of Agricultural and Food Chemistry, 28, p. 1191-1196.

Kapitulnik, J., Wislocki, P. G., Levin, W., Yagi, H., Jerina, D. M. and Conney, A. H. (1978) *Tumorigenicity Studies with Diol-Epoxides of Benzo(a)pyrene which Indicate that (±) trans-7α, 8β-dihydroxy-9α, 10-α-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene is an Ultimate Carcinogen in Newborn Mice.* Cancer Research, 38, p. 354-357.

Karchesy, J. J., Bae, Y., Chalker-Scott, L., Helm, R. F. and Foo, L. Y. (1989) *Chromatography of Proanthocyanidins.* In: Chemistry and Significance of Condensed Tannins, R. W. Hemingway, J. J. Karchesy and S. J. Branham (Editors), Plenum Press, New York, p. 139-154.

Kawabata, T., Oshima, H., Vibu, J., Nakamura, M., Matsui, M., Hamano, M. (1979) *Occurrence, Formation and Precursors of N-nitroso Compounds in Japanese Diet.* In: Naturally Occurring Carcinogens, Mutagens and Modulators of Carcinogenesis, Miller, E. C., Miller, J. A., Sugimura, T., Takayama, S., and Hirono, I. (Editors), University Park Press, Baltimore, p. 195.

King, D. J. and Wiseman, A. (1987) *Yeast Cytochrome P448 Enzymes and the Activation of Mutagens, I. luding Carcinogens.* In: Enzyme Induction, Mutagen Activation and Carcinogen Testing in Yeast, Wiseman, A. (Editor), Ellis Horwood Limited, West Sussex, England, p. 115-157.

Kinsella, J. E., Frankel, E., German, B. and Kanner, J. (1993) *Possible Mechanisms for the Protective Role of Antioxidants in Wine and Plant Foods.* Food Technology, 47(7), p. 85-89.

Kleinhofs, A., Owais, W. M. and Nilan, R. A. (1978) *Azide.* Mutation Research, 55, p. 165-195.

Kowalczykowski, S. C., Paul, L. S., Lonberg, N., Newport, J. W., McSwiggen, J. A. and von Hippel, P. H. (1986) *Cooperative and Noncooperative Binding of Protein Ligands to Nucleic Acid Lattices: Experimental Approaches to the Determination of Thermodynamic Parameters.* Biochemistry, 25, p. 1226-1240.

Kronman, M. J., Sinha, S. K. and Brew, K. (1981) *Characteristics of the Binding of Calcium and Other Divalent Metal Ions to Bovine α-Lactalbumin.* Journal of Biological Chemistry, 256(16), p. 8582-8587.

Kurzer, M. S. (1993) *Planning and Interpreting "Designer Food" Feeding Studies.* Food Technology, 47(7), p. 80-84.

Lackey, D., Walker, G. C., Keng, T. and Linn, S. (1977) *Characterisation of an Endonuclease Associated with the Drug Resistance Plasmid pKM101.* Journal of Bacteriology, 131(2), p. 583-588.

Laks, P. E. (1989) *Chemistry of the Condensed Tannin B-Ring.* In: Chemistry and Significance of Condensed Tannins, R. W. Hemingway, J. J. Karchesy and S. J. Branham (Editors), Plenum Press, New York, p. 249-264.

Langer, P. J., Shanabruch, W. G. and Walker, G. C. (1981) *Functional Organization of Plasmid pKM101.* Journal of Bacteriology, 145(3), p. 1310-1316.

Lehninger, A. L. (1975) Biochemistry, Worth Publishers, Inc., New York, p. 502-503.

Levin, D. E., Yamasaki, E. and Ames, B. N. (1982) *A New Salmonella Tester Strain, TA97, for the Detection of Frameshift Mutagens - A Run of Cytosines as a Mutational Hot-Spot.* Mutation Research, 94, p. 315-330.

Levin, D. E., Hollstein, M. C., Christman, M. F., Schwiers, E. A. and Ames, B. N. (1982b) *A New Salmonella Tester Strain (TA102) with A:T Base Pairs at the Site of Mutation Detects Oxidative Mutagens.* Proceedings of the National Academy of Science (USA), 79, p. 7445-7449.

Lin, S. and Riggs, A. D. (1972) *lac Repressor binding to Non-operator DNA: Detailed Studies and a Comparison of Equilibrium and Rate Competition Methods.* Journal of Molecular Biology, 72, p. 671-691.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1952) *Protein Measurement with the Folin Phenol Reagent.* Journal of Biological Chemistry, 13, p. 275-283.

Lu, A. Y. H. and West, S. B. (1980) *Multiplicity of Mammalian Microsomal Cytochromes P450.* Pharmacological Reviews, 31(4), p. 277-295.

MacPhee, D. G. (1972) *Effect of an R-Factor on Resistance of Salmonella typhimurium to Radiation and Chemical Treatment*. Mutation Research, 14, p. 450-453.

Marmur, J. (1961) *A Procedure for the Isolation of Deoxyribonucleic Acid from Microorganisms*. Journal of Molecular Biology, 21, p. 208-218.

Maron, D. M. and Ames, B. N. (1983) *Revised Methods for the Salmonella Mutagenicity Test*. Mutation Research, 113, p. 173-215.

Martin, R. G., Silbert, D. F., Smith, D. W. E. and Whitfield Jr., H. J. (1966) *Polarity of the Histidine Operon*. Journal of Molecular Biology, 21, p. 357-369.

Matsushima, T., Matsumoto, H., Shirai, A., Swamura, M. and Sugimura, T. (1979) *A Safe Substitute for Polychlorinated Biphenyls as an Inducer of the Metabolic Activation System*. In: In Vitro Metabolic Activation in Mutagenesis Testing, F. J. de Serres, J. R. Fouts, J. R. Bend and R. M. Philpot (Editors). Elsevier/North Holland, Amsterdam, p. 85-88.

Mattice, W. (1989) *Conformational Analysis of Oligomeric Proanthocyanidins*. In: Chemistry and Significance of Condensed Tannins, R. W. Hemingway, J. J. Karchesy and S. J. Branham (Editors), Plenum Press, New York, p. 119-130.

McCann, J., Spingarn, N. E., Kobori, J. and Ames, B. N. (1975) *Detection of Carcinogens as Mutagens: Bacterial Tester Strains with R-Factor Plasmids.* Proceedings of the National Academy of Science (USA), 72(3), p. 979-983.

McCann, J. and Ames, B. (1976) *Detection of Carcinogens as Mutagens in the Salmonella/microsome . Test: Assay of 300 Chemicals: Discussion.* Proceedings of the National Academy of Science (USA), 73(3), p. 950-954.

McGhee, J. D. and von Hippel, P. H. (1974) *Theoretical Aspects of DNA-Protein Interactions: Cooperative and Noncooperative Binding of Large Ligands to a One-Dimensional Homogeneous Lattice.* Journal of Molecular Biology, 86, p. 469-489.

McGrath, R. M., Kaluza, W. Z., Daiber, K. H., van der Riet, W. B. and Glennie, C. W. (1982) *Polyphenols of Sorghum Grain, their Changes during Malting and their Inhibitory Nature.* Journal of Agricultural and Food Chemistry, 30, p. 450-456.

McGrath, R. M., Smith, A., Banister, S., Monoyioudis, J. and Grimmer, H. R. (1993) *The Binding of Large Molecular Mass Procyanidins from Bird Resistant Sorghum Grain to Soluble Proteins.* International Journal of Biochemistry, 25(3), p. 397-402.

McGrath, R. M. and Smith, A. (1990) *The Isolation of Large Molecular Mass Procyanidins from Sorghum Grain and their Interaction with [^{14}C] Methyl Bovine Serum Albumins.* International Journal of Biochemistry, 22(12), p. 1491-1496.

McGraw, G. W. (1989) *Reactions at the A-Ring of Proanthocyanidins.* In: Chemistry and Significance of Condensed Tannins, R. W. Hemmingway, J. J. Karchesy and S. J. Branham (Editors), Plenum Press, New York, p. 227-248.

Mechansho, H., Butler, L. G. and Carlson, D. M. (1987) *Dietary Tannins and Salivary Proline-Rich Proteins: Interactions, Induction and Defense Mechanisms.* Annual Review in Nutrition, 7, p.423-440.

Mitchell, P. (1968). In: Chemiosmotic Coupling and Energy Transduction, Glynn Research Ltd, Bodmin, Cornwall, UK.

Miyamoto, K., Nobuharu, K., Ryozo, K., Yoshida, T., Hatano, T. and Okuda, T. (1987) *Relationship between the Structures and the Antitumour Activities of Tannins.* Chemical and Pharmaceutical Bulletin, 35(2), p. 814-822.

Mollet, P. and Würgler, F. E. (1974) *Detection of Somatic Recombination and Mutation in Drosophila: An Method for Testing Genetic Activity of Chemical Compounds.* Mutation Research, 25, p. 421-424.

Monteith, D.K. (1990) *Catechin Inhibition of Mutagenesis and Alteration of DNA Binding of 2-Acetylaminofluorene in Rat Hepatocytes.* Mutation Research, 240, p. 151-158.

Mortelmans, K. E. and Stocker, B. A. D. (1976) *Ultraviolet Light Protection, Enhancement of Ultraviolet Light Mutagenesis, and Mutator Effect of Plasmid R46 in Salmonella typhimurium.* Journal of Bacteriology, 128(1), p. 271-282.

Morton, J. F. (1989) *Tannin as a Carcinogen in Bush-Tea: Tea, Maté and Khat.* In: Chemistry and Significance of Condensed Tannins, R. W. Hemingway, J. J. Karchesy and S.J. Branham (Editors), Plenum Press, New York, p. 403-416.

Morton, J. F. (1970) *Tentative Correlations of Plant Usage and Oesophageal Cancer Zones.* Economic Botany, 24, p. 217-222.

Mosel, H. D. and Herrmann, K. (1973) *Chromatographisches Verhalten von Pflanzenphenolen an Polyamid.* Journal of Chromatography, 87, p. 280-282.

Miller-Harvey, I., Reed, J. D. and Hartley, R. D. (1987) *Characterisation of Phenolic Compounds, including Flavonoids and Tannins, of Ten Ethiopian Browse Species by High Performance Liquid Chromatography.* Journal of Science, Food and Agriculture, 39, p. 1-14.

Muñoz, N., Wahrendorf, J., Bang, L. J., Crespi, M., Thurnham, D. I., Day, N. E., Ji, Z. H., Grassi, A., Yan, L. W., Lin, G. L., Quan, L. Y., Yun, Z. C., Fang, Z. S., Yao, L. J., Correa, P., O'Conor, G. T. and Bosch, X. (1985) *No Effect of Riboflavine, Retinol and Zinc on Prevalence of Precancerous Lesions of Oesophagus.* The Lancet, p. 111-114.

Muñoz, N., Crepsi, M., Grassi, A., Quin, W. G., Qiong, S. and Cai, L. Z. (1982) *Precursor Lesions of Oesophageal Cancer in High-Risk Populations in Iran and China,* The Lancet, p. 876.

Ogawa, S., Hirayama, T., Nohara, M., Tokuda, M., Hirai, K. and Fukui, S. (1985) *The Effect of Quercetin on the Mutagenicity of 2-Acetylaminofluorene and Benzo(a)pyrene in Salmonella typhimurium Strains.* Mutation Research, 142, p. 103-107.

Ohta, Y., Watanabe, K., Moriya, M., Shirasu, Y. and Kada, T. (1983) *Analysis of the Antimutagenic Effect of Cinnamaldehyde on Chemically Induced Mutagenesis in Escherichia coli.* Molecular and General Genetics, 192, p. 309-315.

Okuda, T., Kazuko, M. and Hayatsu, H. (1984) *Inhibitory effect of Tannins on Direct-Acting Mutagens.* Chemical and Pharmacological Bulletin, 32(9), p. 3755-3758.

Omura, T. and Sato, R. (1964) *The Carbon Monoxide-Binding Pigment of Liver Microsomes.* Journal of Biological Chemistry, 239(7), p. 2370-2376.

Oterdoom, H. J. (1985) *Tannin, Sorghum and Oesophageal Cancer.* The Lancet, p. 330.

Owais, W. M., Kleinhofs, A. and Nilan, R. A. (1979) *In Vitro Conversion of Sodium Azide to a Stable Mutagenic Metabolite in Salmonella typhimurium.* Mutation Research, 68, p. 15-22.

Owais, W. M., Kleinhofs, A., Ronald, R. C. and Nilan, R. A. (1981) *Isolation of an Azide Metabolite in Salmonella typhimurium.* Mutation Research, 91, p. 155-161.

Owais, W. M. and Kleinhofs, A. (1988) *Metabolic Activation of the Mutagen Azide in Biological Systems.* Mutation Research, 197, p. 313-323.

Padan, E., Zilberstein, D. and Rottenberg, H. (1976) *The Proton Electrochemical Gradient in Escherichia coli Cells.* European Journal of Biochemistry, 63, p. 533-541.

Parbhoo, V. (1993) *The Induction of Microsomal S9 Fraction in Rat Liver by Polyphenol-Rich Fractions of Sorghum Grain.* Master of Science Dissertation, University of the Witwatersrand, Johannesburg, South Africa.

Perry, K. L. and Walker, G. C. (1982) *Identification of Plasmid (pKM101)-coded Proteins Involved in Mutagenesis and UV Resistance.* Nature, 300, p. 278–281.

Plumbridge, T. W. and Brown, J. B. (1977) *Spectrophotometric and Fluorescence Polarization Studies of the Binding of Ethidium, Daunomycin and Mepacrine to DNA and to Poly(I·C).* Biochimica et Biophysica Acta, 479, p. 441–449.

Porter, L. J., Newman, R. H., Foo, L. Y., Wong, H. and Hemingway, R. W. (1982) *Polymeric Proanthocyanidins. ^{13}C-NMR Studies of Procyanidins.* Journal of the Chemical Society (Perkin I), p. 1217–1222.

Price, M. L., Hagerman, A. E. and Butler, L. G. (1980) *Tannin in Sorghum Grain. Effect of Cooking on Chemical Assays and on Antinutritional Properties in Rats.* Nutritional Reports International, 21(5), p. 761–767.

Purchase, I. F. H., Longstaff, E., Ashby, J. Styles, J. A., Anderson, D., Lefevre, P. A. and Westwood, F. R. (1976) *Evaluation of Six Short Term Tests for Detecting Organic Chemical Carcinogens and Recommendations for their Use.* Nature, 264, p. 624-627.

Ramos, S., Schuldiner, S. and Kaback, H. R. (1979) *The Use of Flow Dialysis for Determinations of ΔpH and Active Transport.* Methods in Enzymology, Biomembranes. Part F. Bioenergetics: Oxidative Phosphorylation, S. Fleischer and L. Packer (Editors), LV, p. 680-688.

Revzin, A. (1990) *Techniques for Characterising Nonspecific DNA-Protein Interactions.* In: The Biology of Nonspecific DNA-Protein Interactions, CRC Press Inc., Florida, p. 5-21.

Ridlington, J. and Butler, L. G. (1969) *Measurement of Binding of Phosphate Ligands to Ribonuclease A by Direct and Competitive Dialysis Rate Techniques.* Journal of Biological Chemistry, 244(3), p. 777-779.

Riggs, A. D., Bourgeois, S., Newby, R. F. and Cohen, M. (1968) *DNA Binding of the lac Repressor.* Journal of Molecular Biology, 34, p. 365-368.

Roberts, D. B. (1986) *Basic Drosophila Care Techniques.* In: Drosophila - A Practical Approach, IRL Press, Oxford, England, p. 4-6.

Robertson, I. G., Zieger, E. and Goldstein, J. A. (1983) *Specificity of Rat Liver Cytochrome P450 Isozymes in the Mutagenic Activation of Benzo(a)pyrene, Aromatic Amines and Aflatoxin B_1.* Carcinogenesis, 4, p. 93-98.

Rottenberg, H (1979) *The Measurement of Membrane Potential and ΔpH in Cells, Organelles and Vesicles.* Methods in Enzymology, Biomembranes. Part F. Bioenergetics: Oxidative Phosphorylation, S. Fleischer and L Packer (Editors), LV, p. 547-563.

Roux, D. G. and Evelyn, S. R. (1958) *The Correlation Between Structure and Paper Chromatographic Behaviour of Some Flavanoid Compounds and Tannins.* Journal of Chromatography, 1, p. 537-543.

Sakai, S., Reinhold, C. E., Wirth, P. J. and Thorgeirsson, S. S. (1978) *Mechanism of in vitro Mutagenic Activation and Covalent Binding of N-Hydroxy-2-Acetylaminofluorene in Isolated Liver Cell Nuclei from Rat and Mouse.* Cancer Research, 38, p. 2058-2067.

San, R. H. C. and Chan, R. I. M. (1987) *Inhibitory Effect of Phenolic Compounds on Aflatoxin B_1 Metabolism and Induced Mutagenesis.* Mutation Research, 177, p. 229-239.

Scatchard, G. (1949) *The Attractions of Proteins for Small Molecules and Ions.* Annals of the New York Academy of Science, 51, p. 660-672.

Self, R., Eagles, J., Galletti, G. C., Müller-Harvey, I., Hartley, R. D., Lea, A. G. H., Magnolato, D., Richli, U., Gujer, R. and Haslam, E. (1986) *Fast Atom Bombardment Mass spectrophotometry of Polyphenols (syn. Vegetable Tannins).* Biomedical and Environmental Mass Spectrophotometry, 13, p. 449-468.

Shankel, D. M., Hartman, P. E., Kada, T. and Hollaender, A. (1987) *Synopsis of the First International Conference on Antimutagenesis and Anticarcinogenesis: Mechanisms.* Environmental Mutagenesis, 9, p. 87-103.

Shimoi, K., Nakamura, Y., Tomita, I. and Kada, T. (1985) *Bio-antimutagenic Effect of Tannic Acid on UV and Chemically Induced Mutagenesis in Escherichia coli B/r.* Mutation Research, 149, p. 17-23.

Spencer, C. M., Cai, Y., Martin, R., Gaffney, S. H., Goulding, P. N., Magnolato, D., Lilley, T. and Haslam, E. (1988) *Polyphenol Complexation - Some Thoughts and Observations.* Phytochemistry, 27(8), p. 2397-2409.

Stafford, H. A. (1990) *General Aspects of Chemistry and Metabolsim of Flavonoids.* In: Flavonoid Metabolism, CRC Publishers, Florida, p. 10.

Steele, C. M, Lalies, M. and Ioannides, C. (1985) *Inhibition of the Mutagenicity of Aromatic Amines by the Plant Flavonoid (+)-Catechin.* Cancer Research, 45, p. 3573-3577.

Stich, H. F. and Powrie, W. D. (1979) *Plant Phenolics as Genotoxic Agents and as Modulators for the Mutagenicity of Other Food Components.* In: Carcinogens and Mutagens in the Environment, H. F. Stich (Editor), Food Products, CRC Press, Boca Raton, Florida, Volume 1, p. 136-145.

Stich, H. F., Rosin, M. P. and L. Bryson (1982) *Inhibition of Mutagenicity of a Model Nitrosation Reaction by Naturally Occurring Phenolics, Coffee and Tea.* Mutation Research, 95, p. 119-128.

Strumeyer, D. H. and Malin, M. J. (1970) *Resistance of Extracellular Yeast Invertase and other Glycoproteins to Denaturation by Tannins.* Biochemical Journal, 118, p. 899-900.

Stumpf, P. K. and Conn, E. E. (1981) The Biochemistry of Plants, Volume 7. New York Academic Press, USA.

Suwa, Y., Mizukami, Y., Sogawa, K. and Fujii-Kuriyama, Y. (1985)

Gene Structure of a Major Form of Phenobarbital-Inducible Cytochrome P450 in Rat Liver. Journal of Biological Chemistry, 260(13), p. 7980-7984.

Nakechi, M. and Tanaka, Y. (1987) *Binding of 1,2,3,4,6 Pentagalloyl-glucose to Proteins, Lipids, Nucleic Acids and Sugars.* Phytochemistry, 26(1), p. 95-97.

van der Hosven, T. A. and Coon, M. J. (1974) *Preparation and Properties of Partially Purified Cytochrome P450 and Reduced Nicotinamide Adenine Dinucleotide Phosphate-Cytochrome P450 Reductase from Rabbit Liver Microsomes.* Journal of Biological Chemistry, 249(19), p. 6302-6310.

Van Rensburg, S. J., Hall, J. M. and du Bruyn, D. B. (1985) *Effects of Various Dietary Staples on Oesophageal Carcinogenesis Induced in Rats by Subcutaneously Administered N-Nitrosomethylbenzylamine.* Journal of the National Cancer Institute, 75(3), p. 561-566.

Vogel, E. W. and Zijlstra, J. A. (1987) *Mechanistic and Methodological Aspects of Chemically-Induced Somatic Mutation and Recombination in Drosophila melanogaster,* Mutation Research, 182, p. 243-264.

Vogel, E. W., Zijlstra, J. A. and Blijleven, W. G. H. (1983) *Mutagenic Activity of Selected Aromatic Amines and Polycyclic Hydrocarbons in Drosophila melanogaster*. Mutation Research, 107, p. 53-77.

Warminski, E (1989) Personal communication.

Warwick, G. P. and Harington, J. S. (1973) *Some Aspects of the Epidemiology and Etiology of Oesophageal Cancer with Particular Emphasis on the Transk South Africa*. Advances in Cancer Research, 17, p. 81-86.

Watson, J. D. and Crick, F. H. G. (1953) *Molecular Structure of Nuclei Acids*. Nature, 171, p. 737-738.

Watson, W. A. F. (1982) *The Mutagenic Activity of Quercetin and Kaempferol in Drosophila melanogaster*. Mutation Research, 103, p. 145-147.

Wattenberg, L. W. (1985) *Chemoprevention of Cancer*. Cancer Research, 45, p. 1-8.

Wattenberg, L. W. and Leong, J. L. (1970) *Inhibition of the Carcinogenic Action of Benzo(a)pyrene by Flavones*. Cancer Research, 30, p. 1922-1925.

Waxman, D. J. (1986) *Rat Hepatic Cytochrome P450.* In: Cytochrome P450 - Structure, Mechanism and Biochemistry, P. P. Oritz de Montellano, Plenum Press, New York, p. 525-539.

Weeks, C. E., Allaben, W. T., Louie, S. C., Lazear, E. J. and King, C. M. (1978) *Role of Arylhydroxamic Acid Acyltransferase in the Mutagenicity of N-Hydroxy-N-2-fluorenylacetamide in Salmonella typhimurium.* Cancer Research, 38, p. 613-618.

Weinges, K., Bahr, W., Ebert, W., Gortiz, K. and Marx, H. D. (1969a) *Konstitution, Enstehung und Bedeutung der Flavonoid - Gerbstoffe.* Fortschritte in Chemische und Organische Naturstoffe, 27, p. 158-164.

Weinstein, D. and Lewinson, T. M. (1978) *A Statistical Treatment of the Ames Mutagenicity Test.* Mutation Research, 51, p. 433-434.

Weisburger, J. H. and Weisburger, E. K. (1973) *Biochemical Formation and Pharmacological, Toxicological, and Pathological Properties of Hydroxylamines and Hydroxamic Acids.* Pharmacological Reviews, 25(1), p. 2-67.

White, R. E. and Coon, M. J. (1980) *Oxygen Activation by Cytochrome P450.* Annual Review of Biochemistry, 49, p. 315-356.

Whitfield, Jr., H. J., Martin, R. G. and Ames, B. N. (1966) *Classification of Amino Transferase (C gene) Mutants in the Histidine Operon.* Journal of Molecular Biology, 21, p. 335-355.

Wiebel, F. J., Selkirk, J. K., Gelboin, H. V., Haugen, D. A., van der Hoeven, T. A. and Coon, M. J. (1975) *Position-Specific Oxygenation of Benzo(a)pyrene by Different Forms of Purified Cytochrome P450 from Rabbit Liver.* Proceedings of the National Academy of Science (USA), 72, p. 3917-3920.

Winkle, S. A., Rosenberg, L. S. and Krugh, T. R. (1982) *On the Cooperative and Nonccoperative Binding of Ethidium to DNA.* Nucleic Acids Research, 10(24), p. 8211-8223.

Winkler, H. H. and Wilson, T. H. (1966) *The Role of Energy Coupling in the Transport of β-Galactosides by Escherichia coli.* Journal of Biological Chemistry, 10(25), p. 2200-2211.

Wood, A. W., Huang, M., Chang, R. L., Newmark, H. L., Lehr, R. E., Yagi, H., Sayer, J. M., Jerina, D. M. and Conney, A. H. (1982) *Inhibition of the Mutagenicity of Bay-Region Diol Epoxides of Polycyclic Aromatic Hydrocarbons by Naturally Occurring Plant Phenols: Exceptional Activity of Ellagic Acid.* Proceedings of the National Academy of Science (USA), 79, 5513-5517.

Wright, A. S. (1980) *Role of Metabolism in Chemical Mutagenesis and Carcinogenesis.* Mutation Research, 75, p. 215-241.

Würgler, F. E. and Vogel, E. W. (1986) *In Vivo Mutagenicity Testing using Somatic Cells of Drosophila melanogaster.* In: Chemical Mutagens, Vol 10, F. J. de Serres (Editor), Plenum Press, New York, p. 1-59.

Wyss, O., Clark, J. B., Haas, F., Stone, W. S. (1948) *The Role of Peroxide in the Biological Effects of Irradiated Broth.* Journal of Bacteriology, 56, p. 51-57.

Xodo, L. E., Manzini, G. and Ruggiero, J. (1988) *On the Interaction of Daunomycin with Synthetic Alternating DNAs: Sequence Specificity and Polyelectrolyte Effects on the Intercalation Equilibrium.* Biopolymers, 27, p. 1839-1857.

Ya, C., Gaffney, S. H., Lilley, T. H. and Haslam, E. (1989) *Carbohydrate - Polyphenol Complexation.* In: Chemistry and Significance of Condensed Tannins, R. W. Hemmingway, J. J. Karchesy and S. J. Branham (Editors), Plenum Press, New York, p. 307-322.

Yang, C. S., Strickhart, F. S. and Kicha, I. P. (1978) *Interaction between NADPH - Cytochrome P450 Reductase and Hepatic Microsomes.* Biochemica et Biophysica Acta, 509, p. 326-337.

Yoshikawa, K., Nohmi, T., Myata, R., Ishidate Jr., M., Ozawa, N., Isobe, M., Watabe, T., Kada, T. and Kawachi, T. (1982) *Differences in Liver Homogenates from Donryu, Fischer, Sprague-Dawley and Wistar Strains of Rat in the Drug Metabolizing Enzyme Assay and the Salmonella/Hepatic S9 Activation Test.* Mutation Research, 96, p. 167-186.

Zimmermann, F. K. and Taylor-Mayer, R. E. (1985) *Mutagenicity Testing - Some Background Information.* In: Mutagenicity Testing in Environmental Pollution Control, Ellis-Horwood Limited, England, p. 23-46.

Chapter 8. APPENDIX

Table 8.1 Incubation of F_1 and F_2 from sorghum cultivar MK222 with TA97a and TA98 (Standard error of the mean indicated).

Sample	Amount/ml	TA97a	TA98
F_1	1 mg	131 ± 3.5	34 ± 1.5
	100 µg	169 ± 18.5	23 ± 3.5
	10 µg	186 ± 3.5	28 ± 0.5
	1 µg	180 ± 13.0	22 ± 0.5
F_2	1 mg	131 ± 4.0	29 ± 5.0
	100 µg	180 ± 11.5	24 ± 4.0
	10 µg	165 ± 20.0	27 ± 0.5
	1 µg	156 ± 6.0	22 ± 1.0
Daunomycin	60 µg	173 ± 5.5	3121 ± 141
Blank	-	127 ± 3.5	33 ± 0.5

Table 8.2 Incubation of sorghum cultivar SHK3144 - F_1 and F_3 with all four strains without (-9) and with (+S9) metabolic activation at 4% (w//v) in the Ames test. (Standard error of the mean indicated).

Sample	µg/ml	TA97a		TA98		TA100	TA102	
		-S9	+S9	-S9	+S9	-S9	-S9	+S9
F_1	20	163 ± 7.5	192 ± 7.5	23 ± 4.0	27 ± 1.5	270 ± 7.5	276 ± 17.0	299 ± 5.0
	40	167 ± 5.0	143 ± 3.0	24 ± 1.5	29 ± 1.0	229 ± 31.5	290 ± 30.0	331 ± 6.0
	100	143 ± 11.0	91 ± 4.5	18 ± 3.5	41 ± 7.5	150 ± 16.5	168 ± 9.0	117 ± 13.5
F_3	20	127 ± 14.5	117 ± 4.5	1 , 2.5	21 ± 3.5	91 ± 3.0	109 ± 1.5	260 ± 8.0
	40	43 ± 5.5	95 ± 9.5	14 ± 4.5	40 ± 3.5	123 ± 18.5	98 ± 20.0	170 ± 0.0
	100	31 ± 7.5	98 ± 13.5	11 ± 3.0	24 ± 2.5	186 ± 1.0	103 ± 1.5	126 ± 5.5
Blank	-	162 ± 5.0	114 ± 6.0	34 ± 1.0	42 ± 2.0	235 ± 31.0	322 ± 36.0	247 ± 4.5
Mitomycin C	5						2894 ± 53.0	
Sodium Azide	15					2837 ± 4.5		
Daunomycin	60	170 ± 24.0		2440 ± 186.5				
Benzo(a)pyrene	10				126 ± 1.5			311 ± 43.0

Table 8.3 *Fractions F_1, F_2 and F_3 from sorghum cultivar SSK30 were incubated with all four strains without (-S9) and with (+S9) metabolic activation at 4% (w/v) in the Ames test. (Standard error of the mean indicated).*

Sample	µg/ml	TA97a		TA98		TA100		TA102	
		-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
F_1	1	161 ± 0.5	116 ± 24.0	22 ± 2.0	33 ± 4.5	296 ± 6.0	154 ± 6.0	273 ± 33.0	46 ± 4.5
	10	108 ± 0.5	76 ± 24.0	21 ± 4.5	41 ± 5.0	265 ± 24.5	239 ± 49.0	288 ± 62.0	37 ± 13.5
	100	81 ± 5.5	105 ± 7.0	20 ± 1.0	50 ± 7.0	252 ± 4.0	252 ± 8.0	309 ± 41.0	109 ± 5.0
F_2	1	150 ± 36.0	96 ± 22.5	20 ± 2.5	39 ± 4.0	324 ± 10.0	185 ± 15.0	277 ± 13.0	155 ± 1.0
	10	118 ± 17.5	87 ± 0.5	26 ± 1.0	52 ± 9.0	299 ± 27.0	250 ± 25.0	310 ± 10.0	260 ± 40.0
	100	135 ± 7.0	96 ± 9.5	20 ± 4.0	43 ± 16.5	320 ± 0.5	205 ± 5.0	324 ± 10.5	240 ± 60.0
F_3	1	96 ± 11.0	82 ± 10.5	34 ± 1.5	34 ± 8.5	331 ± 15.0	268 ± 18.5	298 ± 34.5	268 ± 18.5
	10	98 ± 15.5	64 ± 8.5	30 ± 8.5	47 ± 3.0	281 ± 29.0	159 ± 11.0	272 ± 8.0	159 ± 11.0
	100	96 ± 5.0	76 ± 2.0	22 ± 3.0	47 ± 3.5	266 ± 46.0	301 ± 51.0	330 ± 16.0	301 ± 51.0
Blank	-	169 ± 5.0	94 ± 10.0	24 ± 0.5	32 ± 0.5	230 ± 31.0	192 ± 30.0	214 ± 36.0	200 ± 4.5
Daunomycin	60	170 ± 24.0		2837 ± 142.0		286 ± 29.5			
Mitomycin C	5							3000 ± 53.0	
2-Aminofluorene	100		1986 ± 38.0				2645 ± 96.0		520 ± 17.5

Table 8.4 Fraction F_1 from sorghum grain cultivar PNR8311 was incubated with TA97a and TA98. (Standard error of the mean indicated).

Sample	Amount/ml	TA97a	TA98
F_1	1 mg	214 ± 10.5	22 ± 0.5
	100 µg	226 ± 8.0	28 ± 3.0
	10 µg	238 ± 0.5	35 ± 4.0
	1 µg	285 ± 5.0	23 ± 2.5
Daunomycin	60 µg	204 ± 17.0	3121 ± 141.0
Blank	-	120 ± 3.5	32 ± 0.5

Table 8.5 Sorghum grain cultivar Barnard Red F_1 mutagenicity testing with TA98 and TA100, and then together with daunomycin at 60 µg/ml (for TA98) and sodium azide at 15 µg/ml (for TA100) in an antimutagenic assay. (Standard error of the mean indicated).

Sample	Amount/ml	TA98		TA100	
		Sample	Daunomycin + Sample	Sample	Sodium azide + Sample
F_1	10 mg	18 ± 0.5	1164 ± 99.5	139 ± 21.0	1426 ± 92.0
	1 mg	22 ± 1.5	1311 ± 6.0	190 ± 15.0	1504 ± 28.0
	100 µg	18 ± 0.5	1412 ± 7.0	217 ± 17.0	1712 ± 9.0
	10 µg	19 ± 2.0	1781 ± 63.5	240 ± 10.0	1613 ± 4.5
	1 µg	23 ± 0.5	2094 ± 91.5	160 ± 12.0	1750 ± 9.5
	0.1 µg	28 ± 0.5	2417 ± 61.5	228 ± 12.0	1802 ± 42.5
Sodium azide	15 µg			2809 ± 4.5	
Daunomycin	60 µg	2838 ± 0.5			
Blank	-	27 ± 2.5		120 ± 0.5	



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