

**THE INFLUENCE OF HOST AND DIETARY FACTORS ON
MUTAGEN METABOLISM IN MICE**

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ABSTRACT

The effects of quantitative and/or qualitative changes to dietary fat and protein on the genotoxicity of dietary carcinogens were evaluated in female BALB/c mice using *in vivo* and *in vitro* bacterial mutation assays. An increase in dietary fat content (from 1% to 20%) increased the *in vitro* activation of MeIQ (2-amino-3,4-dimethylimidazo[4,5-f]quinoline) and Trp-P-2 (3-amino-1-methyl-5H-pyrido[4,3b]indole) with the extent of the increase being dependent on the type of fat used although the activation of aflatoxin B₁ was unaltered. Changes in *in vivo* genotoxicity were also seen but these did not always reflect the *in vitro* results.

As the protein content of the diet increased from 5% to 20% there was a decline in the *in vivo* mutagenicity of aflatoxin B₁ (but not MeIQ and Trp-P-2). Diet related changes to hepatic enzymes involved in the activation and detoxification of aflatoxin B₁ do not explain the reduction in genotoxicity observed in mice fed a high protein diet. Distribution studies indicate that dietary fat and protein may influence the uptake and turnover of mutagens in the liver although fat appeared not to influence the uptake of compounds from the gastro-intestinal tract.

The dietary fibre pectin significantly increased caecal nitrate reductase activity but had no apparent effect on the *in vivo* nitrosation of aminopyrine to the mutagen nitrosodimethylamine. Extreme changes to the gut flora (germ-free mice compared to conventional) however, did result in changes to hepatic metabolism

of mutagens *in vivo*.

As the age of mice used in genotoxicity assays increased from 2 - 24 weeks, the mutagenicity *in vivo* and *in vitro* of MeIQ and Trp-P-2 decreased. Similarly, aflatoxin B₁ genotoxicity was reduced in older animals *in vivo* but not *in vitro*. Changes in hepatic activation and detoxification capacity of the liver appear to explain these age dependent changes. However age had no consistent effect on gut flora metabolism.

To improve the sensitivity of the host-mediated assay, various modifications to the assay were investigated and analysis of this bacterial data was assisted by electron microscopy showing bacterial distribution within the liver.

For my Father,

James Back,

With all my love.

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CONTENTS

| | | |
|--|------|----|
| Abstract | Page | 2 |
| Acknowledgements | | 5 |
| Contents | | 6 |
| List of Figures | | 11 |
| List of Tables | | 15 |
| Abbreviations | | 20 |
| CHAPTER 1 <i>Introduction</i> | | 22 |
| 1. General Introduction | | 22 |
| 2. Formation and metabolism of carcinogens. | | 24 |
| <i>a. Activation and detoxification of carcinogens.</i> | | 24 |
| <i>b. Formation of carcinogens by the gut flora.</i> | | 28 |
| 3. Dietary Carcinogens. | | 34 |
| <i>a. Cooked food mutagens.</i> | | 34 |
| <i>b. Aflatoxin B1.</i> | | 39 |
| 4. Dietary modification of Cancer. | | 41 |
| <i>a. Epidemiological evidence.</i> | | 41 |
| <i>b. Experimental evidence.</i> | | 44 |
| <i>c. Mechanisms involved in dietary modification of carcinogenesis.</i> | | 48 |
| 5. Age and Cancer. | | 53 |
| 6. Methodology. | | 58 |
| CHAPTER 2 <i>Materials and Methods</i> | | 62 |
| 1. Chemicals | | 62 |
| 2. Mutagens | | 62 |
| 3. Animals | | 63 |
| 4. Diets | | 65 |
| 5. Bacterial Strains | | 70 |

| | |
|---|-----------|
| 6. Bacterial Media | 70 |
| <i>a. E. coli D494</i> | 70 |
| <i>b. E. coli K-12/343 strains</i> | 72 |
| <i>c. S.typhimurium TA98 and TA100</i> | 73 |
| 7. Preparation of bacterial cultures | 74 |
| <i>a. Maintenance</i> | 74 |
| <i>b. Growth</i> | 74 |
| <i>c. Tris-EDTA permeabilization of bacteria</i> | 76 |
| 8. Preparation of mouse caecal and hepatic fractions | 76 |
| <i>a. Preparation of caecal fractions</i> | 76 |
| <i>b. Preparation of hepatic fractions</i> | 76 |
| <i>c. Estimation of protein content</i> | 77 |
| 9. Determination of enzyme activities | 78 |
| <i>a. Microbial</i> | 78 |
| <i>b. Mammalian</i> | 81 |
| 10. In vitro mutagenicity and genotoxicity assays | 85 |
| <i>a. Salmonella mutagenicity assay</i> | 85 |
| <i>b. In vitro genotoxicity assay</i> | 86 |
| 11. Host-mediated assays | 87 |
| <i>a. E. coli D494</i> | 87 |
| <i>b. E. coli 343/765 and 343/753</i> | 88 |
| <i>c. Salmonella typhimurium TA98.</i> | 89 |
| 12. Distribution studies | 90 |
| <i>a. Host-mediated assay</i> | 90 |
| <i>b. Absorption of mutagens from the gastro-</i> <i>intestinal tract.</i> | 92 |
| 13. Electron microscopy | 93 |
| 14. Statistical analysis | 93 |

| | | |
|-----------|---|-----|
| CHAPTER 3 | <i>Modulation of mouse hepatic metabolism by dietary fat in vivo and in vitro.</i> | 95 |
| 1. | <i>Salmonella</i> mutagenicity assay | 95 |
| 2. | Host-mediated assays | 102 |
| 3. | Distribution studies | 105 |
| | <i>a. Host-mediated assays</i> | 105 |
| | <i>b. Absorption of [¹⁴C]Trp-P-2 from ligated gut sections</i> | 106 |
| 4. | Effect of dietary fat on the mutagenicity of nitrosodimethylamine in the host-mediated assay | 113 |
| CHAPTER 4 | <i>The effect of dietary protein on the hepatic metabolism of food mutagens</i> | 119 |
| 1. | Host mediated assays | 119 |
| 2. | <i>Salmonella</i> mutagenicity assay | 130 |
| 3. | Cytochrome P450 content and mixed function oxidase activities | 137 |
| 4. | Glutathione and glutathione conjugates | 137 |
| 5. | Effect of hepatic cytosol on the microsomal activation of food mutagens | 142 |
| CHAPTER 5 | <i>The influence of dietary fibre on gut flora metabolism and on the formation and metabolism of nitrosodimethylamine</i> | 148 |
| 1. | Effect of dietary pectin on gut flora metabolism | 148 |
| 2. | Effect of dietary pectin on the mutagenicity of nitrosodimethylamine <i>in vitro</i> and <i>in vivo</i> | 154 |
| 3. | The effect of dietary pectin on the activity of aminopyrine and sodium nitrite in the host-mediated assay | 161 |

| | |
|---|-----|
| 4. Effect of dietary pectin on the activity of aminopyrene and sodium nitrate in the host-mediated assay | 170 |
| 5. The sensitivity of the DNA-repair host-mediated assay to detect changes in the nitrosation of aminopyrene | 173 |
| 6. Effect of a nitrosation catalyst on the genotoxic activity of aminopyrene and nitrite in the host-mediated assay | 174 |
| 7. Effect of germ-free status on the <i>in vivo</i> mutagenicity of food mutagens | 178 |
| | |
| CHAPTER 6 <i>Developmental changes in hepatic and gut flora metabolism</i> | 182 |
| 1. Age-related changes in hepatic metabolism <i>in vivo</i> and <i>in vitro</i> | 183 |
| <i>a. Host-mediated assays</i> | 183 |
| <i>b. Salmonella mutagenicity assay</i> | 187 |
| <i>c. Mixed function oxidase activities</i> | 199 |
| <i>d. Glutathione and glutathione transferase activities</i> | 203 |
| 2. Effect of host age on gut flora metabolism | 203 |
| | |
| CHAPTER 7 <i>Modifications to the DNA-repair host-mediated assay</i> | 218 |
| 1. <i>E. coli</i> strains deficient in cell layer lipopolysaccharide | 219 |
| 2. Bacteria in logarithmic growth | 224 |
| 3. Treatment of <i>E. coli</i> 343/765 and 343/753 with EDTA | 229 |
| 4. Location of <i>E. coli</i> in livers of mice used in intrasanguineous host-mediated assays | 233 |
| 5. Recovery of orally dosed <i>E. coli</i> 343/765 and 343/753 from the mouse gastro-intestinal tract | 236 |

| | |
|--|-----------------|
| CHAPTER 8 Discussion | Page 240 |
| 1. Dietary fat | 240 |
| 2. Dietary protein | 251 |
| 3. Age-related changes in hepatic metabolism | 260 |
| 4. Age-related changes in gut flora metabolism | 264 |
| 5. Dietary pectin and gut flora metabolism | 270 |
| 6. Dietary pectin and nitrosamine formation | 280 |
| 7. The influence of the gut flora on the hepatic activity of mutagens. | 288 |
| 8. <i>In vivo</i> and <i>in vitro</i> bacterial mutation assays | 291 |
| 9. Conclusions. | 306 |
| REFERENCES | 309 |

LIST OF FIGURES

| Figure | | Page |
|--------|--|------|
| 1 | Cytochrome P450 dependent mixed function oxidase system. | 25 |
| " | 2 The structure of glutathione. | 27 |
| " | 3 The nitrosation of aminopyrine in acidic conditions. | 31 |
| " | 4 The activation of nitrosodimethylamine. | 32 |
| " | 5 Three dietary carcinogens used in experiments presented in this thesis. | 35 |
| " | 6 Pathway of metabolic activation of Trp-P-2. | 38 |
| " | 7 Pathways of metabolism of aflatoxin B ₁ . | 40 |
| " | 8 Activation of aflatoxin B ₁ by hepatic S9 fractions from mice fed different fat diets. | 98 |
| " | 9 Activation of Trp-P-2 by hepatic S9 fractions from mice fed different fat diets. | 99 |
| " | 10 Activation of MeIQ by hepatic S9 fractions from mice fed different fat diets. | 100 |
| " | 11 The effect of dietary fat on the uptake of [¹⁴ C] Trp-P-2 from the ligated small intestine. | 109 |
| " | 12 The effect of dietary fat on the uptake of [¹⁴ C] Trp-P-2 from mouse ligated large intestine. | 111 |
| " | 13 Effect of dietary protein concentration on the <i>in vivo</i> mutagenicity of aflatoxin B ₁ . | 120 |
| " | 14 Effect of dietary protein concentration on the <i>in vivo</i> mutagenicity of MeIQ. | 124 |
| " | 15 Effect of dietary protein concentration on the <i>in vivo</i> mutagenicity of Trp-P-2. | 125 |

| | | |
|-----------|--|----------|
| Figure 16 | Activation of aflatoxin B ₁ by different concentrations of hepatic microsomes from mice fed a 5% or 20% protein diet. | Page 133 |
| " | 17 Activation of Trp-P-2 by different concentrations of hepatic microsomes derived from mice fed 5% or 20% protein diets. | 134 |
| " | 18 Activation of MeIQ by different concentrations of hepatic microsomes derived from mice fed 5% or 20% protein diets. | 135 |
| " | 19 Reverse phase HPLC chromatograms of aflatoxin B ₁ and metabolites produced by incubations of quail microsomes and cytosol prepared from mice fed either 5% or 20% lactalbumin. | 140 |
| " | 20 Effect of hepatic cytosol on the microsomal activation of aflatoxin B ₁ , Trp-P-2 and MeIQ. | 144 |
| " | 21 The effect of dietary pectin on mouse caecal enzyme activities. | 151 |
| " | 22 Activation of nitrosodimethylamine by hepatic S9 fractions from mice fed a fibre-free or 5% pectin diet. | 155 |
| " | 23 The influence of dietary pectin on the nitrosation of aminopyrine to a bacterial mutagen. | 163 |
| " | 24 Effect of changes in nitrite concentration on the nitrosation of aminopyrine to a bacterial mutagen. | 169 |
| " | 25 The sensitivity of the DNA-repair host-mediated assay in detecting changes in the nitrosation of aminopyrine to a genotoxin. | 175 |

| | | |
|-----------|---|----------|
| Figure 26 | Determination of optimal S9 concentration for mutagen activation <i>in vitro</i> . | Page 192 |
| " | 27 Activation of MeIQ by hepatic S9 fractions from mice of different ages. | 193 |
| " | 28 Activation of different concentrations of MeIQ by hepatic S9 fractions from mice aged 4 and 24 weeks. | 194 |
| " | 29 Activation of Trp-P-2 by hepatic S9 fractions from mice of different ages. | 195 |
| " | 30 Activation of different concentrations of Trp-P-2 by hepatic S9 fractions from mice aged 4 and 24 weeks. | 196 |
| " | 31 Activation of aflatoxin B ₁ by hepatic S9 fractions from mice of different ages. | 197 |
| " | 32 Activation of different concentrations of aflatoxin B ₁ by hepatic S9 fractions from mice aged 4 and 24 weeks. | 198 |
| " | 33 The effect of age and diet on caecal nitrate reductase activity in mice. | 204 |
| " | 34 The effect of age and diet on caecal nitroreductase activity in mice. | 206 |
| " | 35 The effect of age and diet on caecal β -glucuronidase activity in mice. | 208 |
| " | 36 Comparison of LPS ^{def} and LPS ⁺ <i>E. coli</i> indicator bacteria in their ability to detect the genotoxin hycanthone in an <i>in vitro</i> assay. | 222 |
| " | 37 Growth curves of <i>E. coli</i> 343/765 and 343/753. | 225 |
| " | 38 The effect of growth phase on the ability of <i>E. coli</i> 343/765 and 343/753 to detect the genotoxin nitrosomethylurea. | 228 |

| | | |
|-----------|---|----------|
| Figure 39 | The effect of pretreating <i>E. coli</i> bacteria with EDTA on their ability to detect the genotoxic action of two food mutagens. | Page 232 |
| " | 40 Electron micrographs of liver sections from mice injected intravenously with <i>E. coli</i> D494. | 234 |
| " | 41 The survival and transit of <i>E. coli</i> through the mouse gastro-intestinal tract. | 237 |

LIST OF TABLES

| Table | | Page |
|-------|--|------|
| 1 | Composition of fibre-free and pectin containing diets. | 66 |
| " | 2 Composition of fat diets. | 67 |
| " | 3 Composition of protein diets. | 68 |
| " | 4 Composition of mineral and vitamin mixes used in animal diets. | 69 |
| " | 5 The bacterial strains employed. | 71 |
| " | 6 Body and liver weights of mice fed different fat diets and protein content of hepatic S9 fractions prepared from these mice. | 97 |
| " | 7 Effect of high fat diets on the activity of food mutagens in the host-mediated assay. | 103 |
| " | 8 A representative recovery of <i>S. typhimurium</i> TA98 from the livers of mice in the host-mediated assays of Table 7. | 104 |
| " | 9 Radioactivity in the blood and liver of mice fed different fat diets and dosed with radiolabelled mutagens in the host-mediated assays of Table 7. | 108 |
| " | 10 Effect of a 25% olive oil diet on the uptake of [¹⁴ C]Trp-P-2 from the ligated small and large mouse intestine. | 110 |
| " | 11 Effect of a 30% beef dripping diet on the genotoxicity of nitrosodimethylamine in the host-mediated assay using <i>E. coli</i> D494. | 115 |
| " | 12 Effect of a 30% beef dripping diet on the activity of nitrosodimethylamine in the DNA-repair host-mediated assay. | 116 |

Table 13 The effect of dietary protein concentration on the Page 121

in vivo activity of aflatoxin B₁ (Experiment 2).

" 14 Body and liver weights of mice fed different concentrations of lactalbumin and subsequently used in host-mediated assays. 122

" 15 A representative recovery of *S. typhimurium* TA98 from the livers of mice used in the host-mediated assays of Figures 13 - 15. 127

" 16 Radioactivity in the blood and liver of mice fed a range of protein diets and given [¹⁴C]MeIQ or [¹⁴C]Trp-P-2 in certain host-mediated assays of Figs. 14 - 15. 129

" 17 Body and liver weights and protein concentration of hepatic fractions from mice fed 5% and 20% lactalbumin diets and used in *in vitro* assays (Figs. 16 - 18). 131

" 18 Effect of microsome concentration on the reversion of *S. typhimurium* TA98 to histidine prototrophy in the *Salmonella* mutagenicity assay. 132

" 19 Cytochrome P450 and mixed function oxidase activities of microsomal fractions (used in Figs. 16 - 18) from mice fed 5% and 20% protein diets. 138

" 20 Hepatic glutathione content of mice fed 5% and 20% lactalbumin diets. 141

" 21 Effect of hepatic cytosol on the reversion of *S. typhimurium* TA98 to histidine prototrophy. 146

" 22 Body, liver and caecal weights of mice fed diets containing different amounts of pectin. 150

" 23 The effect of dietary pectin on the genotoxicity of nitrosodimethylamine in the host-mediated assay. 157

| | | |
|----------|--|----------|
| Table 24 | Body and liver weights of mice fed different amounts of pectin and used in the experiments presented in Table 23. | Page 159 |
| " | 25 The <i>in vivo</i> mutagenicity of nitrosodimethylamine in different mouse organs. | 160 |
| " | 26 Effect of dietary pectin on the activity of aminopyrine and sodium nitrite in the host-mediated assay. | 164 |
| " | 27 Dose response of aminopyrine and sodium nitrite in the host-mediated assay. | 165 |
| " | 28 Effect of altering nitrite concentration on the nitrosation of aminopyrine to a bacterial mutagen in the host-mediated assay. | 168 |
| " | 29 Effect of an acute dose of sodium nitrate on the nitrosation of aminopyrine to a bacterial mutagen. | 171 |
| " | 30 Effect of chronic dosing of nitrate on the nitrosation of aminopyrine <i>in vivo</i> . | 172 |
| " | 31 Ability of the DNA-repair host-mediated assay to detect changes in the nitrosation of aminopyrine to a genotoxin. | 176 |
| " | 32 Effect of thiocyanate on the nitrosation of aminopyrine <i>in vivo</i> . | 177 |
| " | 33 Body and liver weights and genotoxicity of aflatoxin B ₁ , MeIQ and Trp-P-2 in conventional and germ-free mice in the host-mediated assay. | 179 |

| | | |
|-----------------|---|-----------------|
| Table 34 | Recovery of <i>S. typhimurium</i> TA98 from the livers of germfree and conventional flora mice in the host-mediated assay of Table 33. | Page 180 |
| " | 35 Body and liver weights of female BALB/c mice of different ages. | 184 |
| " | 36 Effect of age on the activity of food mutagens in the host-mediated assay. | 185 |
| " | 37 Recovery of <i>S. typhimurium</i> TA98 from the livers of mice in the host-mediated assays of Table 36. | 186 |
| " | 38 Protein and cytochrome P450 content of hepatic S9 fractions from mice of different ages. | 188 |
| " | 39 Effect of S9 concentration on the background reversion of <i>S. typhimurium</i> TA98 to histidine prototrophy. | 190 |
| " | 40 Cytochrome P450, cytochrome C reductase and mixed function oxidase activities of microsomal tissue from mice aged 4 and 24 weeks. | 200 |
| " | 41 Determination of glutathione content and glutathione transferase activities in the livers of mice aged 4 and 24 weeks. | 202 |
| " | 42 Caecal enzyme activities of 2 week old preweanling mice and their mothers. | 210 |
| " | 43 Body weight, caecal weight and caecal bacterial numbers of mice of different ages fed a fibre-free or 5% pectin diet. | 212 |
| " | 44 Body weights, caecal weights and caecal enzyme activities of 14 week old mice fed 5% pectin for either 2 or 11 weeks. | 216 |

| | | |
|----------|--|----------|
| Table 45 | Survival of <i>E. coli</i> 343/765 and its repair deficient and LPS-deficient derivatives treated with hycanthone in the <i>in vitro</i> genotoxicity assay. | Page 221 |
| " 46 | Effect of growth phase and exposure to nitrosomethylurea on the survival of <i>E. coli</i> 343/765 and its repair deficient derivative 343/753 in the <i>in vitro</i> genotoxicity assay. | 227 |
| " 47 | The survival of <i>E. coli</i> 343/765 and 343/753 after pretreatment with EDTA in the <i>in vitro</i> genotoxicity assay. | 231 |
| " 48 | Summary of the effects of dietary fat and protein on the activity of food mutagens in bacterial mutation assays. | 241 |
| " 49 | Summary of age and diet related changes to gut flora metabolism. | 265 |

ABBREVIATIONS

| | |
|-------------------|---|
| Anova | analysis of variance |
| Amp ^R | ampicillin resistant bacteria |
| AP | aminopyrine |
| BIBRA | British Industrial Biological Research Association |
| CDNB | 1-chloro-2,4-dinitrobenzene |
| DCNB | 1,2-dichloro-4-nitrobenzene |
| DMBA | 7,12-dimethylbenzanthracene |
| DMSO | dimethylsulphoxide |
| DNA | deoxyribonucleic acid |
| dpm | disintegrations per minute |
| EDTA | ethylenediaminetetraacetic acid |
| ENPP | 1,2-epoxy-3-(p-nitrophenoxy)propane |
| FAD | flavin adenine dinucleotide |
| FMN | flavin mononucleotide |
| His ⁺ | histidine prototrophy |
| HPLC | high performance liquid chromatography |
| IQ | 2-amino-3-methylimidazo[4,5-f]quinoline |
| Lac | phenotype for lactose utilisation |
| LPS | cell membrane lipopolysaccharide |
| LSD | least significant difference |
| MeIQ | 2-amino-3,4-dimethylimidazo[4,5-f]quinoline |
| MeIQx | 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline |
| MRC | Medical Research Council |
| NADP ⁺ | β -nicotinamide adenine dinucleotide phosphate (oxidized form) |
| NADPH | β -nicotinamide adenine dinucleotide phosphate (reduced form) |

| | |
|-----------------|--|
| NB | nutrient broth |
| NMU | nitrosomethylurea |
| NO ₂ | sodium nitrite |
| PBS | phosphate buffered saline |
| PBS-S | phosphate buffered saline supplemented with streptomycin sulphate |
| <i>rec</i> | gene for recombination repair |
| RNA | ribonucleic acid |
| S9 | post-mitochondrial supernatant |
| SD | standard deviation |
| TCA | trichloroacetic acid |
| Trp-P-2 | 3-amino-1-methyl-5H-pyrido[4,3-b]indole |
| <i>uvr</i> | gene for excision DNA repair |

CHAPTER 1

Introduction

1. General Introduction.

Throughout the world one in ten deaths are caused by cancer (Howe 1986). However, cancer incidence at certain anatomical sites displays marked variations between populations. These variations occur between countries, within countries and over time indicating a major role for environmental factors in the etiology of cancer (Howe 1986). If these environmental factors are identified, it may be possible for their role in carcinogenesis to be reduced or even abolished. Some of the factors are well known, such as tobacco, alcohol and asbestos, but others have been more difficult to determine. Ways in which the environment may exert its influence on carcinogenesis are many, for example, by the promotion of tumours, by acting as a source of carcinogens or carcinogenic precursors, or by modifying mammalian and gut flora metabolism (activation or detoxification) of carcinogenic compounds.

There is strong evidence to suggest that among environmental factors, diet may be of particular importance in the development of cancers of the gastro-intestinal tract and breast. Wynder & Gori (1977) estimated the proportion of diet related cancers to be almost 60% for women and over 40% for men. However, of possibly greater concern, is the estimate that changes to dietary practices could result in the prevention of 10 - 70% of cancer (Doll & Peto 1981). A range of dietary components, from macro-nutrients to vitamins and trace elements, are thought to be involved in the enhancement and

inhibition of cancer. Thus far, there is no reliable evidence to indicate which dietary changes would have the greatest preventative effect.

Age is another factor linked with cancer. At most sites the incidence rate of carcinomas per annum (i.e. the probability of developing a new cancer next year) increases in proportion to age (Stenback & Arranto 1985). Cancer accounts, therefore, for a large proportion of deaths in older people and the number of people within these age groups is increasing considerably. According to Siegel (1981), 10.5% of people living in developed countries are over 65 years and by the year 2000 this figure will have increased to 12.1 - 13.1%. Understanding the mechanism that causes cancer incidence to increase with age may aid the treatment of this disease. However, at present, there is no consensus about the mechanisms involved.

The aim of the work presented in this thesis was to gain a greater understanding of the role of diet and other host-related factors in carcinogenesis. The study was primarily concerned with the effects of age and the dietary components, fat, protein and fibre on the metabolism of potential carcinogens. The carcinogens chosen for investigation are those considered to have a role in the initiation process of carcinogenesis in man, in particular those carcinogens found in food.

To investigate the induction and development of carcinogenesis is a time consuming and expensive process. As a result, there has been a move to develop tests which both cost less and provide results after shorter periods. The realization that chemical

carcinogens are frequently mutagenic has given rise to bacterial mutagenicity tests as a tool for assessing the carcinogenic potential of chemicals, since the mechanism of change induced in bacterial DNA, by chemicals, is considered to be the same as that which initiates cancer (Mohn 1981). Because of these advantages, short-term bacterial mutagenicity assays were used in the present study. Since these tests are simple models of highly complex interactions they do have their limitations. For example, the simplified metabolic system (hepatic S9) which is used, reflects neither the pharmacokinetics, nor the metabolic capacity of the intact animal. To help overcome these problems, the *in vivo* host-mediated bacterial mutation assay has also been employed to take into account changes in absorption, excretion and other *in vivo* metabolic systems. To complement this work, experiments have been carried out investigating the effect of diet and host-related factors on various aspects of metabolism that occur *in vivo*, including the uptake of chemicals from the gastro-intestinal tract, the hepatic activation (cytochrome P450 and mixed function oxidases) and detoxification (glutathione and glutathione transferases) of carcinogens and the metabolic activities of the gut microflora that may be involved in the biotransformation of carcinogens or their precursors.

2. Formation and metabolism of carcinogens.

a. Activation and detoxification.

The mechanism of chemical carcinogenesis and mutagenesis is thought usually to involve the covalent binding of electrophilic reactants to nucleophilic sites in cellular macromolecules such as DNA (Bresnick 1980). Certain chemicals are reactive *per se* while

others need to be converted to reactive metabolites (Miller & Miller 1979). Consequently, any modification to this activation process, or to the detoxification of these electrophiles may markedly affect the subsequent development of cancer.

Activation to a reactive intermediate can be carried out by a range of chemical reactions, termed Phase I metabolism, of which oxidation via the cytochrome P450-dependent monooxygenase system is a primary route (Fig. 1).

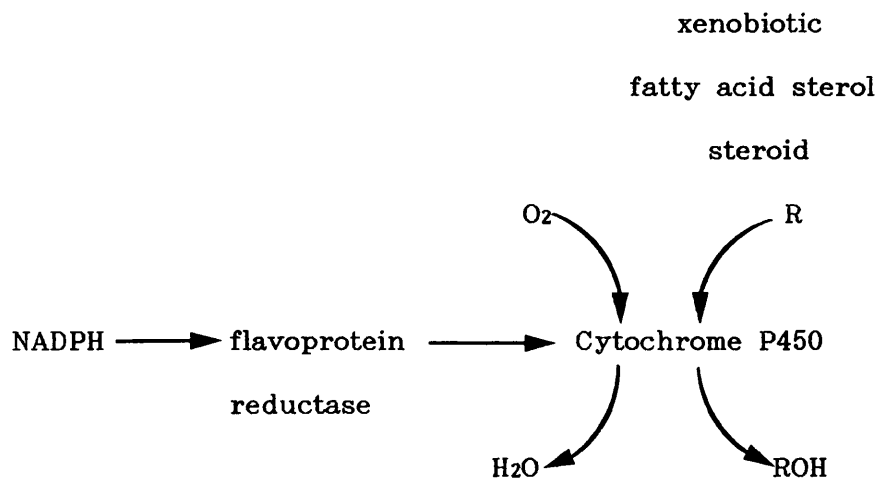


Figure 1. Cytochrome P450 dependent mixed function oxidase system (Parke & Ioannides 1982).

This ubiquitous mixed function oxidase system, comprising of cytochromes P450 and cytochrome P450 reductase is, in mammalian tissues, located in the endoplasmic reticulum, with the greatest activity occurring in the smooth membranes of the liver (Parke & Ioannides 1982). Consequently, this location is the major site of formation of reactive intermediates from environmental chemicals.

The cytochrome P450 component forms the terminal oxidase of this electron transfer system (Fig. 1) and consists of a family of closely related isoenzymes with molecular weights of 48000 - 60000, fourteen of which have been purified so far in rat liver (Yang & Yoo 1988).

The cytochrome P450 reductase is a flavin-containing enzyme consisting of flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) with a monomeric molecular weight of around 78000 (Yang & Yoo 1988). Its role in the mixed function oxidation system is to transfer electrons from NADPH to the cytochromes P450 (Fig. 1). In so doing, the resultant ferro-cytochrome P450 is able to catalyse the activation of molecular oxygen with one of the oxygen atoms being added to the substrate (Guengerich & McDonald 1984).

In addition to the various cytochrome P450 isoenzymes there is another more distinct form of this cytochrome - cytochrome P448 - which may also exist in variant forms. Parke and Gray (1978) have postulated that an increase of cytochrome P448 in the rat liver is an indication of chemical toxicity. Further to this Ioannides *et al* (1984) suggest that compounds which induce cytochrome P448 should be considered as potential carcinogens.

Other Phase I reactions involved in xenobiotic metabolism include reduction, hydrolysis, hydration and isomerization. It is considered that the primary function of this group of reactions is to prepare the compound for the set of reactions termed Phase II metabolism or conjugation, which generally lead to a water soluble

product that can be excreted in bile or urine. As with Phase I, Phase II metabolism also involves a diverse group of enzymes acting on a large range of compounds and includes reactions such as glucuronidation, sulphation, glutathione conjugation, methylation, glucosidation, acetylation and fatty acid conjugation.

Conjugation with glutathione (γ -glutamylcysteinylglycine; Fig. 2) is an important detoxification pathway for a number of mutagens and carcinogens, including the reactive metabolites of aflatoxin B₁, benzo(a)pyrene, methyl-4-aminoazobenzene and nitropyrene (Ketterer 1988).

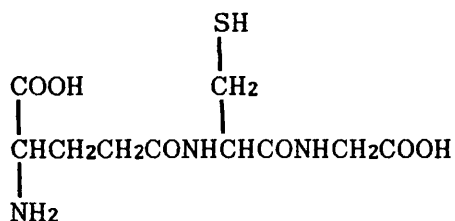


Figure 2. The structure of glutathione

The cysteinyl residue in glutathione's unique tripeptide structure provides a nucleophilic thiol which, together with its net negative charge and hydrophilicity, acts to increase the aqueous solubility of the lipophilic moieties with which it has become conjugated (Ketterer *et al* 1983). The high molecular weight of glutathione ensures that glutathione conjugates are preferentially secreted via the biliary system. Glutathione appears to be universal in nature and often occurs at a high intracellular concentration (5 - 10mM in rat liver; Ketterer 1988). At

physiological pH it exists as two nucleophilic species - glutathione and its thiolate anion, although the latter only represents 1% of the total glutathione present. In mammals, glutathione conjugates are usually metabolized further by hydrolysis and N-acetylation, either in the gut or kidney, to give N-acetylcysteinyl conjugates known as mercapturic acids which are then excreted in the urine (Chasseaud 1976).

The catalysis of the initial conjugation of the metabolite with glutathione is carried out by glutathione transferases (Habig *et al* 1974) which are widespread in both animal and plant tissues (Grover & Sims 1964) occurring usually as a spectrum of isoenzymes characteristic of each particular tissue (Ketterer 1988). In the rat liver, the greatest source of glutathione transferases is in the cytosol (Tipping & Ketterer 1981). There appear to be at least ten soluble isoenzymes (Ketterer 1988) with similar dimeric structures which have broad overlapping substrate specificities and similar physical properties (Habig *et al* 1974).

b. Formation of carcinogens by the gut flora.

The development of cancer may also be influenced by differences in the formation of carcinogens within the body, particularly by the gastro-intestinal flora. Changes to this complex microbial community, which is capable of metabolizing a large variety of xenobiotics and endogenously synthesized compounds, can alter the microbial biotransformation of compounds and so modify any toxicological consequences of this metabolism for the host (Rowland *et al* 1985). For example, many foreign compounds or their metabolites, are conjugated with glucuronic acid in the liver

rendering them water-soluble and usually unreactive and so more suitable for excretion into the bile to be eliminated eventually via the faeces. In the intestinal tract these conjugates may be hydrolysed by bacterial β -glucuronidase enzymes, present in the gut, liberating the parent compound. As a result of this hydrolysis, xenobiotics may be reabsorbed into the portal system giving rise to enterohepatic circulation and delaying excretion, thus potentiating toxic or carcinogenic effects (Rowland & Walker 1983). A number of carcinogens are conjugated with glucuronic acid, for example, N-hydroxy-N-2-fluorenyl^yacetamide (Grantham *et al* 1970), benzo(a)pyrene (Kinoshita & Gelboin 1978), nitropyrene (Morotomi *et al* 1985) and 4-acetylamino^xbiphenyl (Wheeler *et al* 1975).

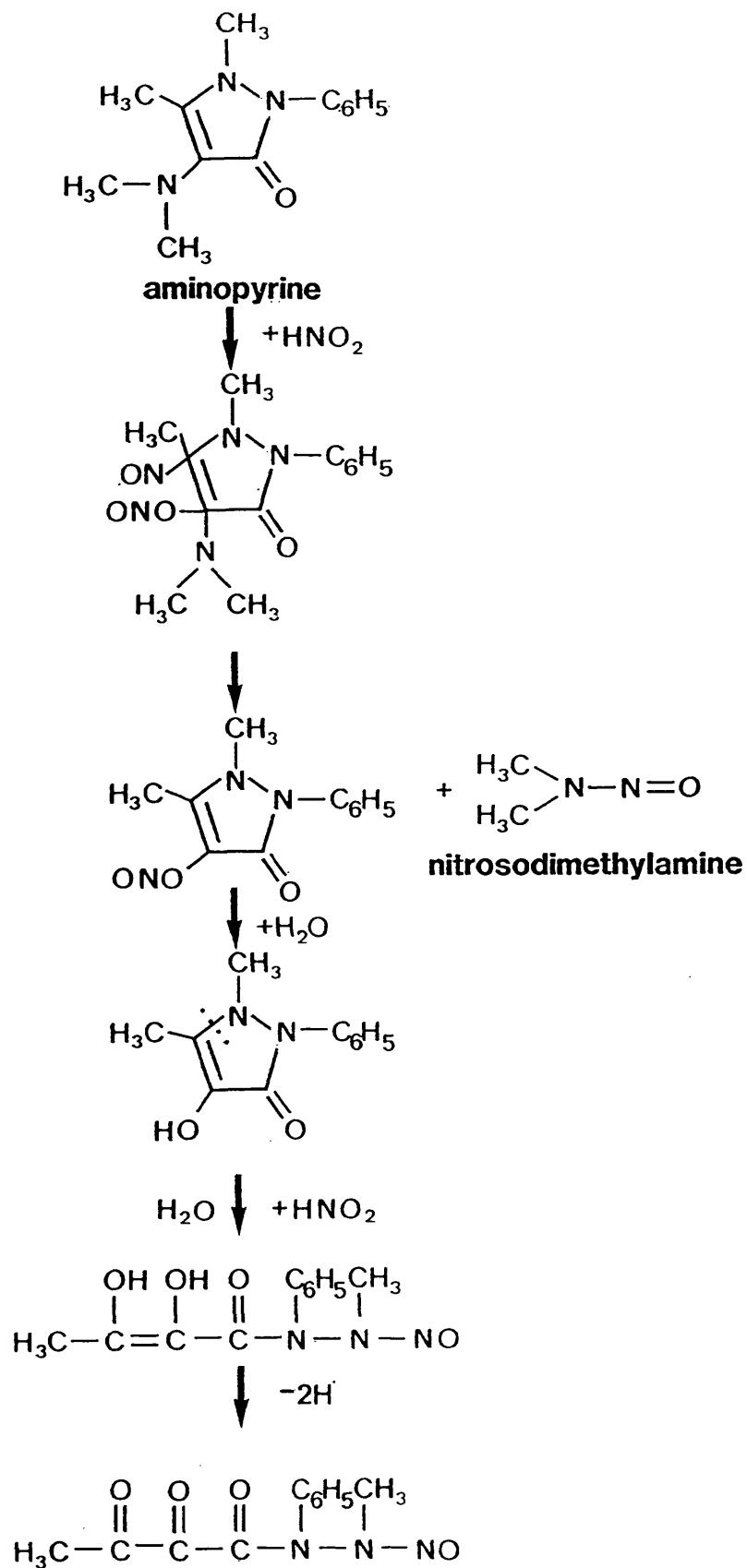
The gastro-intestinal flora can also hydrolyse hepatic conjugates formed with sulphonate^h, amino acids or acetic acid. Glycosides may also be hydrolysed in the gut and many plant glycosides which are ingested in the normal diet are hydrolysed in this way with, in certain cases, toxic effects, for example, when cycasin (the glycoside of methylazoxymethanol) or cyanogenic glycosides are ingested (Rowland & Walker 1983).

A further example of the ability of gastro-intestinal flora to metabolize non-toxic xenobiotics to reactive intermediates is the bacterial reduction of nitrate to nitrite, carried out primarily by the oral microflora (Spiegelhalder *et al* 1976) reducing nitrate recirculated in the saliva. It has been estimated that as much as 25% of ingested nitrate is recirculated into saliva with 20% of this salivary nitrate being reduced to nitrite (Spiegelhalder *et al* 1976)). Nitrate is a normal constituent of food, with vegetables

and drinking water being the principle sources. Nitrite is also present as a preservative in meat and other cured products. The consumption of dietary nitrate and nitrite intake is estimated to lie between 30 - 300mg per day for nitrate, but only 0.3 - 11mg of nitrite per day (ECETOC 1988).

Nitrite itself is not considered to be a carcinogen but it can combine with certain amino groups to yield carcinogenic or mutagenic N-nitroso compounds (nitrosamines or nitrosamides; Green *et al* 1982). This nitrosation reaction usually requires an acid environment (such as in the stomach) in which the nitrite is initially converted to nitrous acid and then converted to an active nitrosating species. The nitrosation of the tertiary amine, aminopyrine (until recently used commonly as an analgesic), as shown in Figure 3, produces the potent carcinogen nitrosodimethylamine, causing the induction of liver tumours in rodents (Taylor & Lijinsky 1975, Barale *et al* 1981). The occurrence of N-nitroso compounds in normal gastric contents has been reported (Mysliwy *et al* 1974) and is increased in the rat following colonization of the stomach by nitrate reductase-positive strains of bacteria (Hashimoto *et al* 1976). Furthermore, N-nitroso compounds detected in human blood are considered to be derived from gastric sources (Fine *et al* 1977, Kowalski *et al* 1980).

As with all nitrosamines, nitrosodimethylamine requires mammalian metabolism, primarily by the hepatic monooxygenase system, to generate its active form (Malling 1966, Gabridge & Legator 1969, review by Montesano & Bartsch 1976). The unstable electrophilic intermediates that result react with cellular constituents, although



1-diketobutyl-1-phenyl-2-methyl-2-nitrosohydrazide

Figure 3. The nitrosation of aminopyrine in acidic conditions
(Barale *et al* 1981)

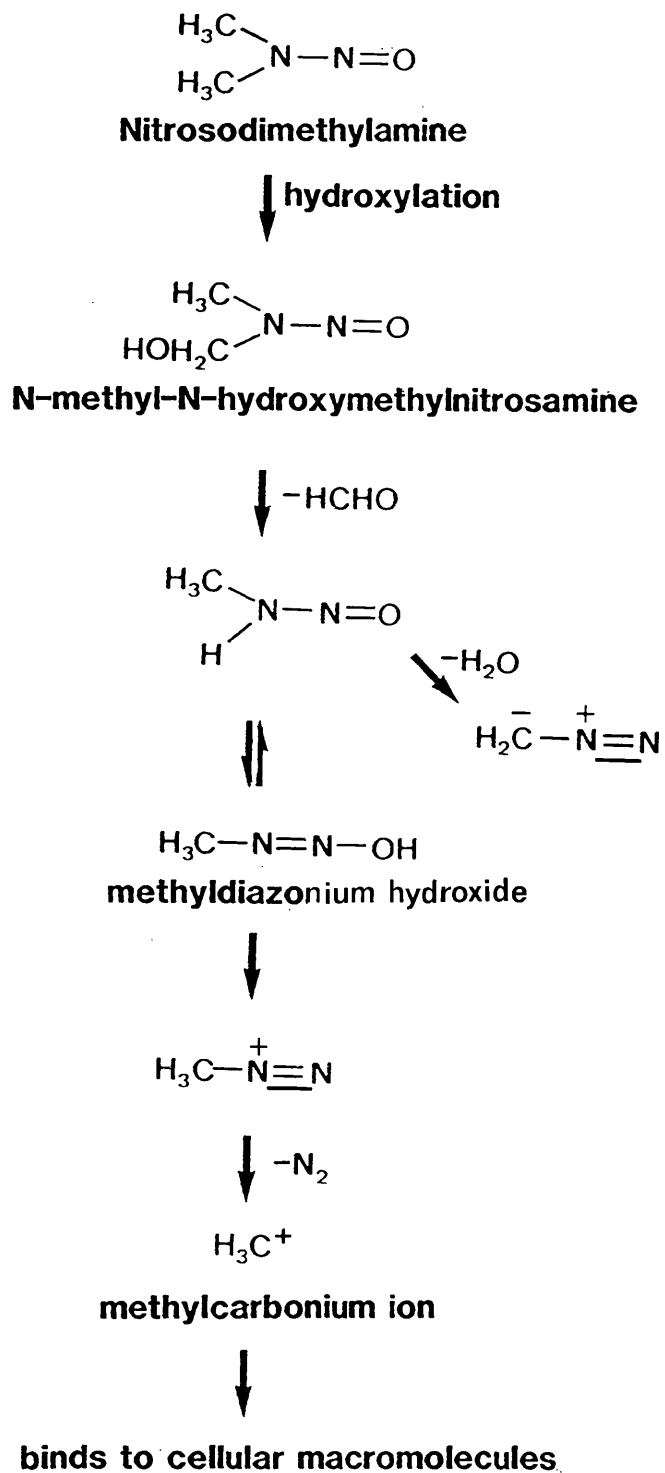


Figure 4. The activation of nitrosodimethylamine
(Lai & Arcos 1980)

it is the methylcarbonium ion (Fig. 4) that is thought to initiate cancer via DNA methylation (Magee & Barnes 1967, Lai & Arcos 1980, Pegg & Perry 1981).

The gut flora has a further crucial role in the metabolism of nitrocompounds. The reduction products of certain aromatic and heterocyclic nitrocompounds - used in both industry and medicine - have been found to be toxic, carcinogenic or mutagenic, as for example, the rat hepatocarcinogen, dinitrotoluene (Rowland *et al* 1985).

The presence of a gut flora can, therefore, have important implications for xenobiotic metabolism within the intestinal tract indicating that the gut flora may have an important role in carcinogenesis. Investigations into the effect of the gut flora on intestinal carcinogenesis were carried out by Laqueur *et al* (1967). They found that while cycasin was a potent intestinal carcinogen in conventional-flora rodents, in their germ-free counterparts, no tumours or hepatotoxicity were apparent. This was attributed to the lack of β -glucosidase in the germ-free animals. Even though this is the only example of a specific bacterial enzyme being clearly incriminated in the causation of tumours, the gut flora has been found, with certain intestinal carcinogens such as dimethylhydrazine, to have a tumour promoting effect (Reddy *et al* 1974) while with other carcinogens the presence of a gut flora has a protective effect, as with azoxymethane (Reddy *et al* 1975), N-methyl-N-nitro-N-nitrosoguanidine and N-methyl-N-nitrosourea (Balish *et al* 1977).

In addition to chemicals present in the intestine, the gut flora appears to have a role in the metabolism of carcinogens at other sites of the body, for instance, Roe & Grant (1970) found germ-free animals to have a lower incidence of dimethylbenz-anthracene-induced hepatic tumours than animals with a conventional flora. To investigate this phenomenon further, an *in vivo* investigation, reported in Chapter 5 of this thesis, was carried out examining the effect of germ-free status on the hepatic mutagenicity of compounds found in food.

3. Dietary Carcinogens.

The presence of carcinogens or their precursors in the diet is likely to be an important factor in the aetiology of human cancer. This group of compounds may arise from a number of sources, such as cooking, microbial degradation of food, artificially added chemicals or from chemical residues derived from pesticides and fungicides. Because of their possible role in the development of cancer, the carcinogens chosen for the present study were often selected from those found in the human diet. In particular two cooked food mutagens 2-amino-3,4-dimethylimidazo-[4,5-f]quinoline (MeIQ) and 3-amino-1-methyl-5H-pyrido[4,3-b]indole (Trp-P-2), representing the classes aminoimidazo azaarenes and aminocarbolines respectively, were used and the fungal metabolite aflatoxin B₁, which is a common contaminant of staple carbohydrate foods in tropical countries (Fig. 5).

a. Cooked-food mutagens.

It was first realized that cooked food could be a source of carcinogens when Lijinsky and Shubik (1964) discovered polycyclic

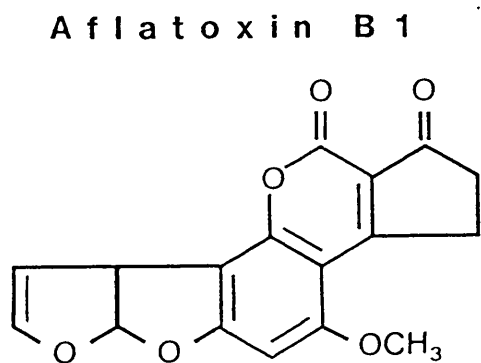
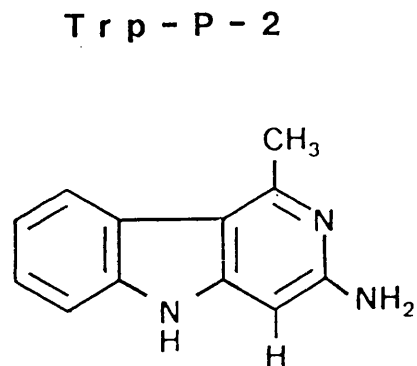
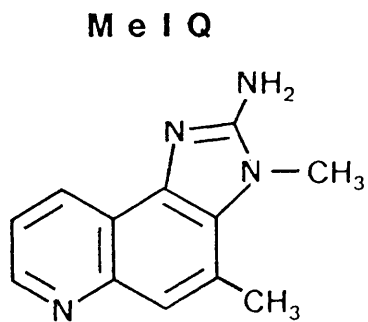


Figure 5 Three dietary carcinogens used in experiments presented in this thesis

aromatic hydrocarbons, such as benzo(a)pyrene, were combustion products of fat on grilled, smoked or fried meat or fish (Lijinsky & Ross 1957). More recently pyrolysis products of protein (heterocyclic aromatic amines) found in charcoal-grilled steaks and smoked food, were found to be highly mutagenic (Sugimura *et al* 1977, Nagao *et al* 1977). The most potent mutagenic activity was observed for pyrolysates of D-L tryptophan, namely 3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole (Trp-P-1) and 3-amino-1-methyl-5H-pyrido[4,3-b]indole (Trp-P-2) (Sugimura *et al* 1977, Kosuge *et al* 1978). However, these compounds formed by grilling fish and beef (Yamaizumi *et al* 1980, Yamaiguchi *et al* 1980) accounted for only a small proportion of the total mutagenic activity present and are only formed at very high temperatures not normally used in cooking. Further mutagenic compounds were isolated from beef (Commoner *et al* 1978) and sardines (Kasai *et al* 1979) cooked at temperatures below 200°C and identified as 2-amino-3-methylimidazo[4,5-f]quinoline (IQ), 2-amino-3,4-dimethylimidazo[4,5-f]quinoline (MeIQ) and 2-amino-3,8-dimethylimidazo[4,5-f] quinoxaline (MeIQx) (Kasai *et al* 1980a,b, 1981b). Sugimura (1985) estimated that the average intake of heterocyclic amines is around 100µg daily, although this may vary considerably depending on the diet of an individual.

Heterocyclic amines are formed via Maillard (non-enzymic browning) reactions (Spingarn & Garvie 1979, Powrie *et al* 1981, Shibamoto 1982). A pathway for the formation of the imidazoquinolines has been proposed by Jagerstad *et al* (1986) in which creatinine, obtained on heating creatine, forms the imidazole part of the molecule. The quinoline moiety is thought to arise primarily from a condensation reaction between pyrimidines (Maillard

reaction products) and aldehydes (formed via Strecker degradation of free amino acids). A number of heterocyclic amines have been shown to be carcinogenic in rodents producing tumours in a range of tissues, although the studies used very high doses of compound. For instance, in the study of Takayama *et al* (1984) 3 - 4mg of IQ were given each day for 35 weeks to rats. IQ and IQ type compounds have been found to be multipotent carcinogens, whereas the tryptophan pyrolysates tend to produce tumours in the liver only (Matsukura *et al* 1981, Hosaka *et al* 1981, Takayama *et al* 1985).

These compounds require metabolic activation to be mutagenic (Sugimura 1982, Felton *et al* 1986), with much evidence implicating the action of cytochrome P448 of the mixed function oxidase pathway (Ishii *et al* 1980a,b, Yamazoe *et al* 1983, Alldrick *et al* 1986, Watanabe *et al* 1982). The proposed pathway of Trp-P-2 activation is shown in Figure 6. The activation of IQ-like compounds by N-oxidation of the exocyclic amino group (Barnes *et al* 1985) involves cytochrome P448 mediated N-hydroxylation (Yamazoe *et al* 1983). This metabolite, like N-OH-Trp-P-2, is subject to further metabolism by enzymes present in the cytosol (Abu-Shakra *et al* 1986), with the ultimate mutagenic species being sulphate esters of N-hydroxides (Nagao *et al* 1983).

Turesky *et al* (1986) identified a non-mutagenic sulphamate derivative as the major biliary metabolite of IQ in rats suggesting therefore, that N-sulphation is a major contributor to the detoxification and elimination of IQ. Another route of detoxification for MeIQ and IQ may involve conjugation with glutathione (Loretz & Pariza 1984, Alldrick *et al* 1986). Although

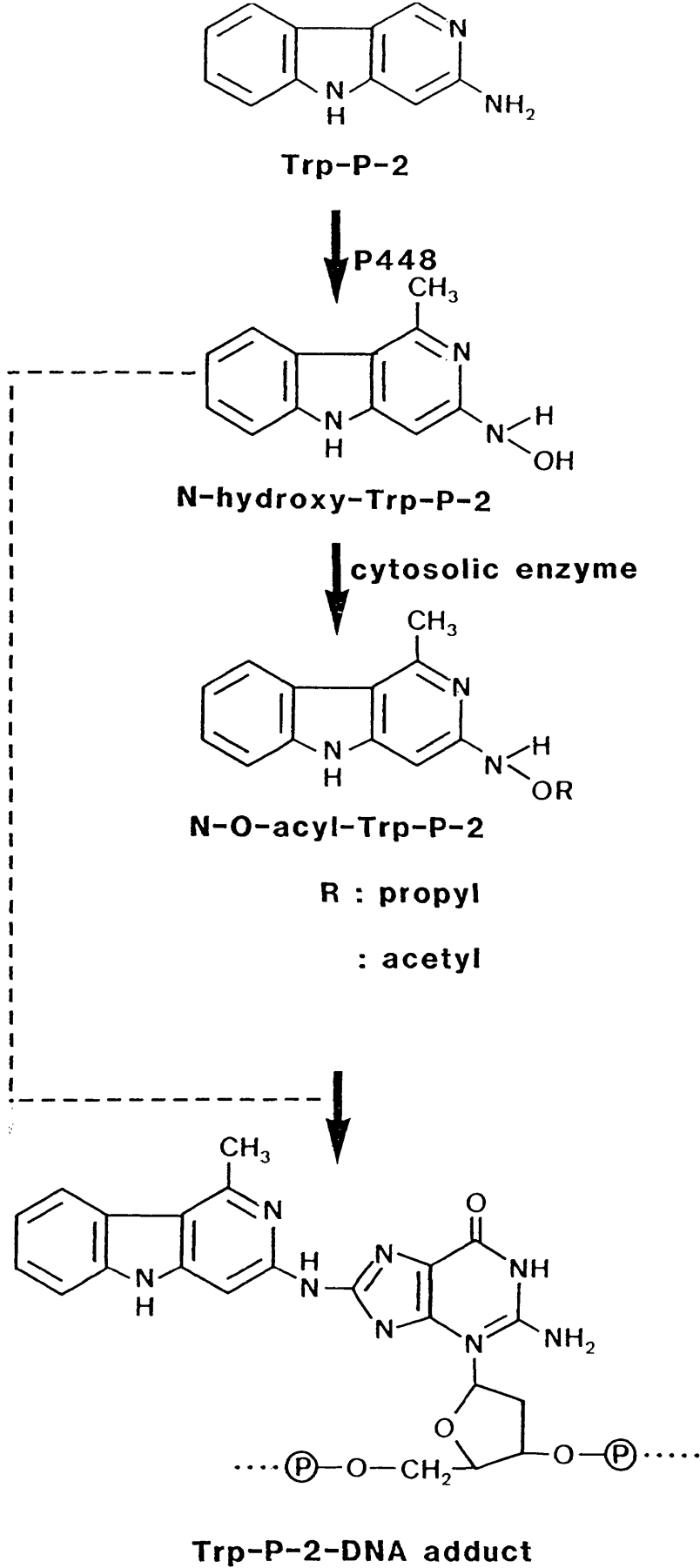


Figure 6. Pathway of metabolic activation of Trp-P-2

(Sato *et al* 1986)

conjugation of N-OH-Trp-P-2 with glutathione has been demonstrated, with the production of at least three different conjugates, one of these was more mutagenic than N-OH-Trp-P-2 (Saito *et al* 1984).

b. Aflatoxin B₁

Aflatoxin B₁ is a product of the fungus *Aspergillus flavus* which commonly contaminates peanuts and other staple carbohydrate foods stored in certain tropical climates and has been strongly associated with the presence of cancer of the liver in these countries (Peers & Linsell 1973, Peers *et al* 1976, review by Natl. Acad. Sci. 1982). Aflatoxin B₁ (Fig 5) is the most potent hepatocarcinogen known, although it can produce tumours in a number of other sites including the stomach (Butler & Barnes 1966), colon (Newberne & Rodgers 1973), kidney (Epstein *et al* 1969) and lung (Newberne *et al* 1967). The mutagenic properties of aflatoxin B₁ are dependent upon its vinyl ether double bond (Coles *et al* 1977) with the reactive metabolite being the epoxide, aflatoxin B₁-2,3-oxide (sometimes numbered 8,9; Garner *et al* 1972, Swenson *et al* 1977). This metabolite covalently binds to N⁷ of guanine in RNA and DNA (Lin *et al* 1977, Croy *et al* 1978). It has been proposed that this activation of aflatoxin B₁ is only a minor pathway in the total metabolism of this compound - the major pathways producing detoxification products (Fig. 7) with the exception perhaps of aflatoxicol, which is readily converted back to aflatoxin B₁, and may serve as a reservoir of aflatoxin since its formation may prolong cellular exposure to the carcinogen (Hsieh 1986).

There is considerable evidence to indicate that the metabolism of aflatoxin to its active form requires the cytochrome

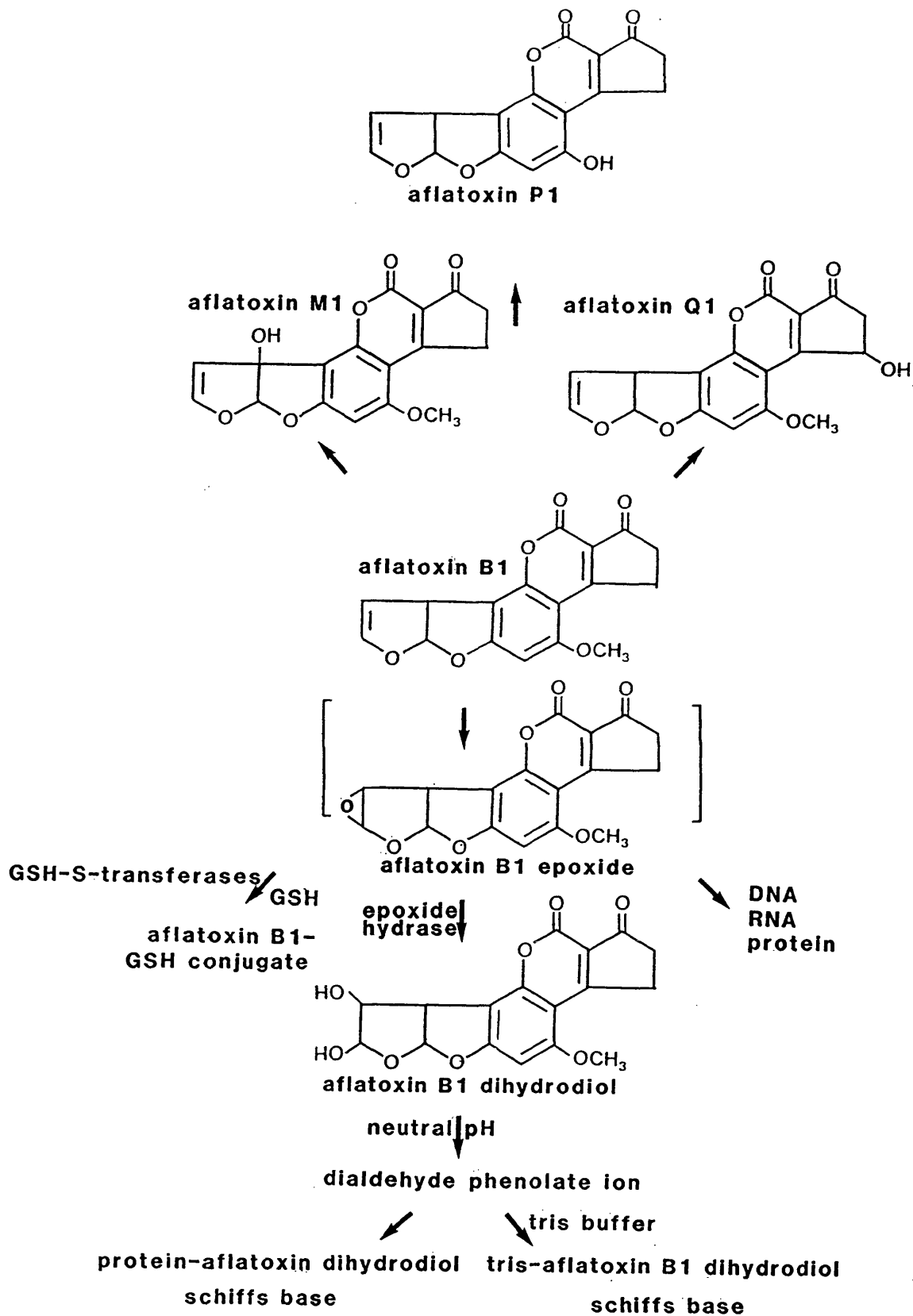


Figure 7. Pathways of metabolism of Aflatoxin B1
(Essigmann *et al* 1982)

P450-dependent mixed function oxidase system (Yoshizawa *et al* 1982, Swenson *et al* 1977, Ames *et al* 1973, Lin *et al* 1977) and several forms of cytochrome P450 appear to be involved, including some P448 species (Ishii *et al* 1986, Shimada *et al* 1987, Yoshizawa *et al* 1982).

The main detoxification pathway for the aflatoxin B₁-epoxide is conjugation to glutathione (Degan & Neumann 1978, 1981) mediated by glutathione transferase (Swenson *et al* 1975, Lotlikar *et al* 1980) although another possible detoxification pathway is hydration to the dihydrodiol followed by conjugation, at one of the hydroxyl groups, with glucuronide or sulphate.

In addition to forming adducts in nucleic acids or conjugation with glutathione, the epoxide may form dihydrodiols or hemiacetals. These may also be active via formation of dialdehyde phenolate intermediate which reacts with primary amines in protein to form a Schiff base as a biochemical lesion (Hsieh & Wong 1982).

It can be seen that dietary components such as cooked food mutagens or aflatoxin B₁ may have an important role in initiating cancer in man. However, other dietary components including nutrients may also have a role in carcinogenesis.

4. Dietary modification of cancer

a. Epidemiological evidence

A dietary component which is thought to have a marked role in altering the development of cancer is dietary fat. Demographic

studies have revealed a strong and consistent link between *per capita* intake of dietary fat and breast cancer incidence and mortality (review by Natl. Acad. Sci. 1982, Drasar & Irving 1973, Gray *et al* 1979). In addition, dietary fat has been associated with cancers of the colon (Drasar & Irving 1973), rectum (Jain *et al* 1980) and prostate (Armstrong & Doll 1975, Rose *et al* 1986). This association has also been supported by investigations of migrant populations, notably the Japanese, where incidence of breast and colon cancer has been found to be higher in the first and second generation of Japanese immigrants to Hawaii and California than in native Japanese living in Japan (Buell 1973, Haenzsal *et al* 1973). It has also become evident that in low risk countries undergoing westernization there has been a rise in breast cancer incidence (Reddy *et al* 1980, Hirayama 1979). A comparison of the levels of cancer between groups of people within a country has also implicated dietary fat as a major cause of colon and breast cancer. For example, Seventh Day Adventists, who restrict their meat intake and so lower their fat consumption, exhibit a lower incidence of breast and colon cancer compared to non-Adventists from the same area (Phillips 1975, Jensen 1983).

There is however, some dispute over the importance of particular aspects of dietary fat and their association with cancer. For example, ^{It is uncertain} whether the factor involved is animal fat, saturated ^{fat} unsaturated fat or ^{dietary} the total ^{fat} content. Furthermore, meat consumption - especially beef - is an important source of dietary fat and has also been linked with cancer of the colon and breast (review by Armstrong & Doll 1975, McKeown-Eyssen & Bright-See 1984). Meat is, of course, also a major dietary source

of protein, so it has not been surprising that epidemiological studies have revealed correlations between the incidence of, and mortality from, cancer of the breast and large bowel of various countries with *per capita* intakes of both total protein and animal protein (Armstrong & Doll 1975, Drasar & Irving 1973). However, it is difficult to distinguish the role of dietary protein and fat in the aetiology of cancer because of the very high association between fat and protein intake in western diets (Natl. Acad. Sci. 1982). In addition, total caloric intake has been linked to tumourigenesis (Kritchevsky 1986) but again the interpretation of this correlation is complicated by the altered distribution of specific nutrients such as fat, carbohydrate and fibre which may independently influence carcinogenesis (Natl. Acad. Sci. 1982).

It is perhaps noteworthy that a number of epidemiological investigations have failed to find a correlation with fat intake and cancers of the breast and colon (Bingham *et al* 1979, Lyon & Sorenson 1978, Smith *et al* 1985, Katsouyanni *et al* 1986, Mettlin 1984, Stemmermann *et al* 1984) or with meat consumption (Enstrom 1975, Graham *et al* 1978, Kinlen 1982). Many of these studies used data derived from a single country. It is possible therefore, that the diets were too uniform to permit associations between diet and disease to be detected (Reddy 1986). Differences in consumption of dietary fibre or other food items that may reduce cancer risk may also have contributed to these conflicting results. This was considered to be the case in a study by MacLennan *et al* (1978) in which the diets of two Scandinavian populations, who had differing risks for colon cancer, had a similar fat content but contained different amounts of fibre.

Dietary fibre has often been purported to have a protective role against cancer, notably cancer of the large bowel (Kritchevsky 1986). The results of epidemiological studies both support (IARC 1977, Modan *et al* 1975, Jenson *et al* 1982, Rozen *et al* 1981, Dales *et al* 1978) and conflict with this hypothesis (Jain *et al* 1980, Phillips 1975, Hill *et al* 1979). For example, Bingham *et al* (1979) found there to be no significant correlation between total fibre intake and corresponding mortality rates for colon and rectal cancers in different regions of Great Britain. However, when the effects of individual components of fibre were examined, there was an inverse correlation between colon cancer incidence and the consumption of the pentosan fraction of fibre (found in whole wheat products) suggesting that further epidemiological studies on fibre should concentrate on analysis of specific dietary fibre components.

Epidemiological investigations have played an important role in determining the association between diet and human cancer. However, these studies are limited in their interpretation as they are conducted on heterogenetic populations consuming complex and varied diets. Problems therefore arise in evaluating precise levels of exposure or in determining the role of specific dietary constituents. In order to provide further evidence to support hypotheses derived from epidemiological investigations on the role of diet and nutrition in the development of cancer, experimental studies on animals were carried out.

b. *Experimental evidence*

As early as 1942, Tannenbaum demonstrated that dietary fat enhanced the development of both chemically and spontaneously

induced mammary tumours in mice, thus supporting the results of epidemiological studies. Since then, numerous investigations (review by Weisburger 1986) have implicated high fat diets with an increase in breast or bowel tumour incidence in rodents induced by carcinogens such as 7,12-dimethylbenzanthracene (DMBA), N-nitroso-N-methylurea, (Dao & Chan 1983, Chan *et al* 1983), azoxymethane or 1,2-dimethylhydrazine (Bansal *et al* 1978). In addition to the quantity of fat consumed, the type of fat has also been shown to influence tumourigenesis. Generally the evidence suggests that unsaturated fat diets are more effective in increasing tumour yield than saturated fat (Carroll & Khor 1971, Reddy *et al* 1977, Newberne *et al* 1979, Longnecker *et al* 1981). This is particularly salient as at present there is an emphasis to reduce saturated fat consumption in western countries, and if necessary, replace it with unsaturated fat. To investigate this relationship further a series of *in vivo* and *in vitro* studies have been carried out (reported in Chapter 3 of this thesis), examining the effect of diets high in saturated or monounsaturated fat on the mutagenicity of potential dietary carcinogens.

An investigation into the effect of quantitative changes in dietary protein on the mutagenicity of carcinogens was also carried out (see Chapter 4). A number of studies have investigated the association between dietary protein and neoplasia in animals, although these have been fewer in number than ^{the studies carried out on fat.} The results indicate that dietary protein may indeed have a role in carcinogenesis with the amount of protein consumed being of particular importance. For instance, Silverstone and Tannenbaum (1951) found that a high protein diet increased the number of

spontaneous hepatomas in mice compared to a low protein diet. High protein diets have also been shown to enhance neoplasia induced by a variety of carcinogens including N-acetyl-2-aminofluorene (Morris *et al* 1948), 1,2-dimethylhydrazine (Topping & Visek 1976), 3-methylcholanthrene (Shay *et al* 1964) and aflatoxin B₁ (Madhavan & Gopalan 1968, Wells *et al* 1976, Temcharoen *et al* 1978), although with DMBA tumour incidence was reduced (Miller *et al* 1941). Furthermore, the relationship between dietary protein and cancer is complicated by studies that have shown levels of protein above 25% do not produce further enhancement and may even inhibit tumourigenesis (Ross & Bras 1973, Tannenbaum & Silverstone 1949, Topping & Visek 1976). Whether this is due to a reduced intake of food and total calories or whether due to other adverse effects, is not yet known (Natl. Acad. Sci. 1982).

Altering the type of protein can also affect neoplasia since an investigation by Nutter *et al* (1983) found increased tumour incidence in mice fed high milk protein diets compared to mice fed diets high in beef protein, although no difference was found when animal and plant protein were compared (Carroll 1975).

Unfortunately, many of the protein diets used in the above studies are questionable since, in certain cases, nutrients other than protein were also varied. As dietary protein may play an important role in carcinogenesis, the present study was carried out using short-term *in vivo* and *in vitro* mutagenicity experiments (as described in Chapter 4) using carefully prepared, balanced, purified diets (with lactalbumin as the protein source) to investigate the hypothesis that different quantities of dietary protein may

influence the activity of potential genotoxins.

As with the epidemiological data, the results of rodent experimental studies investigating the effect of dietary fibre on tumourigenesis, are inconsistent. The results appear to be dependent on a number of factors, such as type of animal species, the carcinogen given, route of administration and site of tumour development as well as the type of fibre consumed. For example, dimethylhydrazine-induced colon cancer was inhibited when rats were fed bran or cellulose (Barbolt & Abraham 1978, Wilson *et al* 1977) but bran had no effect on tumour incidence in the duodenum or caecum and cellulose was not protective when the carcinogen was azoxymethane or nitrosomethylurea (Ward *et al* 1973, Watanabe *et al* 1979).

From these results it is clear that fibre cannot be considered as a single dietary component. It appears that the influence of individual types of fibre on carcinogenesis depends on the extent to which they are fermented. Fibres that are poorly fermented, such as wheatbran, cellulose or lignin, inhibit tumour development (Fleischer *et al* 1978, Nigro *et al* 1979, Reddy *et al* 1983) whereas, the more soluble fermentable fibres including pectin, corn bran and guar gum have been shown to enhance carcinogenesis (Barnes *et al* 1983, Reddy *et al* 1983, Jacobs & Lupton 1986).

Both epidemiological and experimental data strongly suggest that carcinogenesis can be influenced by a variety of diet related factors. In order to gain a greater understanding of this relationship, the influence of diet, with particular reference to

fat, protein and fibre, on *in vivo* and *in vitro* mutagenesis has been investigated (Chapters 3, 4 and 5). Furthermore, the mechanisms involved in the modification of mutagenic activity by diet were examined.

c. Mechanisms involved in dietary modification of carcinogenesis

i. Promotion

It is thought that dietary fat may promote carcinogenesis since Carroll (1980) found increased incidence of mammary tumours when high fat diets were fed after, but not before, DMBA administration. Similar results were obtained by Bull *et al* (1979) with azoxymethane-induced bowel tumours and by de Gerlache *et al* (1987) in hepatocarcinogenesis. However, polyunsaturated fat seemed to effect aflatoxin B₁-induced hepatic tumour formation during both initiation and promotion (Newberne *et al* 1979). Promotion of cancer by dietary fat may be due to an increase in bile acid excretion (bile acids being the primary liver metabolites of cholesterol). An increase in the amount of bile acids has been detected in stools of populations eating a western-style diet compared to populations consuming typical African or Asian diets (Hill *et al* 1971, Reddy & Wynder 1973) and has also been detected in colon cancer patients compared to their healthy counterparts (Hill *et al* 1975, Reddy & Wynder 1977). Increased bile acid excretion together with an increase in azoxymethane-, dimethylhydrazine- and nitrosomethylurea-induced colon tumourigenesis was also apparent in rodents fed either high-fat diets or cholestyramine - a non absorbable resin that increases bile salt excretion (Reddy & Maeura 1984). Promotion of carcinogenesis may also occur through other means such as solvation, by altering the rate of carcinogen transfer

to the large bowel (Vahouny & Kay 1981) or by modulating endocrine balances as has been postulated for breast, ovarian and endometrial cancer (Weisberger 1986).

The stage of carcinogenesis on which dietary protein has the greatest impact is not yet known. Preston *et al* (1976) found aflatoxin B₁ adduct formation was reduced in rodents fed low protein diets, suggesting an involvement of dietary protein at the initiating stage of carcinogenesis. Yet a low protein diet reduced the post-initiation development of both aflatoxin B₁-induced preneoplastic liver lesions (Appleton & Campbell 1982) and neoplastic foci (Appleton & Campbell 1983). This may be due to changes in the proliferative activity of cells, since inhibition of cell replication occurs in the liver of animals with a restricted protein intake (Dallman 1971, Enwonwu & Monro 1970). However, dietary protein may modify other important functions such as immune status and proteolytic enzyme activity (Appleton & Campbell 1983). In the present study however, the role of diet as a promotor has not been considered. The main emphasis has been the effect of diet on the activation of carcinogens.

ii. Activation and detoxification.

Many dietary factors are known to induce specific cytochrome P450 isoenzymes or cause selective inactivation of individual P450 isoenzymes resulting in concomitant changes in the metabolism of certain xenobiotics and alteration of the relative importance of competing metabolic pathways.

Studies indicate ^{that} polyunsaturated fats or essential fatty

acids, such as linoleic acid, vitamin E or cholesterol, are involved in increased metabolism of xenobiotics (review by Natl. Acad. Sci. 1982). Dietary fat, in particular unsaturated fat, has been shown to alter the structure of the microsomal membrane (Lang 1976, Bidlack *et al* 1986) and a lack of essential fatty acids in the diet causes microsomal membranes to become more saturated (Bidlack *et al* 1986).

Dietary protein also appears to affect the cytochrome P450-dependent monooxygenase system. In humans consuming a low protein diet, a lower metabolic clearance of aminopyrine and theophylline was observed (Anderson *et al* 1982, Mucklow *et al* 1980, Kappas *et al* 1976) and in animals the microsomal cytochrome P450 content and cytochrome P450 reductase activity was reduced together with a decline in the mixed function oxidase activities towards a number of substrates when the quality or quantity of protein was reduced (Campbell & Hayes 1976, review by Meydani 1987).

Less information is available on the effect of diet on glutathione and glutathione-transferases. Norred and Marzuki (1984) have demonstrated that diets devoid of or low in polyunsaturated fat and high in saturated fat produced a lower level of glutathione-transferase activity in rat hepatocytes. Clinton *et al* (1984) found no differences in glutathione transferases in high unsaturated and saturated fat diets, although these activities were induced by phenobarbitone when the rats were fed a source of polyunsaturated fatty acids (Wade *et al* 1978). Sulphur amino acids of dietary protein participate in the biosynthesis of glutathione and it was found that reducing dietary protein intake reduced the

concentration of hepatic glutathione (Mainigi & Campbell 1981).

iii. Formation of carcinogens by the gut flora.

The amount and type of nutrients present in the gut is obviously dependent upon diet. Changes in the nutrient content can change the composition and metabolic activity of the gut microbial community. On the whole it would appear that changes to either the protein or the fat content of the human diet do not affect, to any great extent, the composition of faecal flora (Rowland *et al* 1985). However, examination of the metabolic activity of the human faecal flora has indicated that certain bacterial enzyme activities increase, namely β -glucuronidase, nitroreductase and azoreductase, when a high meat containing diet is consumed (Reddy *et al* 1978, Reddy & Wynder 1973, Goldin *et al* 1978) but this is offset when the consumption of dietary fibre is raised. Similarly, experimental studies have shown that rodents exhibit altered microbial metabolic activities in response to both changes in dietary fat and meat (Rowland *et al* 1985). In general feeding high fat diets reduced nitrate reductase activity and when the fat used was beef dripping, an increase in β -glucuronidase activity was observed.

Marked changes in the metabolic activity of the rat hindgut flora have also been reported with changes in dietary fibre. Bauer *et al* (1979) found that the faecal β -glucuronidase activity in rats was increased when they were fed a diet supplemented in citrus pectin. This increase was associated with a significant rise in dimethylhydrazine-induced colo-rectal tumours, indicating that glucuronide deconjugation released an active carcinogen within the gut. Similar increases in β -glucuronidase activity have been

observed for rats fed 5 - 10% pectin by Rowland *et al* (1983a) and DeBethizy *et al* (1983). Such changes in glucuronide hydrolysis may increase enterohepatic circulation and explain the observation of Chadwick *et al* (1978) that 10% citrus pectin increased the excretion of radiolabelled products and the concentration of label in hepatic tissue of rats treated with [¹⁴C] lindane.

Dietary pectin with a high methoxyl content, has also been reported to increase nitrate reductase activity of rat gut flora. Pectin, a fermentable fibre, consists mainly of polymerized galacturonic acid with additional rhamnose, arabinose and galactose subunits (Rowland *et al* 1985). The uronic acid backbone can further react to varying extents with metal hydroxides or cations, or ^{be}esterified with methoxyl groups, resulting in a variety of different structures which can affect the microbial communities of the gut in different ways (Rowland *et al* 1985). The pectin related increase in nitrate reductase activity could lead to an increase in the *in vivo* production of nitrite. The bacterial reduction of nitrate to nitrite is also associated with nitrosamine formation as described above (see section 2b). Anaerobic incubation of caecal contents from rats fed a 5% (high methoxyl) pectin diet generates significant amounts of N-nitrosoproline from nitrate and [¹⁴C] proline than incubations containing caecal contents from rats fed a fibre-free diet (Mallett *et al* 1982). In addition, caecal contents from pectin-fed animals also show increased production of Ames Test positive mutagens (presumably nitrosodimethylamine) when incubated with dimethylamine and nitrate (Mallett *et al* 1982). Work presented in Chapter 5 of this thesis investigates the effect of dietary pectin on nitrosation *in vivo* by examining formation of

nitrosodimethylamine from aminopyrine and nitrite.

As well as being a source of nutrients for the gut flora, dietary fibre may have a number of other effects on the gastro-intestinal environment. For instance, it may act as a bulking agent causing the bacteria to be diluted and so decreasing the total bacterial activity of the gut contents, or the concentration of carcinogens or promoters present in the colon (Reddy *et al* 1978). Dietary fibre can alter the digestion and absorption of some nutrients which may lead to changes in the amount of non-fibrous substrates available to the bacteria. Furthermore, fibre may affect production of the intestinal mucosa and mucus which again can influence the bacterial population of the gastro-intestinal tract (Brown *et al* 1979).

5. Age and cancer.

The incidence of many types of cancer, in particular common epithelial tumours such as lung cancer, increases progressively with age. Numerous hypotheses exist in explaining age-related increases in tumour incidence, although in general they fall into two categories (Anisimov 1985). The first attributes the relationship to an accumulation of carcinogenic reactions with age and/or an increasing length of exposure, whereas the second postulates that age-related changes in the human organism increases the probability of tumour appearance and promotes tumour growth and progression (Anisimov 1985).

In experimental studies with rodents, the age at which animals are exposed to carcinogens has a marked effect on tumour incidence.

It has been found that animals treated as neonates or infants develop significantly more tumours within a shorter period of time than animals treated as young adults. This has been shown to be the case for hepatic tumours in mice induced by a range of N-nitroso compounds such as N-nitrosodiethylamine and N-nitrosoethylurea (Vesselinovitch *et al* 1974, 1984) and for nitrosodimethylamine- and nitrosodiethylamine-treated rats (Hard 1979, Peto *et al* 1984). In the latter study, it was reported that when nitrosodiethylamine treatment began at 3 weeks of age, the liver tumour incidence rate was six times higher than when treatment began at 20 weeks. The authors concluded that temporary factors existed in very young animals which greatly enhance the sensitivity of the liver to nitrosodiethylamine. Similar age-related differences were observed in mammary tumour development in rats treated with DMBA (Nagasawa *et al* 1976, Sinha & Dao 1980), 3-methylcholanthrene (Huggins *et al* 1961) or nitrosomethylurea (Grubbs *et al* 1983). However, this increase in young animals was not observed for nitrosodiethylamine-induced oesophageal tumourigenesis (Peto *et al* 1984).

Not only does age influence the incidence of tumours, it can also affect the type of tumour induced. Hard (1979) reported a bimodal distribution of renal tumour incidence with respect to age in rats given nitrosodimethylamine. This distribution was the consequence of the induction of two types of kidney tumours. Mesenchymal tumours were characteristic of animals exposed to nitrosodimethylamine during the neonatal and immature periods, with the greatest susceptibility to tumours occurring in 1 month old rats. By contrast, tumours induced by nitrosodimethylamine in

older (2 - 4 months) rats were generally in the cortical epithelium.

Marked age-related changes have also been reported in the activities of the cytochrome P450 mixed function oxidase system (Short *et al* 1976) which may, as a consequence, alter the disposition of toxic or carcinogenic substrates *in vivo*. In the foetus and the neonate, the haemoprotein and flavoprotein components of the mixed function oxidase are initially absent or very low but generally increase together with their respective activities during the immediate perinatal period (Neims *et al* 1976). However, the various isoenzymes which comprise the cytochrome P450 exhibit different developmental patterns (Klinger 1982, Mannering 1985). For example, the hydroxylation of 4-methylcoumarin in rats reaches a peak 3 to 5 days post-partum and then declines to adult levels (Feuer & Liscio 1970), while the metabolism of other substrates are maximal 2 weeks (Gram *et al* 1969), 1 month (Basu *et al* 1974) or 6 months (Kamataki *et al* 1985, Kaur & Gill 1985) after birth. Certain mixed function oxidase activities stabilise in adult animals and give approximately constant values at 1 or 2 years of age (Koizumi *et al* 1987) while the expression of other functions decreases in adult or geriatric animals (Short *et al* 1976, Kamataki *et al* 1985). A coherent pattern for the development of cytochrome P450-associated pathways is clearly lacking and may reflect the influence of diet (Vesell 1984, Bidlack *et al* 1986), hormones (Kamataki *et al* 1985, Bandiera *et al* 1986), species and strain differences on enzyme synthesis and expression.

A large number of carcinogens and mutagens are dependent on mammalian xenobiotic-metabolising enzymes for their genotoxic

activity, which may therefore exhibit age-related changes, as demonstrated by Raineri *et al* (1986). For example, the activation of 3-methylcholanthrene or benzo(a)pyrene by aroclor-induced rat hepatic S9, and activation of N-nitrosopyrrolidine by male hamster preparations, decreased with increasing donor age (Raineri *et al* 1986). This dependence may in turn lead to differential susceptibility of animals at various stages of their development to the mutagenic or carcinogenic effects of such compounds.

Electrophilic intermediates of xenobiotics produced by the mixed function oxidase system can be metabolized by conjugation to glutathione. No difference in the hepatic glutathione content has been found between neonatal and adult mice or rats (Stohs *et al* 1982, James & Pheasant 1978) but marked age-related changes can be observed in the activities of hepatic glutathione transferases, the group of enzymes which catalyse the conjugation with glutathione (Gregus *et al* 1985). Generally, hepatic glutathione transferase activities in mice and rats increase from a basal neonatal level and reach adult levels by 5 weeks of age but the rate of increase varies between the different transferases, as reflected by the substrate used (Klinger 1982, Henderson 1978). As described above (see sections 3a & 3b) glutathione and glutathione transferases are thought to provide an important detoxification pathway for aflatoxin B₁ (Coles *et al* 1985) and are also considered to have a role in heterocyclic amine metabolism.

It has previously been described in section 4ciii of this chapter, how the activities of intestinal flora are sensitive to dietary modification and the possible effect this may have on

carcinogenesis. It was considered, therefore, that age-related changes to gut flora enzyme activities would also influence tumourigenesis. At birth, the mammalian gut is sterile but rapidly becomes colonised by a wide variety of organisms derived from the mother's skin, vagina and faeces as well as from air and food (Schaedler *et al* 1965). Many of these bacteria are unable to colonize the neonatal gastro-intestinal tract and so appear only transiently, however others can colonise ecological niches in the gut and eventually form the climax community of the adult (Savage 1981). Such communities are formed through succession (Savage 1977). For instance, in suckling mice, *Lactobacilli* appear throughout the gut a few days after birth (Schaedler *et al* 1965). Populations of facultative anaerobes, such as *Escherichia coli* and *Streptococci* sp. also develop and reach high levels prior to weaning (Savage 1977). During weaning, when the animals begin to sample solid food, a major change in population occurs and strictly anaerobic bacteria begin to colonise the large intestine (Lee & Gemmell 1972) and quickly increase in numbers. As the numbers of the strict anaerobes rise there is a concomitant decline in the population of facultative anaerobes (Lee & Gemmell 1972, Schaedler *et al* 1965). Similar successions of intestinal bacterial populations occur in other animals including man (Morishita & Miyaki 1979, Bullen *et al* 1977, Tomkins *et al* 1981, Mata & Urrutia 1971).

In view of these major changes in bacterial composition that occur in the developing mammal, it seems likely that concomitant changes occur in the metabolic activity of the microflora. Although this has not been systematically studied, age-related differences have been reported for the bacterial metabolism of bile

acids and cholesterol (Huang *et al* 1981) with infants excreting much lower amounts of microbially-modified steroids in faeces than adults. Goldin *et al* (1978) found that caecal microbial β -glucuronidase activity was greater in older rats than in young. It has also been demonstrated in mice and humans that the rate of demethylation of methylmercury by the gut flora increases at the time of weaning (Rowland *et al* 1983b). Such changes in bacterial enzyme activities can lead to alterations in the metabolism of ingested compounds and, in turn, to differential susceptibility of adult and infant to the toxic effects of such compounds.

Developmental changes in three bacterial enzymes associated with the caecal flora of mice over the period 2 to 24 weeks after birth have been investigated during this project (Chapter 6). Since the developing gut flora is in a state of flux, it is possible that it is more sensitive to modification by diet. Consequently the sensitivity of the gut flora of mice at different ages to modification by pectin was also studied.

6. Methodology.

To investigate the effects of age and diet on the hepatic metabolism of carcinogens to mutagenic species, the *Salmonella* mutagenicity assay and the host-mediated assay were employed. The *Salmonella* mutagenicity assay (the Ames Test; Ames *et al* 1973) was developed, and currently used, as a simple and rapid screening test to detect potential carcinogens. The assay consists of histidine auxotrophs of *Salmonella typhimurium*, test substance and a representative fraction of mammalian metabolism (for example, liver homogenate) being poured, together with top agar, on to agar

selective for histidine revertants (Ames *et al* 1975). The *Salmonella* strains are partly deficient in their lipopolysaccharide layer through an *rfa* mutation which results in increased cell membrane permeability to chemicals of large molecular weight (Ames *et al* 1973). To increase the strains' sensitivity to certain mutagens DNA error prone repair deficiency has been introduced mediated by the mutation plasmid pkM101 (McCann *et al* 1975). A further modification of this assay, which can again improve its sensitivity (and used in the present study), is a liquid preincubation in which the various components are incubated at 37°C for 30 minutes prior to pouring on to the selective agar.

A disadvantage of the Ames test, however, is that it is unable to simulate accurately the whole range of metabolic pathways present in the intact animal. The use of liver homogenates places emphasis on activating reactions but disregards tissue distribution, extrahepatic activation and detoxification. The host-mediated assay was one test developed to overcome this problem and provided the first immediate proof that some mutagens, such as nitrosodimethylamine, require metabolic activation by mammals before exerting their effect.

Initially an intraperitoneal host-mediated assay was performed by Gabridge and Legator in 1969, but administration of the indicator organism by this method resulted in the assay being relatively insensitive. Intravenous administration of bacteria is now preferred, since this method has the advantage of a large fraction of the bacteria being retained in the liver - the main site of xenobiotic metabolism - thereby increasing the chance that

short-lived reactive metabolites reach the target bacteria (Glatt *et al* 1985). A further advantage of the host-mediated assay is that the carcinogen can be administered via a number of routes, including *per os*, intravenously and subcutaneously, which can provide a greater understanding of the metabolism of the compound in question.

Stationary phase, rough-walled bacteria, such as *Escherichia coli* and *Salmonella typhimurium*, are often but not solely, used in the host-mediated assay since they are small enough to get through fine blood capillaries after intravenous injection, although other indicator cell types include yeast (Frezza *et al* 1979), *Neurospora* (Whong & Ong 1979), bacteriophage (Mohn *et al* 1977) and mammalian cells (Capizzi *et al* 1974, Hsie *et al* 1978). Consequently, a number of genetic endpoints are available including forward mutation markers such as antibiotic resistance in *E. coli* D494 which has the additional advantage of reducing host-dependent plate contamination (Neale & Solt 1980), backward mutation, mitotic gene conversion and recombination, recessive lethal mutations and chromosomal aberrations (Legator *et al* 1982).

In 1984 Mohn developed a DNA-repair host-mediated assay. This assay compares the survival of DNA repair-proficient and repair-deficient (*uvrB*, *recA*) *E. coli* strains injected in control and treated animals. Mohn & van Zeeland (1985) considered this assay to have several advantages over gene mutation tests since the target size for this former endpoint (lethal damage if not repaired) is much larger than for mutations at specific loci, encompassing a large number of DNA alterations. Furthermore, in this type of host-mediated assay, the phenotypic expression of lethality is

immediate and only a few hundred cells are required to perform the assay. In order to assess the differential bacterial survival Mohn (1984) rendered the strains different in their ability to ferment lactose, so that survival of both strains can be determined on a single agar medium containing lactose and neutral red as a pH indicator. Finally, streptomycin-dependent mutations were introduced to prevent uncontrolled growth of the bacterial cells within the various organs and to avoid contamination of the agar medium (Mohn 1984).

In the present study, three host-mediated assays were used - the DNA-repair, ampicillin resistance in *E. coli* and finally, a host-mediated assay using *Salmonella typhimurium* TA98 developed by Arni *et al* (1977). An advantage of the latter host-mediated assay is that the results can be compared with those from the *in vitro* *Salmonella* mutagenicity assay. Several experiments, as detailed in Chapter 7, were also carried out to try and further improve the sensitivity of the host-mediated assay, in particular the DNA-repair host-mediated assay.

In addition to bacterial mutation tests, diet- and age-related changes to other aspects of hepatic and extrahepatic xenobiotic metabolism were investigated, including measurements of cytochrome P450, mixed function oxidase activities, glutathione content and glutathione-transferase activities and alterations ^{the metabolism of the} to gut microflora.

The primary aim of these experiments was to obtain a more complete ^p view of the effects of major dietary components (fat, protein and fibre) as well as host-age, on carcinogen metabolism.

CHAPTER 2

Materials and Methods

1. Chemicals.

All chemicals were AR grade and stored at room temperature unless otherwise stated. The main suppliers were BDH Chemicals, Poole, UK; Fisons Scientific Apparatus, Loughborough, UK and Sigma Chemical Company Ltd., Poole, UK.

2. Mutagens.

MeIQ (MW: 212.25) and IQ (MW: 198.22) were generous gifts from Prof. K. Olsson and Dr S. Grivas, Swedish University of Agricultural Science, Uppsala, Sweden and gave single peaks when subjected to HPLC on a Waters C18 μ -Bondapack column (personal communication A. Abu-Shakra). Trp-P-2 was purchased in its acetate form (MW: 257.28) from Wako Chemicals GmbH, Neuss, FRG and migrated as a single spot on silica gel G25 (solvent system: isobutanol-acetic acid-water, 80:10:10 by volume). Aflatoxin B₁ (MW: 312.30) was obtained from Sigma Chemical Co., Poole, UK. These four mutagens were stored at -20°C and dissolved to the required concentrations in pure dimethylsulphoxide (DMSO). Radioactively labelled mutagens [2-¹⁴C]MeIQ (specific activity, 10mCi/mmol) and [3-¹⁴C]Trp-P-2 (specific activity, 10mCi/mmol) were purchased from Toronto Research Chemicals Inc., Ontario, Canada and stored frozen in DMSO at a concentration of 2.13mg/ml, 100 μ Ci/ml for MeIQ, or 2.57mg/ml, 100 μ Ci/ml for Trp-P-2. [U-³H]Aflatoxin B₁ (specific activity, 10Ci/mmol) was kindly given by Dr G. Neal, Medical Research Council (MRC), Carshalton, Surrey, and stored frozen at -20°C in 100%

methanol at a concentration of 15.62 μ g/ml, 500 μ Ci/ml.

Dimethylaminoantipyrine (aminopyrine; MW: 231.30) was purchased from Sigma Chemical Co., and stored desiccated in the dark, at room temperature and dissolved in 0.9% saline to the required concentration immediately prior to use.

Nitrosodimethylamine (MW: 74.08) was obtained from Eastman Organic Chemicals, Rochester, NY, USA, stored desiccated in the dark at 4°C and diluted in saline to the concentration required just before use.

Nitrosomethylurea (MW: 103.10) was purchased from Schuhardt, Germany, recrystallized by Dr P. Swann of this medical school and stored desiccated in the dark at -20°C. Stock solutions of nitrosomethylurea were prepared in 0.9% saline pH5 at 1.04 mg/ml (10mM) and stored in covered bottles at -20°C. Individual bottles were thawed once only, immediately prior to use.

Hycanthon (MW: 452.6) was purchased as the mesylate form (Etrenol) from Winthrop Inc., UK, stored at room temperature in the dark and dissolved in saline to the required concentration immediately prior to use.

3. Animals.

Female BALB/c inbred mice, aged 3 weeks were purchased from Harlan Olac (1976) Ltd., Bicester, Oxon., kept in grid-bottomed cages and fed on the appropriate diets until use (usually at 7 - 8

weeks of age). To change the diet of newly purchased mice from a stock commercial diet to a purified diet, as described below, will affect the amount and type of nutrients present in the gastro-intestinal tract and hence, may alter the composition of the gut flora. Mice were, therefore, maintained on experimental diets for a period of at least 3 weeks, to ensure that the microbial flora had stabilized (Mallett *et al* 1983).

For the study investigating the effect of age on hepatic and gut flora metabolism in mice (Chapter 6), pregnant female inbred BALB/c mice were obtained, from Harlan Olac Ltd., approximately 3 weeks after mating and fed a stock breeding diet (Rat and Mouse Diet No. 3, Special Diet Services, Witham). After giving birth, the mothers were transferred onto a purified, fibre-free diet (Table 1) and the offspring were weaned onto this or a 5% pectin diet (Table 1) at 3 weeks of age and maintained on these diets until the time of sacrifice.

Germ-free female BALB/c mice were born and maintained germ-free in plastic film isolators at the MRC Toxicology Unit, Carshalton, Surrey. The mice were given sterile tap water and fed a sterile fibre-free diet (Table 1) from weaning, when 3 weeks old, until use at 7 - 8 weeks of age.

Food and water were provided *ad libitum* to all animals.

4. Diets.

Olive oil was purchased from Boots plc, Nottingham. Citrus fruit pectin with an 8% methoxyl content, α -cellulose, safflower oil and lactalbumin were purchased from Sigma Chemical Co., Poole, UK. Beef dripping (food grade) and corn oil were obtained from a local retailer.

The diets were prepared, (at BIBRA, as detailed in Tables 1 - 4), so that nutrients were supplied in the proportions recommended by the National Research Council (1978). Diets containing different amounts of pectin were prepared by supplementing a fibre-free diet with 0.5% (w/w), 2% (w/w) or 5% (w/w) pectin as specified in Table 1.

High fat diets were prepared by substituting the maize starch in a 1% safflower oil diet (low fat) with either olive oil, beef dripping or safflower oil until the required fat content was achieved (Table 2). The remaining nutritional components were adjusted so that the diets had similar protein, vitamin and mineral densities i.e. the ratio of nutrient mass to total energy available was constant for all diets. As a result of problems in feeding the unsaturated oil diets, due to their liquid consistency, α -cellulose (10% w/w) was included in later batches of diet as a solidifying agent (Table 2).

Diets deficient in protein were obtained by replacing the protein component, lactalbumin, of a 20% (w/w) protein diet with maize starch (Table 3). Lactalbumin contains 4% lactose, so additional quantities of lactose were added to the diets deficient

Table 1 Composition of fibre-free
and pectin containing diets

| Diet | Ingredient | Amount (g/kg) |
|-------------|--------------------------|----------------------------------|
| Fibre-free | maize starch | 755 |
| | casein | 150 |
| | mineral mix ¹ | 35 |
| | vitamin mix ¹ | 10 |
| | corn oil ² | 50 |
| | | Amount (g/kg fibre-free diet) |
| 0.5% pectin | citrus fruit pectin | 5 |
| 2% pectin | citrus fruit pectin | 21 |
| 5% pectin | citrus fruit pectin | 53 |

From Wise *et al* (1982).

¹ see Table 4 for composition.

² contained 1g/l dl- α -tocopheryl acetate. This was increased to 3g/l when fed to germ-free mice.

Table 2 Composition of fat diets

| Ingredient | Quantity (g/kg diet) | | | |
|---|----------------------|-----------------|-----------------|-------------------|
| | Low fat 1 | 35% Fat diet | Low fat 2 | 25% Fat diet |
| casein | 150 | 216 | 200 | 270 |
| maize starch | 795 | 355 | 645 | 306 |
| mineral mix ¹ | 35 | 51 | 35 | 47 |
| vitamin mix ¹ | 10 | 14 | 10 | 13.5 |
| safflower oil | 10 ³ | 14 ³ | 10 ² | 13.5 ² |
| α -cellulose | - | - | 100 | 100 |
| fat (beef dripping, olive oil or safflower oil) - | | 350 | - | 250 |

Initially, 35% fat diets were used and compared to diets containing only 1% fat (Recipes: Low fat 1 & 35% Fat diet; Mallett *et al* 1984). In order to increase the solidity of the high unsaturated fat diets, 10% cellulose was added to the diets and compared to an appropriate low fat diet (Recipes: Low fat 2 & 25% Fat diets).

¹ see Table 4 for composition.

² contained 5g/l dl- α -tocopheryl acetate.

³ contained 3g/l dl- α -tocopheryl acetate.

Table 3 Composition of protein diets

| Ingredient | Quantity (g/kg diet) | | | |
|--|---------------------------|------|-------|------|
| | Lactalbumin content (w/w) | | | |
| | 5% | 7.5% | 12.5% | 20% |
| lactalbumin | 50 | 75 | 125 | 200 |
| maize starch | 849 | 824 | 774 | 699 |
| lactose | 2 | 1.8 | 0.8 | - |
| L-methionine | 0.8 | 1.2 | 2.0 | 3.2 |
| vitamin mix ¹ | 10 | 10 | 10 | 10 |
| mineral mix ¹ | 0.4 | 0.4 | 0.4 | 0.4 |
| mineral mix ² supplement | 37.9 | 37.9 | 37.9 | 37.9 |
| corn oil ³ | 50 | 50 | 50 | 50 |

From Wise *et al* (1983)

¹ see Table 4 for composition.

² contained per kg diet 15.8g calcium tetrahydrogen diorthophosphate, 10g potassium citrate, 4.8g magnesium sulphate, 1.3g sodium chloride, 0.2g sodium metasilicate and 5.8g calcium carbonate.

³ contained 1g/l dl- α -tocopheryl acetate.

Table 4 Composition of Mineral and Vitamin mixes
used in animal diets

| Mineral mix g/kg | | Vitamin mix g/kg ¹ | |
|---|---------|----------------------------------|-----------------|
| potassium citrate | 290.1 | maize starch | 837.7 (812.25) |
| calcium tetrahydrogen diorthophosphate | 275.7 | choline chloride | 134.0 (134.0) |
| calcium carbonate | 241.6 | inositol | 20.0 (20.0) |
| magnesium sulphate | 139.3 | nicotinamide | 3.0 (12.0) |
| sodium chloride | 37.34 | retinyl palmitate (4000000iu) | 1.6 (4.80) |
| sodium metasilicate | 5.34 | calcium pantothenate | 1.6 (4.80) |
| ferric citrate | 6.08 | pyridoxine | |
| manganous carbonate | 3.03 | hydrochloride | 0.7 (4.20) |
| zinc carbonate | 0.61 | riboflavin | 0.6 (3.60) |
| nickel sulphate | 0.39 | thiamin | |
| cupric carbonate | 0.28 | hydrochloride | 0.6 (3.60) |
| stannic chloride | 0.083 | folic acid | 0.2 (0.60) |
| sodium fluoride | 0.065 | biotin | 0.02 (0.06) |
| ammonium metavanadate | 0.032 | menadione | 0.005 (0.015) |
| chromic chloride | 0.028 | cholecalciferol | 0.0025 (0.0075) |
| sodium selenate | 0.0096 | cyanocobalamin | 0.001 (0.003) |
| potassium iodide | 0.0056 | | |
| ammonium molybdate | 0.00208 | | |

From Wise *et al* (1982).

¹ The numbers in parenthesis are the levels of vitamins added to diets fed to germ-free animals.

in protein. Since lactalbumin is deficient in the amino acid methionine, additional methionine was added to the diets at a concentration equivalent to 1.6% of the lactalbumin concentration.

Germ-free animals were provided with a sterile fibre-free diet (Table 1). As a precaution, the vitamin supplements were increased (Table 4) to compensate for possible losses during sterilisation and for the absence of those vitamins normally contributed by microbial synthesis. The diet was sterilized by exposure to γ -irradiation (5 Mrad from a ^{60}Co source) and the drinking water was sterilized by autoclaving. Control mice, with a conventional flora, were fed this sterile diet also.

To provide the essential fatty acids, safflower oil or corn oil was included in all diets and was supplemented with dl- α -tocopheryl acetate (Vitamin E). Diets were stored for a maximum of 3 months at 4°C.

5. Bacterial Strains.

The bacterial strains used and their genotypes are noted in Table 5.

6. Bacterial Media.

a. *E. coli* D494

232 salt solution provided basic mineral requirements and contained, per litre of double strength solution, 8.16g KH_2PO_4 , 2.0g NaCl , 4.0g NH_4Cl , 0.004g FeSO_4 in twice distilled water adjusted to pH 7.2 with 1M NaOH .

Table 5 The bacterial strains employed

| Strain | Genotype |
|---|---|
| <i>Escherichia coli</i> D494 08 ¹ | <i>pant</i> ⁻ , <i>met</i> ⁻ , <i>pro</i> ⁻ , B ₁ ⁻ , complement |
| <i>Escherichia coli</i> K-12/343/765 ² | <i>galR</i> ^s 18, <i>arg</i> -56, <i>nad</i> -113, <i>uvr</i> ⁺ , <i>rec</i> ⁺ , Str-D, Δ(Lac, <i>pro</i>) |
| <i>Escherichia coli</i> K-12/343/753 ² | <i>galR</i> ^s 18, <i>arg</i> -56, <i>nad</i> -113, <i>lys</i> -60, <i>recA</i> 13, Δ(<i>uvrB</i> , <i>bio</i>) Str-D, Lac ⁺ |
| <i>Escherichia coli</i> K-12/343/801 ² | <i>galR</i> ^s 18, <i>arg</i> -56, <i>nad</i> -113, <i>lys</i> -60, <i>uvr</i> ⁺ , <i>rec</i> ⁺ , Str-D, Δ(Lac, <i>pro</i>) LPS ⁻ |
| <i>Escherichia coli</i> K-12/343/772 ² | <i>galR</i> ^s 18, <i>arg</i> -56, <i>nad</i> -113, <i>lys</i> -60, <i>recA</i> 13, Δ(<i>uvrB</i> , <i>bio</i>) Str-D, Lac ⁺ , LPS ⁻ |
| <i>Salmonella typhimurium</i> TA98 ³ | <i>rfa</i> , Δ(<i>uvrB</i> , <i>chl</i> , <i>bio</i> , <i>gal</i>) <i>hisD</i> 3052 and contains R factor plasmid pKM101 |
| <i>Salmonella typhimurium</i> TA100 ³ | <i>rfa</i> , Δ(<i>uvrB</i> , <i>chl</i> , <i>bio</i> , <i>gal</i>) <i>hisG</i> 46 and R factor plasmid pKM101 |

¹ from G. Schmidt, Max Plank Institut for Immunbiologie, Frieberg, W.Germany.

² from G. Mohn, Dept of Radiation Genetics and Chemical Mutagenesis, University of Leiden, Leiden, Netherlands.

³ from B. Ames, Biochemistry Dept, University of California, Berkeley, USA.

Abbreviations

| | |
|---|-----------------------------------|
| <i>pant</i> - pantothenic acid | <i>bio</i> - biotin |
| <i>met</i> - methionine | <i>lys</i> - lysine |
| <i>pro</i> - proline | <i>rec</i> - recombination repair |
| B ₁ - vitamin B ₁ | <i>uvr</i> - excision repair |
| <i>his</i> - histidine | Str-D - streptomycin dependent |
| <i>gal</i> - galactose | <i>rfa</i> - deep rough mutation |
| <i>lac</i> - lactose | <i>chl</i> - nitrate reductase |
| <i>arg</i> - arginine | LPS - membrane lipopoly- |
| <i>nad</i> - niacin | saccharide |

PEP medium contained, per litre, 500ml 232 salts, 10g Bacto-Peptone (Difco Labs), 5g D-glucose in twice distilled water, sterilized at 121°C, 15lb/in² for 15 mins.

1% Nutrient Broth (NB) was prepared with 1g Oxoid Nutrient Broth No. 2 in 100ml distilled water and sterilized at 15lb/in² for 15 mins.

Minimal medium agar plates contained, per litre, 500ml 232 salts and 15g Bacto-Agar (Difco Labs) sterilized together with twice distilled water by autoclaving in an Agar-Matic AS-3 benchtop agar sterilizer (New Brunswick Scientific Co. Inc., Edison, NY, USA) at 15lb/in² for 20 min and held at 50°C until poured. This was supplemented prior to pouring with glucose and amino acids sterilized separately at 10lb/in² for 10 min and added to a final concentration of 0.5% D-glucose, 30µg/ml L-methionine and L-proline, 10µg/ml D-pantothenic acid and thiamine (Vitamin B1).

Minimal medium agar plates supplemented with ampicillin (Beechams Pharmaceuticals, 'standard' material stored desiccated at 4°C) to a final concentration of 4mg/l. The ampicillin was prepared as a 2.5mg/ml solution and sterilized through a 0.2µm filter (Schleicher and Schuell, Dassel, W.Germany), with 1.6ml added, per litre of agar, immediately prior to pouring. These plates were prepared 1 - 2 days before use as ampicillin is gradually deactivated when in solution or at temperatures above 4°C.

b. *E. coli* K-12/343 strains

PEPS-bouillon contained, per litre, 20g Bacto-Peptone, 5g

Bacto-Tryptone (Difco Labs) and 5g NaCl sterilized at 15lb/in² for 15 mins. This was supplemented with lactose (Difco Labs) to a final concentration of 10mg/ml, an amino acid solution providing thiamine (1µg/ml final concentration), d-biotin (5µg/ml), nicotinic acid (20µg/ml), L-histidine (30µg/ml), L-proline (30µg/ml), L-arginine (30µg/ml) and L-lysine (30µg/ml) both sterilized at 10lb/in² for 10 min and with streptomycin sulphate (filter sterilized) to a final concentration of 50µg/ml.

NR-S agar contained, per litre, PEPS-bouillon supplemented with 15g Bacto-Agar and 30mg/l of the filter-sterilized pH indicator neutral red (Difco Labs). Plates were used within one week as the ability of neutral red, when in solution, to detect acidic pH diminishes.

Phosphate buffered saline (PBS) pH 7.2, contained 4g NaCl, 7g Na₂HPO₄·2H₂O and 3g KH₂PO₄ per litre distilled water. This was usually supplemented with 50mg/l streptomycin sulphate (PBS-S).

c. *S. typhimurium* TA98 and TA100

2.5% Nutrient Broth (NB) consisted of 2.5g Oxoid Nutrient Broth in 100ml twice distilled water and sterilized at 15lb/in² for 15 min.

Vogel Bonner E medium contained 10g MgSO₄·7H₂O, 100g citric acid monohydrate, 500g K₂HPO₄ and 175g NaH₂NH₄PO₄·4H₂O.

Minimal glucose agar plates contained, per litre, 20ml Vogel Bonner E medium and 15g Bacto-Agar sterilized together at 15lb/in²

for 20 min, supplemented with D-glucose to a final concentration of 20mg/ml sterilized at 10lb/in² for 10 min.

Nutrient agar plates contained, per litre, 25g NB, 15g Bacto-Agar in twice distilled water, autoclaved at 15lb/in² and supplemented with filter sterilized ampicillin to a concentration of 4µg/ml.

Tris/KCl contained, per litre, 3.025g Tris [(hydroxymethyl) methylamine], adjusted to pH 7.4 with 5N HCl, and 11.5g KCl and sterilized at 15lb/in² for 15 min.

7. Preparation of bacterial cultures.

a. *Maintenance.*

Aliquots (0.8ml) of cultures grown in either NB (*E. coli* D494 and *S. typhimurium* TA100) or PEPS-bouillon (*E. coli* 343 strains) to mid logarithmic phase were distributed into sterile, glass bijou bottles containing 0.07ml DMSO, frozen rapidly with dry ice and ethanol and stored at -70°C.

A stationary culture of *S. typhimurium* TA98 grown in 2.5% NB supplemented with 0.2% glycerol was sedimented at 3000g for 25 min, resuspended to its original volume with a 1:1 (v/v) mixture of PBS and 40% glycerol, dispensed into bijou bottles and frozen at -20°C. Each culture was tested for ampicillin resistance and crystal violet sensitivity. Frozen cultures were discarded after 1 month.

b. *Growth*

To initiate bacterial growth a few crystals of the frozen

cultures were added to 8ml PEP medium (*E. coli* D494 and *S. typhimurium* TA100) or PEPS-bouillon (*E. coli* 343 strains) and incubated at 37°C for 6 - 8 hours without shaking. This turbid culture was then used to inoculate flasks containing NB (for *E. coli* D494 and *S. typhimurium* TA100) or PEPS-bouillon (*E. coli* 343 strains) and maintained at 37°C in a shaking water bath overnight.

A few drops of thawed frozen culture of *S. typhimurium* TA98 were added to flasks containing 2.5% NB and shaken in a water bath at 37°C overnight.

For experiments requiring bacteria in exponential growth, an aliquot of stationary overnight culture was inoculated into two flasks containing 50ml fresh growth medium. The volume of the inoculum was that estimated to give an optical density of 0.1 as read on an EEL Portable Colorimeter using a blue filter 0B10. The culture was incubated shaking at 37°C and samples from one of the flasks were removed and the optical density measured. When the optical density reading was observed to be increasing at a logarithmic rate when plotted against time, the second flask of culture was rapidly cooled by placing in a bath of iced water.

If required the cultures were sedimented in a MSE High Speed 18 or an IEC Centra-3RS High Speed benchtop centrifuge at 10,000g for 10 mins at 4°C. Each strain was resuspended in 0.9% saline (pH7.2) or PBS as appropriate (see sections 10 & 11).

c. *Tris-EDTA permeabilization of bacteria.*

Cultures of the paired strains *E. coli* 343/765 and 343/753 were grown overnight to stationary phase in PEPS-bouillon containing 5mM MgCl₂. The cells were washed by sedimenting the bacteria and resuspending in an equivalent volume of 0.12M Tris-HCl buffer pH 8.0 (containing per litre, 14.52g Tris adjusted to pH 8.0 with HCl) then gently sedimented again and resuspended to approximately 1 - 2 x 10⁸ bacteria/ml in 0.12M Tris/HCl pH 8.0. A concentrated solution of EDTA (0.01M) was added to the bacterial suspension to a final concentration of 0.5mM, mixed well, incubated for 3 min at 37°C before addition of 0.04M MgCl₂ (to a final concentration of 2mM) to stop EDTA activity (Leive, 1968). The bacteria were then centrifuged and resuspended as required for the *in vitro* genotoxicity assay.

8. Preparation of mouse caecal and hepatic fractions.

a. *Preparation of caecal fractions.*

Mice were killed by cervical dislocation, the entire caecum excised and a crude suspension of the pooled caecal contents from at least 3 mice prepared, using a known volume (between 3 - 7ml) of sterile cold 0.1M KH₂PO₄ buffer pH 7.2 (containing, per litre, 13.61g KH₂PO₄ and sufficient 2N NaOH to give the required pH). Non-bacterial debris was sedimented at 700g for 2 min in a MSE Superminor centrifuge. The supernatant was decanted and used immediately (see section 9).

b. *Preparation of hepatic fractions.*

Liver whole homogenate. Mice were killed by cervical dislocation and the livers from at least 3 animals were pooled and

homogenized aseptically in 50mM Tris/150mM KCl, pH 7.4 (3 volumes/g liver) using five reciprocal strokes of a Potter-Eveljhem tissue homogenizer.

Hepatic post-mitochondrial (S9) fractions were prepared by differential centrifugation (9000g for 20 min) of the whole homogenate using a MSE High Speed 18 centrifuge. Aliquots (2ml) of the supernatant (S9) were immediately snap-frozen in sterile tubes by immersion in liquid nitrogen and stored at -80°C until required.

Hepatic microsomal fractions were prepared by a second centrifugation of fresh S9 at 104,000g for 1 hour using a MSE Europa 65M ultracentrifuge. The resulting supernatant (cytosol) was snap-frozen and stored at -80°C until required. The microsomal pellet was resuspended to the original liver weight with Tris buffer (10mM Tris-HCl pH 7.4, 1mM EDTA, 20% glycerol, 1.15% KCl). If the samples were not to be used immediately they were snap-frozen in liquid nitrogen and stored at -80°C.

c. Estimation of protein content.

The protein content of hepatic fractions was estimated by the method of Lowry *et al* (1951). Samples were diluted to approximately 150µg protein/ml with 0.5M NaOH. Aliquots of 0.5ml were then diluted with 5ml of fresh Lowry reagent (2% Na₂CO₃:1% CuSO₄: 2% Na/K tartrate 100:1:1 by volume) and allowed to stand for 10 min at room temperature. After this time, 0.5ml of 50% (v/v) Folin-Ciocalteu phenol reagent was added to each sample and well mixed. Standards (bovine serum albumin, 0 - 250µg/ml in 0.5M NaOH) were treated in parallel. After 40 min the absorbance at 720nm was

determined on a Cecil 272 spectrophotometer (Cecil Instruments Ltd., Cambridge). A linear standard curve was obtained from which, values for the experimental samples were determined.

9. Determination of enzyme activities.

a. Microbial

Determination of bacterial enzyme activities were carried out on mouse caecal suspensions prepared ^{as described} in section 8a. All assays were performed at 37°C in glass bijoux bottles fitted with a screw cap and rubber septum. Anaerobic conditions were maintained by bubbling a mixture of H₂/CO₂/N₂ (3:10:87 by volume) through the system. The suspension of caecal contents, together with 0.1M KH₂PO₄ buffer pH 7.2 to a final volume of 3.9ml, were pre-incubated for 10 min at 37°C before injection of the substrate through the septum. Aliquots of the reaction mixture were withdrawn in a similar manner at specified time points. Cellular debris was sedimented by centrifugation at 5000g for 5 min in a MSE bench top centrifuge.

Nitrate reductase activity was determined as described by Wise *et al* (1982). To start the reaction 0.1ml sodium nitrate (final concentration 20mM) was added to the incubation. Aliquots (0.5ml) of the reaction mixture were then withdrawn from the system at various intervals and centrifuged. The concentration of nitrite in the supernatant was determined by the method of the Association of Official Analytical Chemists (1970), in which 0.5ml of Greiss reagents 1 and 2 was added to 0.1ml supernatant and made up to 4ml with deionized water. After 20 min the absorbance at 542nm was measured using a Cecil 272 spectrophotometer. If required, the

volume of supernatant was adjusted to obtain a more accurate reading on the spectrophotometer. Standards were prepared using a range of volumes (0.0 - 1.0ml) of 0.1mM sodium nitrite diluted to a volume of 4ml with deionized water and treated with Greiss Reagents as described previously.

Nitroreductase activity was determined by the method of Wise *et al* (1982). The reaction was started by introducing 0.1ml p-nitrobenzoic acid solution (final concentration 1.5mM) to the incubation. Aliquots (0.5ml) were removed at regular intervals after addition of the substrate and mixed with 0.5ml 10% trichloroacetic acid (TCA). The denatured protein was sedimented by centrifugation. The concentration of p-aminobenzoic acid in the supernatant fraction was determined by the method of Bratton & Marshall (1939). To 0.5ml of the supernatant was added 0.125ml 0.1% sodium nitrite, 0.125ml 0.5% ammonium sulphate and 0.125ml 0.1% naphthylethylenediamine hydrochloride (causing diazotisation of the p-aminobenzoic acid), and made up to 2.5ml with deionized water. After 30 min at room temperature the absorbance at 545nm was determined using a Cecil 272 spectrophotometer. Standards were prepared using 0.0 - 0.1ml of 0.5mM p-aminobenzoic acid together with 0.25ml 10% TCA and made up to 0.5ml with water before^{being} treated in an identical manner to the supernatants.

β -glucuronidase and β -glucosidase activities were determined by the method of Rowland *et al* (1983a) using as substrates 0.1ml p-nitrophenol- β -glucuronide and p-nitrophenol- β -D-glucopyranoside respectively (final concentration 3mM). Aliquots (0.5ml) of the reaction mixture were removed at various time points after the

addition of the substrate. After centrifugation, the absorbance of the supernatant at 402nm was determined using a Cecil 272 spectrophotometer. The reaction rates were then calculated using the extinction coefficient of 18.30cm²/mmol.

The activity of hydrolytic enzymes, such as β -glucuronidase is usually not decreased by freezing as is the case of the reductive enzymes nitro- and nitrate reductase (Coates *et al* 1988), so on occasions when not enough time was available to determine all the assays on fresh caecal material, the caecal suspensions were frozen at -80°C and β -glucuronidase and β -glucosidase activities determined the following day. To confirm the stability of the enzyme, β -glucuronidase activity was determined in fresh and frozen caecal suspensions prepared from mice fed the fibre-free diet. A slight increase in activity was observed, rising from 103 μ mol of p-nitrophenol produced/hr/g caecal contents (μ mol/hr/g) in fresh material to 116 μ mol/hr/g on frozen caecal suspension. Similar increases have been observed for β -glucosidase (personal communication I.R. Rowland) and are presumably due to cell lysis allowing greater access of substrates to enzymes (Vince *et al* 1976). Although freezing was not the preferred method, in any particular experiment the caecal contents were treated identically and for each experiment (presented in Chapters 5 & 6) it is stated whether fresh or frozen caecal suspensions were used.

Determination of microbial numbers in caecal contents.

An estimation of the total number of bacteria (viable and nonviable) was obtained from a direct microscopic count on a heat-fixed, Gram-stained preparation as described by Holdeman &

Moore (1975). An aliquot (10 μ l) of an appropriate dilution in saline (usually 10⁻³) of caecal suspension (as prepared in section 8a) was spread evenly over a 1cm square marked on a slide, air dried, heat-fixed and Gram-stained. The slide was again allowed to dry in air prior to counting under a light microscope (Gillett & Sibert Ltd., London, UK). For each sample at least 10 fields (2 edge fields and 8 centre fields) or 100 organisms were counted, whichever was greater. According to the method of Holdeman & Moore (1975) any chains of bacteria were counted as "1". The mean number of bacteria per ml of caecal suspension or per gram caecal contents was then calculated from the mean number of counts per field and from the number of fields per cm² - as measured using a calibrated microscope slide.

b. Mammalian.

Cytochrome P450.

The cytochrome P450 content of the hepatic S9 and microsomal fractions was determined from the carbon monoxide saturated dithionite difference spectrum as described by Omura & Sato (1964). Samples of hepatic S9 and microsomal fractions were diluted in 0.2M KH₂PO₄ buffer pH 7.4 so as to contain the equivalent of 0.06 - 0.08g of fresh tissue/ml. A small quantity of solid sodium dithionite was added to the sample and mixed gently before dividing the samples into two matched spectrophotometer cuvettes. Using a Cecil CE599 automatic scanning spectrophotometer (Cecil Instruments Ltd., Cambridge) the cuvettes were scanned initially between 400 and 500nm to determine the baseline level. Carbon monoxide was bubbled through the contents of one cuvette for 1 min prior to a second scan in which the difference spectrum between 400 and 500nm of the two

cuvettes was recorded. From the results the A(450-490)nm for each sample was determined and using the extinction coefficient of cytochrome P450 as $91\text{cm}^2/\text{mmol}$ (Omura & Sato 1964) the cytochrome P450 concentration (nmol/ml) of each sample was calculated. Cytochrome P450 is so named because of the characteristic spectral absorption maximum at 450nm when reduced and complexed with carbon dioxide.

Cytochrome c reductase.

The determination of this microsomal flavoprotein enzyme was based on the principle that when oxidized cytochrome c is converted to reduced cytochrome c it has, unlike the former, a characteristic absorption maximum of 550nm (Lake 1987). Microsomal suspension (0.1ml of a 50mg fresh tissue/ml suspension) was added to two matched spectrophotometer cuvettes, each containing 1ml 0.125mM cytochrome c (stored at 4°C) in 0.1M KH_2PO_4 buffer pH 7.6 and 0.2ml 15mM KCN and made up to 2.4ml or 2.5ml with buffer for the test and reference cuvettes respectively. The cuvettes were incubated at 37°C for 10 mins prior to addition of 0.1ml β -nicotinamide adenine dinucleotide phosphate reduced form (NADPH) to the test cuvette. The increase in absorbance at 550nm with time was recorded using a Cecil CE599 automatic scanning spectrophotometer. The initial rate of cytochrome c reduction (A550nm/min) was determined and using the extinction coefficient of reduced cytochrome c at 550nm of $21\text{cm}^2/\text{mmol}$ (Williams & Kamin 1962) the NADPH-cytochrome c reductase activity (nmol/min/ml microsomal sample) was calculated.

Mixed function oxidase enzyme activities.

i. 7-ethoxycoumarin- and 7-ethoxyresorufin O-deethylases.

Activities of 7-ethoxycoumarin- and 7-ethoxyresorufin-O-deethylase in hepatic microsomal fractions were measured by fluorescence intensity as described by Lake (1987). Aliquots (equivalent to 5mg microsomal tissue) were added to tubes containing 0.5mM β -nicotinamide adenine dinucleotide phosphate (NADP⁺), 7.5mM DL-isocitric acid, 5mM MgSO₄ and 1 unit isocitric dehydrogenase made up to a final volume of 2ml with 50mM Tris-HCl buffer¹. The samples were allowed to equilibrate to 37°C prior to addition of the appropriate substrate (2mM 7-ethoxycoumarin or 5 μ M 7-ethoxyresorufin) dissolved in Tris-HCl¹ and incubated at 37°C for 10 min before placing on ice and addition of 1ml 5% ZnSO₄ and 1ml saturated barium hydroxide to each tube. After centrifugation at 2000g for 15 min using a MSE coolspin centrifuge, 1ml of the deproteinized supernatant was mixed with 2ml 0.5M glycine/NaOH buffer² and the fluorescence recorded using a Perkin-Elmer fluorescence spectrophotometer³. The background fluorescence was assessed in samples in which substrate was added to replica tubes after the incubation period. Standards were prepared using 0.1mM 7-hydroxycoumarin or 2 μ M resorufin in replace of the microsomal tissue. The enzyme activity expressed as nmol/min/mg tissue was determined.

¹ pH 7.6 for ethoxycoumarin-O-deethylase

pH 8.2 for ethoxyresorufin-O-deethylase

² pH 10.5 for ethoxycoumarin-O-deethylase

pH 8.5 for ethoxyresorufin-O-deethylase

³ excitation and emission monochromators set at 380 and 452nm

respectively for ethoxycoumarin-O-deethylase and 535 and 582nm for ethoxyresorufin-O-deethylase.

ii. Benzphetamine N-demethylation.

The determination of benzphetamine N-demethylation was based on the method of Lu *et al* (1969) which measures formaldehyde production. Tubes containing 0.7mg microsomal tissue, 3.33mM semicarbazide, 1mM benzphetamine, 0.5mM NADP+, 6mM glucose-6-phosphate and 3 units glucose-6-phosphate dehydrogenase were made up to a final volume of 1.5ml with 0.05M Tris-HCl buffer (pH 8.8) and incubated for 20 mins at 37°C. The reaction was stopped by adding 1ml 10% TCA and samples centrifuged at 2000g for 10 mins. An aliquot of supernatant (1ml) was mixed with 1ml Nash reagent (containing per litre, 150g ammonium acetate, 3ml glacial acetic acid, 2ml acetylacetone) and incubated for 10 mins at 60°C before recording the absorption at 412nm using a Cecil 272 spectrophotometer. Tissue blanks were also prepared using replica samples but containing no substrate. Standards containing 0.0 - 0.4µmol formaldehyde/ml were treated in parallel.

Glutathione and glutathione-transferase activities.

Levels of reduced glutathione and oxidized glutathione were determined by the Dept of Biochemistry at BIBRA using the whole liver homogenate as described by Adams *et al* (1983).

The activities of glutathione-transferases in the hepatic cytosol were estimated by the method of Habig *et al* (1974) using 1-chloro-2,4-dinitrobenzene (CDNB), 1,2-dichloro-4-nitrobenzene (DCNB) and 1,2-epoxy-3-(p-nitrophenoxy)propane (ENPP) as substrates.

Determination of aflatoxin B₁-glutathione conjugation.

The ability of mouse hepatic cytosol to convert aflatoxin B₁-2,3 epoxide to a glutathione conjugate was determined by Dr G. Neal, MRC, Carshalton, Surrey using the method of O'Brien *et al* (1983). Hepatic cytosol was incubated with aflatoxin B₁ activated to aflatoxin B₁-2,3 epoxide *in situ*, by quail microsomes supplemented with 5mM reduced glutathione. The production of aflatoxin B₁-glutathione conjugates was measured using reverse phase HPLC.

10. *In vitro* mutagenicity and genotoxicity assays.

a. *Salmonella* mutagenicity assay.

In all experiments, the *Salmonella* mutagenicity assay was performed at 37°C with a preincubation step of 30 min, based on the method of Maron & Ames (1983). For assays using hepatic S9 fractions (as prepared in section 8b) the incubation tubes contained (in a total volume of 0.8ml) 2µmol NADP⁺, 2.5µmol D-glucose-6-phosphate, 16.5µmol KCl, 4µmol MgCl₂, 50µmol NaH₂PO₄ buffer (pH 7.4), 2 units glucose-6-phosphate dehydrogenase, 0.1ml stationary phase (overnight culture) *S. typhimurium* TA98 or TA100 (containing 1 - 2 x 10⁹ bacteria/ml) and appropriate concentrations of mutagen. Assays using hepatic microsomal tissue contained per incubation tube (in a final volume of 0.6ml) 2µmol NADP⁺, 4.5µmol DL-isocitric acid, 5.5 units isocitric dehydrogenase, 16.5µmol KCl, 4µmol MgCl₂, 50µmol NaH₂PO₄ buffer (pH 7.4), 0.1ml stationary phase *S. typhimurium* TA98 and mutagen. Tris/KCl was used to adjust volumes as necessary in both microsome- and S9-containing assays. Concentrations of mutagen and tissue fractions used are described under each individual experiment. After the 30 min incubation period, 2ml molten top agar (50°C) containing 0.1µmol histidine and

0.1µmol biotin was added to each tube and the mixture overlaid onto 20ml Vogel Bonner agar plates. The plates were incubated for 48hr at 37°C and the number of histidine revertants was scored using an 'Artek' colony counter (Artek Systems Corp., New York, USA.). All assays were carried out in triplicate.

b. In vitro genotoxicity assay.

This assay is similar to the *Salmonella* mutagenicity assay, but uses the paired *E. coli* K-12/343 strains (Table 5) based on the method of Mohn (1984). Stationary phase cultures of each strain to be used were centrifuged and resuspended in PBS-S to an optical density of 5¹. A small volume (0.2ml) of the resuspended bacteria was incubated for 30 mins at 37°C in PBS-S with the appropriate concentrations of mutagen in a final 1ml volume. If a promutagen was used, hepatic S9 fractions and cofactors (2µmol NADP⁺, 2.5µmol D-glucose-6-phosphate, 16.5µmol KCl, 4µmol MgCl₂ and 50µmol NaH₂PO₄ buffer, pH 7.4) were included in the incubation mix. At the end of the incubation period an aliquot (0.1ml) was removed and diluted 100-fold in ice cold saline to stop any further reaction. Appropriate further dilutions were carried out and aliquots (0.1ml) plated on to NR-S agar and incubated for 24hr at 37°C. The number of surviving red repair deficient *E. coli* 343/753 or 343/772 (Lac⁺, *uvrB*, *recA*) and white *E. coli* 343/765 or 343/801 (Lac⁺, *uvr+*, *rec+*) was then determined by colony counting. The survival of the repair deficient strain relative to the repair proficient strain was then calculated and expressed as a percentage.

¹ approximately $3 - 4 \times 10^9$ bacteria/ml for 343/765

5 - 7×10^8 bacteria/ml for 343/753

4 - 5×10^9 bacteria/ml for 343/801

4 - 5×10^8 bacteria/ml for 343/772

11. Host-mediated assays.

a. *E. coli* D494

This method was based on the intrasanguineous host-mediated assay described by Solt & Neale (1980). A stationary phase culture of *E. coli* D494 was resuspended in 0.9% saline (pH 7.2) to an optical density, at A^{420} , of 50 equivalent to a bacterial density of between $7 - 8 \times 10^{10}$ cells/ml. Mice were injected intravenously via the tail vein with 0.2ml of this bacterial suspension. The mutagens to be tested were either administered intravenously (with the bacteria if a promutagen) or orally, at appropriate times before or following the bacterial injection depending on the aims of the experiment. Control animals received a bacterial injection and an appropriate volume of solvent.

Three hours after administering the bacteria the mice were killed by cervical dislocation, the liver removed, washed in sterile saline and homogenized in 1ml saline using a Polytron homogenizer (Kinematica, GmbH, Switzerland). If required other tissues (for example, lung, kidney or spleen) were removed and homogenized in pairs in 0.5ml saline.

To determine the number of ampicillin resistant (Amp^R) mutants 0.1ml aliquots of crude homogenate were spread, in triplicate, on to minimal agar containing $4\mu\text{g/ml}$ ampicillin. Total viable numbers

were determined by plating suitable dilutions on to minimal agar plates. All plates were incubated at 37°C for 24 hours. The mutation rate was calculated as the number of Amp^R mutants present per 10⁹ survivors.

b. E. coli 343/765 and 343/753

The method is based on that of Mohn (1984). Stationary phase cultures of *E.coli* 343/765 and 343/753 grown up in PEPS-bouillon were mixed in the ratio 1:20 (v/v) respectively, prior to harvesting. The bacterial mix was resuspended in saline to an optical density of 80 (equivalent to 2 - 3 x 10¹⁰ bacteria of each strain/ml). Aliquots (0.1ml) of the resuspended bacterial mix were injected into mice, either intravenously via the tail vein and/or *per os*. Mutagen solutions were administered as in the *E. coli* D494 host-mediated assay. Ninety minutes after injection of the bacteria, the mice were killed by cervical dislocation, the liver removed, washed in saline and homogenised, in 1ml PBS-S using a Polytron homogenizer. The small intestine and caecal sac were excised from those animals given bacteria orally and homogenised in 2ml PBS-S. If required, faecal pellets were also collected from the mouse cages and homogenized in 2ml PBS-S. Appropriate dilutions of the homogenates were plated on to NR-S agar and subsequently incubated for 24hr at 37°C. The frequency of red *E.coli* 343/753 (Lac⁺, *uvrB/recA*) and white *E.coli* 343/765 (Lac⁻ *uvr+*/*rec+*) colonies were then determined as for the *in vitro* genotoxicity assay in order to calculate the induction of DNA damage.

c. Salmonella typhimurium TA98.

A modification of the intrasanguineous host-mediated assay by Arni *et al* (1977) was used. A stationary phase culture of *S. typhimurium* TA98 was resuspended to 1/50th of its original volume in 0.9% saline (5×10^{10} cells/ml). An aliquot (0.1ml) of this bacterial suspension was injected intravenously into mice via the tail vein immediately prior to an oral dose of mutagen. The mutagen was dissolved in pure DMSO and a total volume of 0.01ml/g body weight was given. Control animals received 0.01ml DMSO/g body weight. In those experiments where only one concentration of mutagen was used, the chosen concentration was derived from results of recent host-mediated assays using the compound in question and found to give around 500 - 700 revertants/plate. This was designed to maximize the sensitivity of the assay so as to be able to detect increases or decreases in mutagenicity between treatment groups. One hour after bacterial injection the mice were killed by cervical dislocation, their livers removed, washed and homogenized in 10ml Tris/KCl using an Ultra-Turrax homogenizer (IKA-Labortechnik, Switzerland). After initial centrifugation for 10 min at 100g (to sediment cell debris) the supernatant was centrifuged at 1700g for 30 mins and the sedimented bacterial cells then resuspended in 1ml Tris/KCl. To determine the number of histidine revertants, 0.1ml bacterial suspension was mixed with 2.5ml top agar (50°C) containing 0.1µmol histidine, 0.1µmol biotin and 25µg/ml ampicillin and poured onto Vogel Bonner agar plates. Three replicate plates were made for each sample.

Total numbers of viable bacteria present in each liver were calculated from the liver weight and by plating a suitable dilution

of the suspension on to nutrient agar plates. From this value, it was possible to calculate the numbers of bacteria poured on to Vogel Bonner plates. Howes (1987) recently showed that when a range of 2×10^7 to 1×10^9 bacteria were plated on to Vogel Bonner plates, the mean number of revertants differed by only 2 colonies, with counts of 17.0 ± 4.6 (mean \pm SD) and 18.6 ± 2.5 colonies respectively recorded. Mutation data from host-mediated assays using *S. typhimurium* TA98 were used if the numbers of bacteria plated fell within this range. An aliquot of the bacterial dosing suspension was also diluted appropriately and plated on to nutrient agar, in order to determine the numbers of bacteria administered to each mouse. Using this value it was possible to calculate the percentage of dosed viable bacteria recovered in the liver. All plates were incubated at 37°C for 48 hr before being counted, using an Artek automatic colony counter.

12. Distribution studies.

a. Host-mediated assay.

In selected host-mediated assays (using *S. typhimurium* TA98 as the indicator organism, section 11c), the mice were dosed with radiolabelled mutagen, either $[2\text{-}^{14}\text{C}]$ MeIQ, $[3\text{-}^{14}\text{C}]$ Trp-P-2 or $[\text{U}\text{-}^3\text{H}]$ aflatoxin B₁ at a concentration such that each mouse in general received $7.5\mu\text{Ci/kg}$ of $[^{14}\text{C}]$ or $20\mu\text{Ci/kg}$ of $[^3\text{H}]$ per gram body weight. Immediately after cervical dislocation of the mice, the hearts were cut open and a sample (approximately 0.1ml) of blood collected into a heparinized syringe and placed into a scintillation vial and weighed. The liver was removed, weighed and homogenized in 5ml Tris/KCl using an Ultra-Turrax homogenizer. An aliquot (0.5ml) of the homogenate was placed in a scintillation vial. From

the remaining homogenate, to which a further 5ml of Tris/KCl had been added, *S. typhimurium* cells were recovered and the number of revertant bacteria determined as described in section 11c above.

To solubilize the blood and liver samples, 1ml Soluene mix (1:1 soluene 350:propan-2-ol) was added to each vial and incubated at 37°C for 30 - 60 mins until solubilization was completed. The samples of blood were then decolourized with 35% hydrogen peroxide added drop-wise. Hionic-fluor scintillation fluid (10ml) was added to each solubilized sample, the samples were placed in the dark at 4°C overnight before measuring the level of radioactivity present in each sample using a Packard liquid scintillation counter. The radioactivity of appropriate dilutions of the labelled mutagen solution was also determined in the same manner as the tissue samples.

Radioactivity was calculated in disintegrations per minute (dpm). Initially, the radioactivity in the blood was calculated as dpm/g blood. Since mice have approximately 80ml of blood/kg body weight (ICN Nutritional Biochemicals 1979) and the specific gravity of blood is 1.057 (Ditmer, 1961), it was possible to estimate the amount of activity in the total blood volume and express this as a percentage of the dose administered. For the liver, radioactivity was initially calculated as dpm for the whole organ, adjusting the value to account for that volume of liver that comprises blood (approximately 0.332g/g liver weight). Those values were then expressed as a percentage of the total [¹⁴C] dose administered to each mouse.

b. Absorption of mutagens from the gastro-intestinal tract.

BALB/c mice, 7 - 8 weeks of age, fed on appropriate diets were anaesthetized with Sagatal (pentobarbitone sodium BP; 60mg/kg). The intestine of each mouse was exposed via a small abdominal incision and ligated, either close to the pyloric sphincter and at the ileo-caecal junction (small intestine) or at the ileo-caecal junction and close to the rectum (large intestine). A volume (0.2ml or 0.1ml for the ligated small and large intestine respectively) of [¹⁴C]Trp-P-2 in DMSO (0.15mg/ml; 0.75 μ Ci/ml) was injected into the ligated section using a fine hypodermic ^{Syringe.} The intestine was returned to the abdomen, the cavity was closed with a suture and a pad of saline-soaked tissue laid over the abdomen. Mice with ^{ligatures of the} large intestine were left for 30 and 60 minutes, whereas mice which had received the radiolabelled mutagen in the small intestine were left for 3, 6, 9 and 15 minutes. During these times mice were kept warm on an electric blanket.

After the appropriate times, the anaesthetized mice were killed by cardiac puncture. A sample (approximately 0.1ml) of blood was collected from the chest cavity in a heparinized syringe, placed in a scintillation vial and weighed. The liver and ligated gut section were each removed, weighed and homogenized in 5ml Tris/KCl using an Ultrax-Turrax homogenizer. A 0.5ml aliquot of the liver homogenate, and a 0.2ml aliquot of the homogenized intestine, were placed into scintillation vials. These samples were then treated as described in the above section 9.

13. Electron microscopy.

Livers were removed from mice, divided into three lobes, and submerged in a 1% solution of osmium tetroxide in phosphate buffer pH 7.2, prior to a rapid 'chopping' of the lobes into approximately 1mm cubes. For complete fixation of the liver, the tissue remained in osmium for at least 2 hours.

The tissue was then prepared for electron microscopic examination. This was carried out at the Dept of Pathology at University College and Middlesex Hospital, London. Osmium-fixed tissue was dehydrated through a graded series (25% to absolute) of analytical standard ethanol, embedded in Spurr resin (Agar Aids, Stanstead, Essex.) and polymerized at 60°C for 16 hr. Thick (1µm) resin-embedded sections were cut on an LKB Ultratome III microtome and stained with 1% toluidine blue. Thin, primrose-yellow-silver sections (700 - 500A) were cut using a Reichert Ultracut ultramicrotome and stained with an alcoholic solution of uranyl acetate followed by Reynolds' (1963) lead citrate. Thick, resin-embedded sections were examined with a Zeiss Photomicroscope while thin, resin-embedded sections were examined in a Jeol 100B electron microscope at 60 or 80 KV and recorded on EM Technical Film (Ilford Ltd, Mobberley, Cheshire.).

14. Statistical analysis.

Initially, the individual results were tested for homoscedasticity using the Bartlett Test (Snedecor & Cochran 1967) and, if required, the data were transformed to either log₁₀ or square root values to achieve this (Altman *et al* 1983). Analysis of the results was then carried out by analysis of variance using either

the Minitab (Minitals Inc., Pennsylvania, USA; Ryan *et al* 1976) or Genstat Statistical Package (Alvey *et al* 1982). Significant differences between mean values were assessed using the least significant difference criterion (Snedecor & Cochran 1967).

CHAPTER 3

Modulation of mouse hepatic metabolism by dietary fat

The studies reported in this chapter investigate the influence of dietary fat on the activation of food mutagens aflatoxin B₁, MeIQ and Trp-P-2 by mouse hepatic S9 preparations in the *Salmonella* mutagenicity assay using *S. typhimurium* TA98 (see Methods section 10a). These results are compared to the *in vivo* mutagenic activity of the three chemicals in the host-mediated bacterial mutation assay also using *S. typhimurium* TA98 as indicator (Methods section 11c). In addition, the effect of dietary fat on the uptake of [¹⁴C]Trp-P-2 from the mouse gastro-intestinal tract was investigated (see Methods section 12b). The diets consisted of a 1% safflower oil diet (low fat) or that diet supplemented with 25% (w/w) beef dripping or 25% (w/w) olive oil representing high saturated and monounsaturated fat diets respectively (see Methods section 4, Table 2). Mice were fed these diets for a 3 - 4 week period and appeared healthy and alert throughout this period. In the final section of this chapter are reported the results of studies examining the effect of dietary fat on the mutagenicity of nitrosodimethylamine *in vivo* in mice (see Methods, section 11a).

1. *Salmonella* mutagenicity assay

The hepatic S9 fractions used in this study were prepared from a single batch of mice and stored at -80°C until immediately prior to use (see Methods section 8b). Despite all three diets being isocaloric, the body and liver weights of mice fed the two high fat

diets were, on average, 6% and 11.5% larger (for body and liver weights respectively) compared to mice fed the low fat diet (Table 6a). However, ^{it was} only the increase in the body weights of mice fed the beef dripping ^{compared to the} low fat diet ^{which statistically} was significant ($p < 0.05$; Table 6a). For mice fed the low fat diet, the relative liver weight ratio (weight of liver per 10g body weight) was 0.55 increasing to 0.58 for mice fed the beef dripping diet and olive oil diets (Table 6a).

The mean protein content of the hepatic S9 fractions prepared from mice fed the control diet was 32.1mg protein/ml S9 (Table 6a). The protein content of hepatic fractions from mice fed the beef dripping and olive oil diets was lower (by 12 and 20% respectively), although the differences were not significant (Table 6a).

In the bacterial mutation assay the number of histidine revertants recorded per plate for the control samples, in which DMSO was added to the incubation in place of mutagen, ranged from 14 ± 3 His⁺/plate (mean \pm SD) in those assays containing no S9, to 17 ± 4 revertants at 3.0mg S9 protein/ml incubation mix (Fig. 8). In these controls, the diet of the mice from which the hepatic fractions were derived, had no significant influence upon the reversion of *S. typhimurium* TA98 to histidine prototrophy (Fig. 8).

In incubations containing 0.8nmol aflatoxin B₁ (Fig. 8) there was an almost linear increase in the numbers of induced histidine revertants concomitant with the increase in S9 protein concentration, rising from 93 ± 17 (mean \pm SD) His⁺/plate at 0.2mg S9 protein/ml incubation mix to 973 ± 197 His⁺ revertants at 3.0mg protein/ml - a doubling in the numbers of revertants per plate for

Table 6 Body and liver weights of mice fed different fat diets and protein content of hepatic S9 fractions prepared from these mice.

| | Diet | | |
|---|--------------|----------------|-----------------|
| | Low fat | Beef dripping | Olive oil |
| <i>Body weight (g)</i> | | | |
| a | 17.38 ± 1.54 | 18.58 ± 1.62* | 18.20 ± 1.50 |
| b | 17.90 ± 1.24 | 18.82 ± 1.53** | 18.66 ± 1.26* |
| c | 15.00 ± 0.71 | - | 17.67 ± 1.84*** |
| d | 17.13 ± 1.88 | 18.25 ± 0.74 | - |
| <i>Liver weight (g)</i> | | | |
| a | 0.96 ± 0.10 | 1.08 ± 0.09 | 1.06 ± 0.03 |
| b | 0.81 ± 0.06 | 0.86 ± 0.10* | 0.97 ± 0.09*** |
| c | 0.69 ± 0.08 | - | 0.91 ± 0.10*** |
| d | 0.73 ± 0.08 | 0.81 ± 0.06* | - |
| <i>Relative liver weight</i> | | | |
| a | 0.55 ± 0.03 | 0.58 ± 0.03 | 0.58 ± 0.01 |
| b | 0.45 ± 0.04 | 0.46 ± 0.05 | 0.52 ± 0.04*** |
| c | 0.43 ± 0.04 | - | 0.51 ± 0.08* |
| d | 0.43 ± 0.02 | 0.44 ± 0.03 | - |
| <i>Protein content (mg protein/ml S9)</i> | | | |
| a | 32.10 ± 4.05 | 28.23 ± 1.91 | 25.75 ± 6.57 |

- a Mice used for preparation of hepatic S9 fractions, see section 1, Figs. 8 - 10.
b Mice used in host-mediated assays using *S. typhimurium* TA98, see section 2, Table 7.
c Mice used in distribution studies, see section 3b.
d Mice used in host-mediated assays using *E. coli*, see section 4, Tables 11 & 12.

Livers from 5 mice were pooled for each S9 fraction, with 4 fractions prepared for each diet (see Methods section 8b). Relative liver weight was calculated as the weight of liver per 10g body weight. Results are given as means ± SD. Asterisks indicate significant differences from the low fat group *** p<0.001; ** p<0.01; * p<0.05, (Anova).

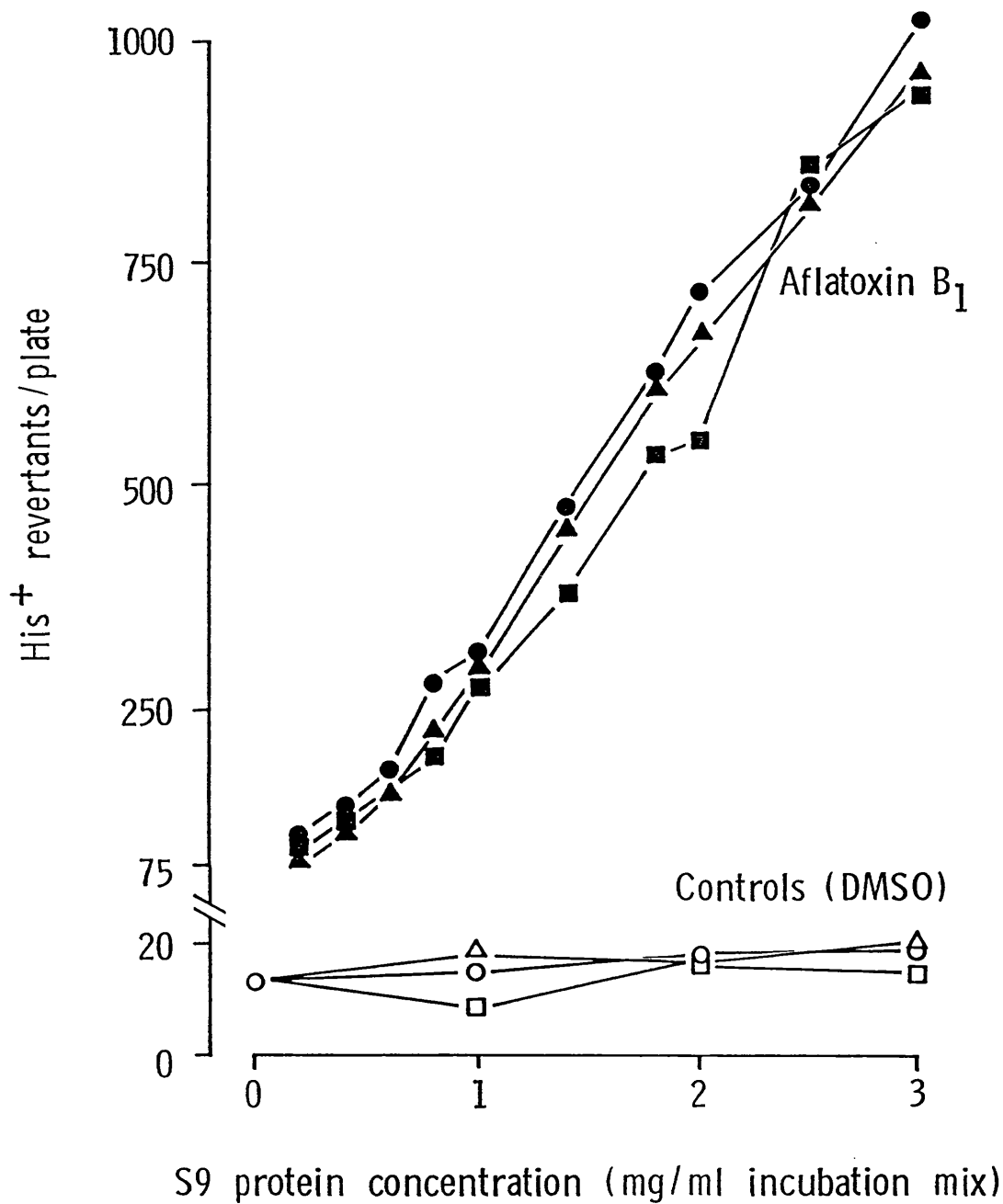


Figure 8 Activation of aflatoxin B₁ by hepatic S9 fractions from mice fed different fat diets. For each S9 fraction, livers from 5 mice were pooled and 4 S9 fractions (at a concentration of 0.25g liver/ml S9) were prepared for each diet. Various concentrations of each hepatic fraction were incubated in a final volume of 0.6ml, containing 0.8nmol aflatoxin B₁ (or 25 μ l DMSO; Controls), *S. typhimurium* TA98 and appropriate cofactors for 30 mins at 37°C before being poured on to Vogel Bonner agar: all assays were carried out in triplicate (see Methods section 10a). Results are given as means for each diet: Low fat (●); Beef dripping (▲); Olive oil (■).

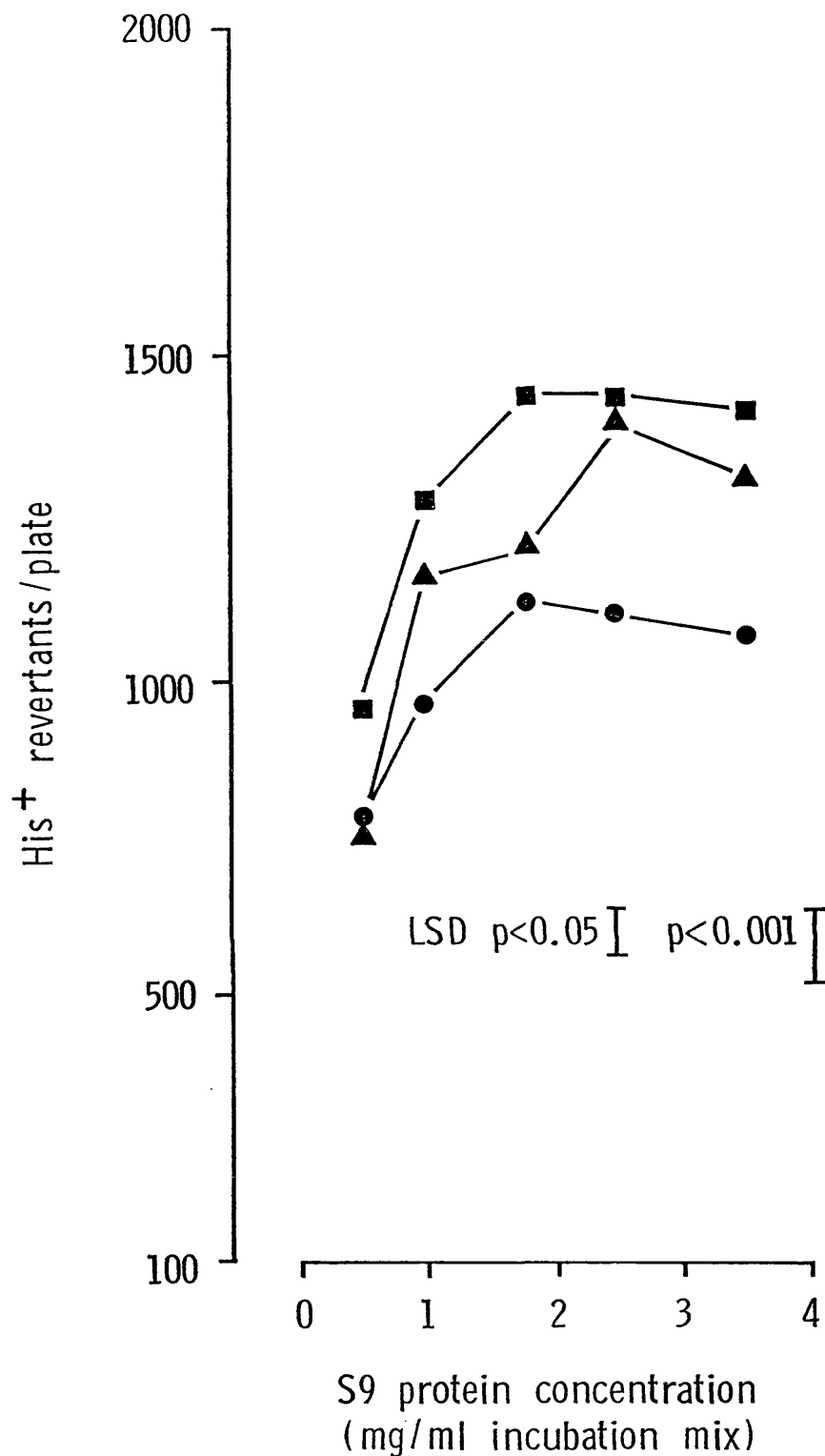


Figure 9. Activation of Trp-P-2 by hepatic S9 fractions from mice fed different fat diets. Various concentrations of hepatic fractions were incubated in 0.6ml with 0.08nmol Trp-P-2. For further details see Fig. 7 and Methods section 10a. Results are given as means for each diet with comparisons between values carried out using Anova and the LSD criterion. Diets: Low fat (●); beef dripping (▲); olive oil (■).

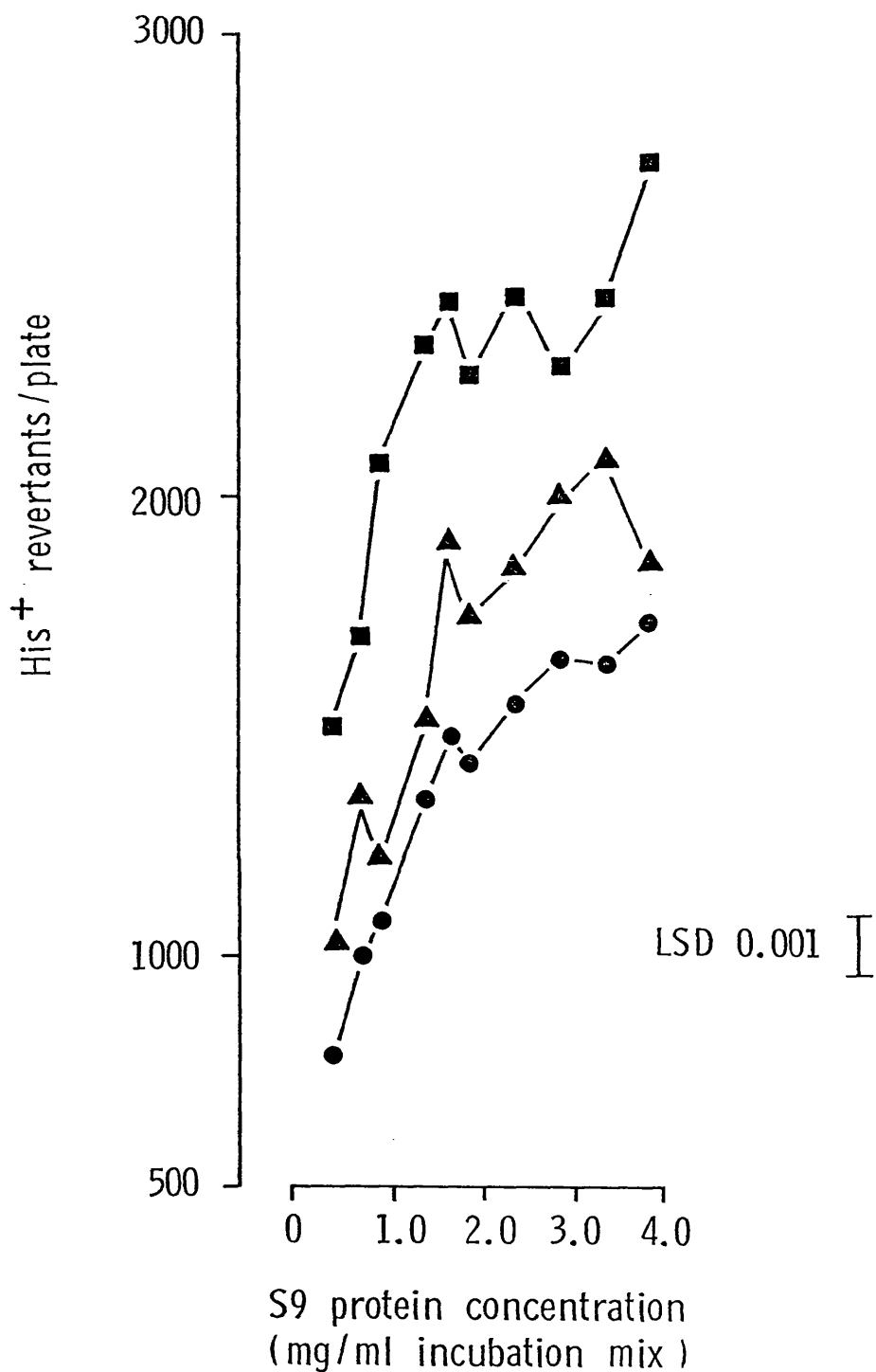


Figure 10. Activation of MeIQ by hepatic S9 fractions from mice fed different fat diets. Various concentrations of each S9 fraction were incubated in 0.6ml with 0.05nmol MeIQ. For further details see Fig. 7 and Methods section 10a. Results are given as means for each diet with comparisons between values carried out using Anova and the LSD criterion. Diets: Low fat (●); beef dripping (▲); olive oil (■).

each 1.7 fold rise in the S9 protein concentration (Fig. 8). Diet had no apparent influence on the mutagenicity of aflatoxin B₁, at any concentration of S9 used (Fig. 8).

However, with the heterocyclic amines, Trp-P-2 (0.08nmol/assay) and MeIQ (0.05nmol/assay), hepatic S9 fractions from mice fed either high fat diet had a greater ability to activate these compounds to bacterial mutagens than S9 from mice fed the low fat diet (Figs. 9 & 10). Significantly higher numbers of revertants, at $p < 0.001$, were induced by virtually all concentrations of S9 used from mice fed the beef dripping or olive oil diets than S9 from mice fed the control low fat diet (Figs. 9 & 10). ^{animals receiving the diet containing olive oil consistently} S9 from _{amines.} induced the greatest number of revertants for both _{heterocyclic} amines. For example, when mice were dosed with Trp-P-2 increases in the number of mutations per plate, of up to 1.25 and 1.4 fold for mice fed the beef dripping and olive oil diets respectively, were observed (Fig. 9). The activity of MeIQ increased initially with increasing S9 protein concentration at a similar rate for all three diets, so that for each 2 fold increase in the concentration of S9 protein there was approximately a 1.3 fold rise in the numbers of histidine revertants. At concentrations above 1.75mg S9 protein/ml incubation mix the rate of increasing mutagenic activity slowed (Fig. 10). Similar S9 protein/activation curves were obtained for Trp-P-2, although the initial rate of increase in revertant numbers was greater for samples containing S9 from mice fed the two high fat diets (Fig. 9). At S9 protein concentrations above 2mg protein/ml incubation mix, the numbers of induced mutants reached a plateau for those hepatic fractions prepared from mice fed the low fat and olive oil diets (Fig. 9).

2. Host-mediated assays

As for the *in vitro* studies, the body and liver weights of mice fed the high fat diets were greater than those of mice fed the low fat diet (Table 6b) resulting in ^abody weight ^{increase} of around 5%.

Mice fed the olive oil diet exhibited a 20% increase in liver weight compared to the controls. However, only a 6.2% rise in liver weight was found for those animals fed the beef dripping diet (Table 6b). Consequently the relative liver weight of mice given olive oil was greater ($p < 0.001$) than that of both other diet groups, being 0.45, 0.46 and 0.52 for mice fed low fat, beef dripping and olive oil diets respectively (Table 6b).

The results of the host-mediated assays (Table 7) showed that in mice fed the different fat diets and administered the solvent (DMSO) in place of the mutagen, the numbers of His⁺ revertants were between 10 and 32 per plate for all three diet groups with no significant diet related differences (Table 7). This range of values was comparable to the background reversion to histidine prototrophy of the *S. typhimurium* TA98 culture used in these experiments (20 ± 4 ; mean \pm SD revertants per plate).

In mice exposed to mutagens the *in vivo* activity of the heterocyclic amines Trp-P-2 (1.5mg[5.8 μ mol]/kg) and MeIQ (0.4mg[1.88 μ mol]/kg) was raised only slightly (by approximately 10%) when mice were fed a 25% beef dripping diet in place of the control, low fat diet. As in the *in vitro* studies, ^{treatment with the olive oil-containing high fat diet} induced a greater number of mutants than beef dripping, giving values

Table 7 Effect of high fat diets on the activity of food mutagens in the host-mediated assay

| Diet | His ⁺ revertants/plate | | | |
|--|-------------------------------------|---------------------|-------------------|----------------------|
| | Aflatoxin B ₁ 10mg/kg | Trp-P-2 1.5mg/kg | MeIQ 0.4mg/kg | Control DMSO only |
| Low fat | 653 ± 109 (8) | 610 ± 262 (4) | 633 ± 209 (9) | 22 ± 5 (7) |
| Beef dripping | 533 ± 175* (8) | 683 ± 169 (7) | 681 ± 234 (9) | 21 ± 4 (8) |
| Olive oil | 475 ± 234** (8) | 818 ± 302* (7) | 802 ± 295* (9) | 23 ± 7 (9) |
| <i>S. typhimurium</i> TA98 dosing suspension (background reversion) 20 ± 4 | | | | |

Female BALB/c mice were exposed to mutagens and *S. typhimurium* TA98 in the host-mediated assay described in Methods section 11c. Results are given as means ± SD, n in parenthesis. Asterisks indicate significant differences from the low fat group, ** p<0.01, * p<0.05 (Anova).

Table 8 A representative recovery of *S. typhimurium* TA98 from the livers of mice in the host-mediated assays of Table 7

| Mutagen | Diet | Mean Liver wt(g) | Viable bacteria per | |
|-----------------------------|---------------|------------------|---|----------------|
| | | | 0.1ml resuspended pellet ($\times 10^6$) ¹ | liver (% dose) |
| <i>MeIQ (0.4mg/kg)</i> | | | | |
| | Low fat | 0.79 | 70 ± 21 | 13.0 ± 3.5 |
| | Beef dripping | 0.82 | 81 ± 21 | 15.1 ± 3.5 |
| | Olive oil | 0.94 | 76 ± 27 | 15.3 ± 5.1 |
| <i>Controls (DMSO only)</i> | | | | |
| | Low fat | 0.77 | 99 ± 18 | 18.5 ± 2.4 |
| | Beef dripping | 0.83 | 99 ± 30 | 19.1 ± 5.5 |
| | Olive oil | 0.93 | 8 ± 30 | 21.8 ± 5.1 |

S. typhimurium TA98 dosed per mouse: 9.7×10^9 cells

Mice were injected iv with 0.1ml suspension of *S. typhimurium* TA98 immediately followed by mutagen or the solvent (DMSO) p.o. in the host-mediated assay (see Methods section 11c). After 1 hour, mice were sacrificed, the liver removed and homogenized. Bacteria were recovered by centrifugation and resuspended in 1ml Tris/KCl. Aliquots (0.1ml of appropriate dilutions) of the resuspended pellets were plated on to nutrient agar and incubated for 48 hours at 37°C (see Methods section 11c).

¹ This set of results represents the number of viable bacteria counted on nutrient agar plates and the number of bacteria poured onto each Vogel Bonner plate.

approximately 1.3 fold greater than the controls but significant at only $p < 0.05$ (Table 7).

In contrast, the mutagenicity of aflatoxin B₁ (10mg [32 μ mol]/kg) *in vivo* in mice fed either high fat diet was significantly less than that in mice fed the low fat diet. On average, 653 revertants were recorded per plate from mice fed the low fat diet compared to 533 and 475 revertants for mice fed beef dripping and olive oil respectively (Table 7).

The numbers of viable *S. typhimurium* TA98 recovered from the livers of mice fed the different fat diets, varied from 8% - 27% of the bacterial dose injected with the numbers of bacteria poured on to the Vogel Bonner plates (from a 0.1ml sample of liver homogenate) ranging between 4×10^7 and 2×10^8 cells. Table 8 gives the results from the host-mediated assay with the mutagen MeIQ together with a set of control mice (given DMSO in place of the mutagen) which is representative of data obtained in all three studies. The recovery of *S. typhimurium* TA98 from the liver was not influenced by the diet fed to the mice, although bacterial numbers were, on average, 1.4 fold higher in the control mice than in the mutagen-treated animals.

3. Distribution studies

a. Host-mediated assays

In the host-mediated assays of Table 7, radiolabelled mutagens were administered to the animals (see Methods section 12a). The mutagens [2-¹⁴C]MeIQ, [3-¹⁴C]Trp-P-2 and [U-³H]aflatoxin B₁ were given by oral intubation to the mice at doses of 0.4mg;1.88 μ Ci/kg

body weight for MeIQ, 1.5mg;7.5µCi/kg for Trp-P-2 and 10mg;25µCi/kg for aflatoxin B₁. Since the body weights of the mice ranged between 15 and 21g (Table 6) and the radiolabelled mutagens were administered on a per kg body weight basis (Methods section 12a & 11c), the amount of radioactivity administered to each mouse varied. The results were, therefore, calculated as radioactivity (dpm) present in total blood (estimated as 80ml of blood per kg mouse body weight), dpm/liver (based on wet weight) and as the proportion of the administered dose (Table 9). At the time of sacrifice, one hour after the mice had been given [¹⁴C]Trp-P-2 or [¹⁴C]MeIQ, approximately 1 - 2% of the administered radioactivity was present in the blood, irrespective of diet (Table 9). In mice given [³H]aflatoxin, 2 - 4% of the administered radiolabel was detected in the blood but, as with the heterocyclic amines, there were no significant diet-related differences. In contrast, the amount of radioactivity in the liver (Table 9) was as great as 14% of the administered dose. However, for those mice fed the two high fat diets, the amount of radioactivity, irrespective of the mutagen used, was consistently lower (by 21 - 45%) than that recorded in the livers of mice on the low fat diet. This reduction was particularly marked for Trp-P-2 and aflatoxin B₁ (Table 9).

b. Absorption of [¹⁴C] Trp-P-2 from ligated gut sections

The reduced amount of radioactivity in the livers of mice fed the beef dripping or olive oil diets (see section a. above) may ^{have been} a consequence of diet-induced differences in the absorption of radio-labelled mutagen from the gastro-intestinal tract.

To determine the effect of dietary fat on the absorption of

compounds from the gastro-intestinal tract [¹⁴C]Trp-P-2 was injected into either the ligated small or large intestine of mice fed the low fat or olive oil diets (see Methods section 12b). The uptake of [¹⁴C] radiolabel into the blood and liver from the ligated sections was then measured (see Methods section 12b).

Over the 15 minute period that the absorption of radiolabel from the small intestine was recorded, there was a near linear rise in the amount of label present in the blood, increasing by approximately 0.046% (of the injected [¹⁴C] dose) per minute irrespective of diet (Table 10; Fig. 11). This resulted in 0.7% of the radioactivity being in the blood after 15 minutes (Fig. 11; Table 10). A concomitant increase in the amount of [¹⁴C] in the liver was apparent. In the liver, the amount of radioactivity increased at a rate of 0.56% (of the administered dose) per minute for the initial six minutes. Thereafter the rate of increase appeared to slow resulting in about 6% of the administered [¹⁴C] dose being in the liver 15 minutes after dosing (Fig. 11; Table 10). As for the blood, no significant diet-related differences in the amount of label in the liver were apparent (Fig. 11; Table 10). It was noted, however, that as before, the liver weights of mice fed the olive oil diet were significantly greater (by 31%) than those in mice fed the low fat diet (Table 6c). Consequently, the amount of radioactivity present per g liver was lower in mice fed the high fat diet.

The initial absorption of radiolabel from the small intestine was very rapid as only 40 - 50% of the administered dose was detected in the small intestine (and its contents) 3 minutes after

Table 9 Radioactivity in the blood and liver of mice fed different fat diets and dosed with radiolabelled mutagens in the host-mediated assays of Table 7

| Mutagen & Diet | Radioactivity dosed/mouse (dpm x 10 ⁴) | Mean Liver wt(g) | Radioactivity per liver | | |
|-----------------------------------|--|------------------|--------------------------|--------------|-----------|
| | | | dpm(x10 ⁴) | % of dose | |
| a. Aflatoxin B₁ | | | | | |
| Low fat (8) | 66.38±4.36 | 0.85 | 6.75±1.48 | 10.21±2.41 | |
| Beef dripping (8) | 65.45±5.65 | 0.82 | 4.44±1.48 | 6.71±1.91** | |
| Olive oil (7) | 69.19±6.00 | 1.00 | 5.53±2.27 | 7.88±2.99 | |
| b. MeIQ | | | | | |
| Low fat (9) | 5.79±0.57 | 0.79 | 0.34±0.16 | 5.85±2.48 | |
| Beef dripping (9) | 6.33±0.52 | 0.82 | 0.30±0.10 | 4.61±1.41 | |
| Olive oil (9) | 6.22±0.37 | 0.94 | 0.27±0.08 | 4.34±1.32 | |
| c. Trp-P-2 | | | | | |
| Low fat (5) | 31.74±0.73 | 0.79 | 2.88±0.51 | 9.07±1.64 | |
| Beef dripping (7) | 34.58±2.02 | 0.89 | 2.04±0.94 | 5.81±2.54* | |
| Olive oil (8) | 32.04±1.90 | 0.96 | 1.56±0.69 | 4.88±2.22*** | |
| Radioactivity in blood | | | | | |
| | | | /g | /mouse | % of dose |
| | | | (dpm x 10 ⁴) | | |
| a. Aflatoxin B₁ | | | | | |
| Low fat | | | 1.32±0.44 | 1.81±0.62 | 2.72±0.90 |
| Beef dripping | | | 1.19±0.31 | 1.59±0.44 | 2.43±0.65 |
| Olive oil | | | 1.13±0.38 | 1.64±0.63 | 2.33±0.78 |
| b. MeIQ | | | | | |
| Low fat | | | 0.06±0.02 | 0.09±0.03 | 1.44±0.47 |
| Beef dripping | | | 0.06±0.02 | 0.08±0.03 | 1.31±0.48 |
| Olive oil | | | 0.07±0.03 | 0.10±0.04 | 1.53±0.59 |
| c. Trp-P-2 | | | | | |
| Low fat | | | 0.29±0.11 | 0.40±0.16 | 1.24±0.49 |
| Beef dripping | | | 0.28±0.08 | 0.42±0.14 | 1.20±0.35 |
| Olive oil | | | 0.32±0.09 | 0.44±0.11 | 1.40±0.38 |

After a bacterial (iv) injection mice were given, p.o., a dose of [³H]aflatoxin B₁, [¹⁴C]MeIQ or [¹⁴C]Trp-P-2 in the host-mediated assay (see Methods section 11c). After 1 hr, mice were sacrificed, the liver and an aliquot (approx 0.1ml) of blood removed from each mouse and the amount of radioactivity determined (see Methods section 12a). Results are given as mean ± SD (n in parentheses). Asterisks indicate significant differences from mice fed the low fat diet * p<0.05; ** p<0.01; *** p<0.001 (Anova).

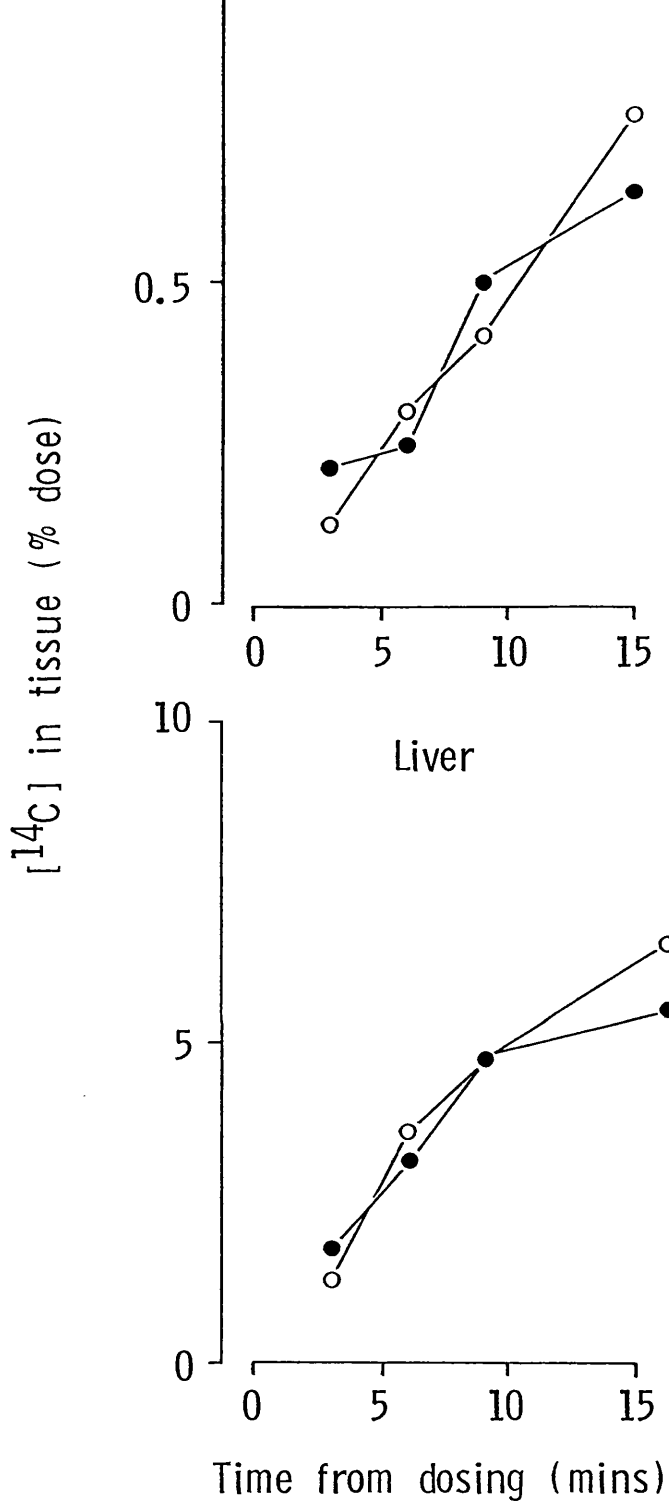


Figure 11. The effect of dietary fat on the uptake of $[^{14}\text{C}]$ Trp-P-2 from the ligated small intestine. Into the ligated small intestine of anaesthetized mice was injected 0.2ml of $[^{14}\text{C}]$ Trp-P-2 in DMSO (0.15mg;0.75 $\mu\text{Ci/ml}$). At various times after dosing, mice were killed, the liver and an aliquot of blood removed and the amount of radioactivity in these tissues was determined (see Table 10 and Methods section 12b). Results are given as means of 3 samples. Analysis of statistical significance was carried out using Anova. Diets: Low fat (●); Olive oil (○).

Table 10 Effect of a 25% olive oil diet on the uptake of [¹⁴C]Trp-P-2 from the ligated small and large mouse intestine

| Tissue & Diet | Radioactivity (dpm x 10 ⁴ /tissue or /ml blood) | | | |
|--|--|-------------|-------------|------------|
| | Time after dosing (mins) | | | |
| | 3 | 6 | 9 | 15 |
| 1. Ligated small intestine | | | | |
| Radioactivity administered /mouse : 44.3 x 10 ⁴ dpm | | | | |
| <i>a. Liver</i> | | | | |
| Low fat | 0.82±0.20 | 1.40±0.19 | 2.12±0.41 | 2.48±0.83 |
| Olive oil | 0.57±0.15 | 1.58±0.40 | 2.15±0.23 | 2.91±0.66 |
| <i>b. Blood</i> | | | | |
| Low fat | 0.05±0.01 | 0.07±0.02 | 0.13±0.02 | 0.17±0.08 |
| Olive oil | 0.03±0.01 | 0.08±0.03 | 0.11±0.04 | 0.20±0.05 |
| <i>c. Small intestine (+ contents)</i> | | | | |
| Low fat | 17.29±3.98 | 20.49±1.11 | 20.01±0.78 | 17.91±1.65 |
| Olive oil | 21.00±1.61 | 15.97±1.12* | 14.83±4.01* | 14.49±1.78 |
| | | 30 | 60 | |

2. Ligated large intestine

Radioactivity administered /mouse : 22.1 x 10⁴ dpm

| | | |
|--|-----------|-------------|
| <i>a. Liver</i> | | |
| Low fat | 1.48±0.49 | 1.71±0.05 |
| Olive oil | 2.05±0.52 | 1.94±0.10* |
| <i>b. Blood</i> | | |
| Low fat | 0.16±0.09 | 0.25±0.16 |
| Olive oil | 0.23±0.02 | 0.29±0.06 |
| <i>c. Large intestine (+ contents)</i> | | |
| Low fat | 7.66±0.14 | 4.71±0.53 |
| Olive oil | 5.62±1.53 | 4.19±1.40 |
| <i>d. Small intestine (+ contents)</i> | | |
| Low fat | 0.55±0.20 | 2.43±0.08 |
| Olive oil | 0.81±0.22 | 1.30±0.31** |

Mice were anaesthetized prior to the small or the large intestine being ligated. An aliquot (0.1 or 0.2ml for small and large intestine respectively) of [¹⁴C]Trp-P-2 (0.15mg; 0.75 µCi/ml) was injected into the ligated section. At various times after dosing, 3 - 4 mice were killed, the liver, intestine (and contents) and an aliquot (approximately 0.1ml) of blood removed and the amount of radioactivity present in these tissues determined (see Methods section 12b). Results are given as means ± SD. Asterisks indicate significant differences from mice fed the low-fat diet, * p<0.05, ** p<0.01 (Anova).

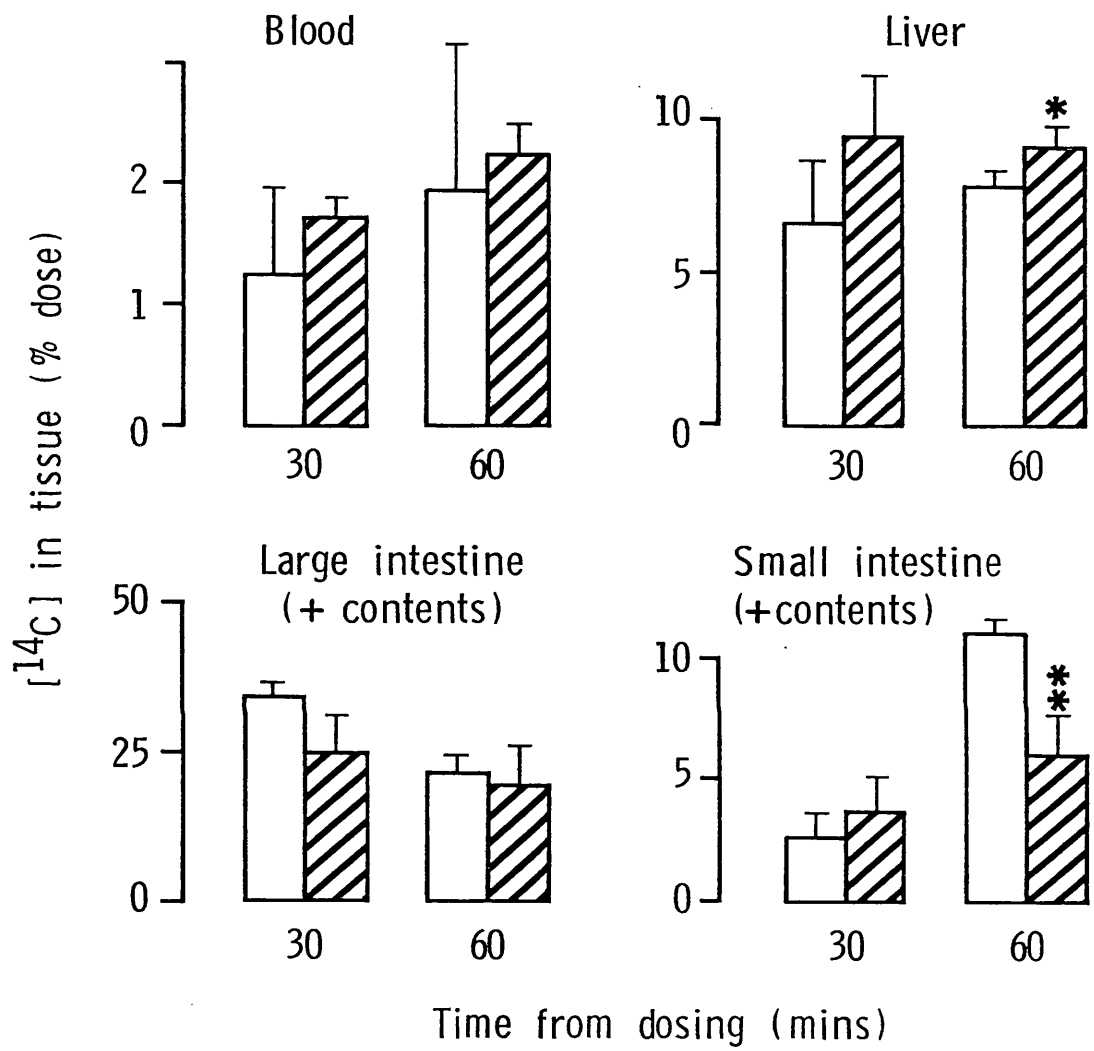


Figure 12 The effect of dietary fat on the uptake of [¹⁴C]Trp-P-2 from mouse ligated large intestine. Into the ligated large intestine of anaesthetized mice was injected 0.1ml of [¹⁴C]Trp-P-2 in DMSO (0.15mg;0.75 μ Ci/ml). At 30 and 60 minutes after dosing mice were killed, and the liver, an aliquot of blood and the small and large intestine removed. The amounts of radioactivity in these tissues were determined (see Table 10 and Methods section 12b). Results are given as means of 3 - 4 samples \pm SD. Asterisks indicate significant differences from the low fat group ** p<0.01; * p<0.05 (Anova). Diets: Low fat (\square); olive oil (hatched).

the mice had been dosed (Table 10). Subsequently, the rate of uptake diminished since 26% of the injected radiolabel remained in the small intestine of mice fed the olive oil at the last timepoint (15 minutes after dosing; Table 10). Furthermore, for mice fed the low fat diet, no further absorption of radioactivity was apparent between 3 and 15 minutes after the animals were dosed (Table 10).

In experiments in which the large intestine was ligated (from the caecum to the rectum) approximately 1.5% of the administered radioactivity was found in the blood 30 minutes after injecting [¹⁴C]Trp-P-2. This occurred irrespective of the diet fed to the mice (Fig. 12; Table 10). This increased to 2% at the 1 hour timepoint. The livers of mice fed the low fat diet contained around 6 - 8% of the radiolabel at both the 30 and 60 minute timepoints. In contrast, mice fed the olive oil diet contained up to 1.4 fold more radiolabel than the control mice (Fig.12; Table 10). It can be seen in Figure 12 that 30 minutes after injecting [¹⁴C]Trp-P-2 more than 60% of the radioactivity had been absorbed from the ligated large intestine in animals fed the low fat diet and around 75% in mice fed olive oil. The uptake of [¹⁴C] from the large intestine during the next 30 minutes was not so marked, as approximately 22% of the administered dose

remained in the ligated section at the 1 hour timepoint. A significant amount of radioactivity was also measured in the small intestine, between 2.5% and 3.5% of the administered dose after 30 minutes. The amount of radioactivity increased to 11% for mice fed the low fat diet and to around 6% for mice fed the olive oil diet 1 hour after dosing (Fig. 12; Table 10).

4. Effect of dietary fat on the mutagenicity of nitrosodimethylamine in the host-mediated assay

In the host-mediated assay using *S. typhimurium* TA98 (described in section 2 above) dietary fat had only a moderate influence on the mutagenicity of Trp-P-2 and MeIQ. A similar result was obtained in previous experiments when the effect of dietary fat on nitrosodimethylamine activity was investigated.

The diets fed to mice in this study were prepared to slightly different recipes (see Methods section 4; Table 2) to those described in the previous sections 1 - 3, in that they contained no cellulose and the low fat diet was supplemented with 30% (w/w) beef dripping or 30% (w/w) safflower oil. Unfortunately, no reliable data were obtained from mice fed the 30% safflower oil diet. This diet had a liquid consistency and, as a result, mice tended to become covered in the diet leading to eczema and also appeared to have a reduced capacity to maintain body temperature. To overcome this problem, in the subsequent studies on the effect of dietary fat on the mutagenicity of aflatoxin B₁, MeIQ and Trp-P-2 (described in the above sections 1 - 3), the diets were modified by inclusion of cellulose (10% w/w) which increased the solidity of those diets containing high levels of safflower oil or olive oil.

The body weights of mice fed the beef dripping diet were approximately 1g (6.5%) heavier than those of mice fed the low fat diet (Table 6d). This difference, however, was not significant. The livers of mice fed the high fat diet were also heavier (by 11%) significant at the $p < 0.05$ level than those mice fed the low fat

diet, with values of $0.81 \pm 0.06\text{g}$ and $0.73\text{g} \pm 0.08\text{g}$ respectively recorded. The relative liver weight was also greater in mice fed the high fat diet with values calculated as 0.42 for mice fed the low fat diet and 0.46 for mice fed beef dripping (Table 6).

Initially, the host-mediated assay with the complement-resistant *E. coli* D494 and ampicillin resistance as a forward mutation marker was used to determine the effect of dietary fat on the mutagenicity of nitrosodimethylamine ($10\text{mg}[135\mu\text{mol}]/\text{kg}$) *in vivo* (see Methods section 11a). The results from three separate experiments consistently showed a small, but statistically insignificant increase (of 1.4 fold) in the numbers of ampicillin resistant mutants recovered from the livers of mice fed the 30% beef dripping diet (Table 11). A typical example of such an increase is seen in Experiment 1 of Table 11 where a mutation rate of 6289 Amp^R mutants/ 10^9 bacteria was obtained from mice fed the low fat diet, whereas 8459 mutants/ 10^9 bacteria were recorded in the livers of mice fed the high fat (beef dripping) diet. Control mice (given an injection of bacteria only) expressed a mutation rate to ampicillin resistance of around 13 mutants/ 10^9 bacteria (Table 11).

The recovery of *E. coli* D494 from the mouse livers was found to vary considerably from 3% to 76% of the administered dose (Table 11). Although no significant differences in the recovery of bacteria was apparent between mutagen-treated animals and controls (given saline in place of nitrosodimethylamine) mice fed the high beef dripping diet consistently exhibited a lower recovery of bacteria from the mouse livers than mice fed the low fat diet (Table 11).

Table 11 Effect of a 30% beef dripping diet on the genotoxicity of nitrosodimethylamine in the host-mediated assay using *E. coli* D494

| Diet | n | Mean Liver wt(g) | Viable <i>E. coli</i> per | | | Mutation Rate (Amp ^R /10 ⁹ viable cells) |
|---|---|------------------|---------------------------|------------------|-----------|--|
| | | | ml homogenate | | liver | |
| | | | Total(x10 ⁸) | Amp ^R | % dose | |
| 1. Nitrosodimethylamine (10mg/kg) | | | | | | |
| a.Lowfat | 3 | 0.69 | 4.44±1.82 | 2543±893 | 49.0±24.2 | 6289±1065 |
| Beef | 6 | 0.83 | 2.72±0.80 | 2050±510 | 33.4± 6.9 | 8459±2564 |
| b.Lowfat | 6 | 0.75 | 10.30±5.50 | 1432±346 | 32.5±17.6 | 1726± 805 |
| Beef | 9 | 0.79 | 6.00±1.93 | 1487±546 | 19.9± 4.7 | 2555± 945 |
| c.Lowfat | 8 | 0.65 | 4.65±2.60 | 1013±710 | 14.4± 7.3 | 2158± 637 |
| Beef | 3 | 0.83 | 3.37±1.84 | 1001±565 | 10.6± 7.4 | 3172±1390 |
| 2. Controls (saline in place of mutagen)¹ | | | | | | |
| Lowfat | 4 | 0.76 | 7.08±4.57 | 5± 5 | 22.0± 6.5 | 11±7 |
| Beef | 4 | 0.80 | 6.75±1.47 | 5±14 | 21.2± 4.3 | 15±7 |

E. coli injected/mouse: 1.5 x 10¹⁰ cells

In 3 separate experiments (a,b and c) a single intravenous (0.2ml) injection (0.3ml) containing a combination of *E. coli* (0.2ml) and nitrosodimethylamine (0.1ml) in saline was given to mice (fed either a low fat diet or that diet supplemented with 30% beef dripping) in the host-mediated assay (Methods section 11a). After 3 hours mice were sacrificed, livers removed, homogenized and aliquots (0.1ml of appropriate dilutions) plated on to non-selective agar and agar selective for ampicillin resistance (Amp^R). Results are expressed as means ± SD. Comparison between diets was carried out using Anova.

¹ A representative mutation rate (from experiment b.) for mice injected with bacteria only.

Table 12 Effect of a 30% beef dripping diet on the activity of nitrosodimethylamine in the DNA-repair host-mediated assay

| Diet | Dose mg/kg | n | Mean liver wt(g) | Viable <i>E. coli</i> /ml homogenate (x10 ⁷) | | Relative survival (%) ¹ |
|----------------------------------|------------|---|------------------|--|-------------|------------------------------------|
| | | | | 343/765 | 343/753 | |
| 1. Nitrosodimethylamine | | | | | | |
| a.Lowfat | 1 | 5 | 0.74 | 21.9 ± 2.7 | 13.90 ± 4.2 | 62.7 ± 14.5 |
| | 5 | 4 | 0.79 | 22.6 ± 3.2 | 7.45 ± 4.3 | 30.6 ± 11.8 |
| | 7.5 | 5 | 0.78 | 21.1 ± 2.6 | 1.81 ± 0.5 | 8.8 ± 3.4 |
| Beef | 1 | 5 | 0.88 | 17.2 ± 3.3 | 11.81 ± 2.0 | 70.4 ± 14.4 |
| | 5 | 3 | 0.82 | 17.5 ± 4.9 | 3.91 ± 2.5 | 28.8 ± 6.4 |
| | 7.5 | 3 | 0.83 | 27.4 ± 12 | 5.57 ± 2.6 | 17.9 ± 8.01*** |
| b.Lowfat | 1 | 4 | 0.74 | 20.2 ± 4.1 | 15.65 ± 2.7 | 78.8 ± 12.4 |
| | 10 | 4 | 0.68 | 24.4 ± 4.1 | 2.81 ± 1.3 | 11.5 ± 4.6 |
| | 15 | 5 | 0.70 | 19.2 ± 4.0 | 1.54 ± 0.5 | 8.5 ± 3.7 |
| Beef | 1 | 4 | 0.86 | 20.2 ± 2.1 | 15.10 ± 3.0 | 74.7 ± 13.7 |
| | 10 | 3 | 0.79 | 22.9 ± 2.0 | 2.83 ± 1.6 | 12.0 ± 4.9 |
| | 15 | 3 | 0.84 | 16.8 ± 4.1 | 2.00 ± 7.5 | 12.1 ± 4.9 |
| 2. Controls (saline only) | | | | | | |
| Lowfat | | 3 | 0.74 | 24.3 ± 3.06 | 25.4 ± 4.1 | 100.7 ± 8.5 |
| Beef | | 3 | 0.86 | 24.6 ± 2.62 | 25.8 ± 3.2 | 105.8 ± 8.5 |

E. coli administered/mouse: 1 x 10⁹ cells of each strain

In two separate experiments (a and b) a single intravenous injection (0.2ml) containing a combination of bacteria (*E. coli* 343/765 and 343/753) and nitrosodimethylamine in saline was given to mice (fed either a lowfat diet of that diet supplemented with 30% beef dripping) in the host-mediated assay (see Methods section 11b). After 90 mins. mice were sacrificed and each liver homogenized in 1ml PBS-S. Aliquots (0.1ml) of appropriate dilutions (10⁻⁵) were then plated onto NRS-S agar. Comparisons between diets were carried out using Anova and the LSD criterion, *** p<0.001.

¹ Survival of the repair deficient strain (343/753) relative to the repair proficient strain (343/765) and expressed as a percentage.

These results were then compared to those from the DNA repair host-mediated assay using the paired *E. coli* strains 343/765 and 343/753 (Table 12; see Methods section 11b). In control mice (given saline in place of nitrosodimethylamine) there was no decline in the relative survival of the repair deficient strain (*E. coli* 343/753). Furthermore, in mice given different concentrations of nitrosodimethylamine there was no apparent reduction in the survival of *E. coli* 343/765 recovered from the livers of mice fed either of the two diets. However, the survival of *E. coli* 343/753 was reduced markedly even at the lowest concentration of nitrosodimethylamine (1mg[13.5 μ mol]/kg) (Table 12). As the concentration of nitrosodimethylamine increased (up to 15mg/kg, equivalent to 202 μ mol/kg) there was a concomitant reduction in the relative survival of *E. coli* 343/753. However, in the two experiments given in Table 12, the extent of the loss of *E. coli* 343/753 differed between the two experiments. For instance, in experiment 1a of Table 12, mice were given 7.5mg nitrosodimethylamine/kg, resulting in a relative survival of 343/753 of, on average, 8% of that of 343/765. In comparison, in experiment 1b (Table 12) a similar survival rate (of 8%) was obtained in mice dosed with 15mg nitrosodimethylamine/kg. No marked difference in liver or body weight of these mice was apparent. There was no consistent diet-related difference in the relative survival of *E. coli* 343/753 observed. The only significant diet-related difference in the relative survival of 343/753 was in those mice dosed with 7.5mg nitrosodimethylamine/kg, (Table 12) in which the activity of the mutagen was 10% less in mice fed the beef dripping diet compared to the low fat diet.

To summarize, the results of this chapter have demonstrated that *in vitro* hepatic fractions from mice fed diets with a high fat content exhibit a greater capacity to produce mutagenic metabolites from the heterocyclic amines MeIQ and Trp-P-2 than those from mice fed a low fat diet. The mutagenicity of aflatoxin B₁ however, was unaffected by diet. Furthermore, these results were not reproduced in the *in vivo* studies. Both MeIQ and Trp-P-2 showed only small increases in mutagenic activity *in vivo* in mice fed high fat diets, whereas the activity of aflatoxin B₁ *in vivo* was reduced in these mice compared to that of mice fed the low fat diets. In addition, dietary fat had little apparent effect on the mutagenicity of nitrosodimethylamine *in vivo*. Distribution studies indicated that these *in vivo/in vitro* differences were not due to diet-related changes in the uptake of compounds from the gastro-intestinal tract.

CHAPTER 4

The effect of dietary protein on the hepatic metabolism of food mutagens.

The importance of dietary protein concentration on the genotoxicity of food mutagens aflatoxin B₁, MeIQ and Trp-P-2 was investigated *in vivo* in the host-mediated assay and *in vitro*, using the *Salmonella* mutagenicity assay, with *S. typhimurium* TA98 as the indicator strain for both tests. The mice used in these experiments were fed, for 3 - 4 weeks, on diets containing 5%, 7.5%, 12.5% or 20% (w/w) lactalbumin as the sole source of dietary protein (see Methods section 4; Table 3) with no apparent detrimental effect on the health of the mice. Further investigations into the effect of 5% and 20% dietary lactalbumin on the content and function of hepatic cytochrome P450 and glutathione are also reported in this chapter.

1. Host-mediated assays.

Mice fed different amounts of dietary protein were dosed *per os* with aflatoxin B₁ (10mg[32µmol]/kg), MeIQ (1.5mg[7µmol]/kg, 0.5mg[2.34µmol]/kg or 0.25mg[1.18µmol]/kg), Trp-P-2 (10mg[40µmol]/kg or 1mg[4µmol]/kg) or the solvent DMSO (controls). In control mice (given DMSO in place of mutagen) the number of histidine revertants recorded (approximately 20 revertants per plate irrespective of the diet fed to mice; Fig. 13), was similar to the background reversion to histidine prototrophy of *S. typhimurium* TA98, determined as 18 ± 9 revertants/plate.

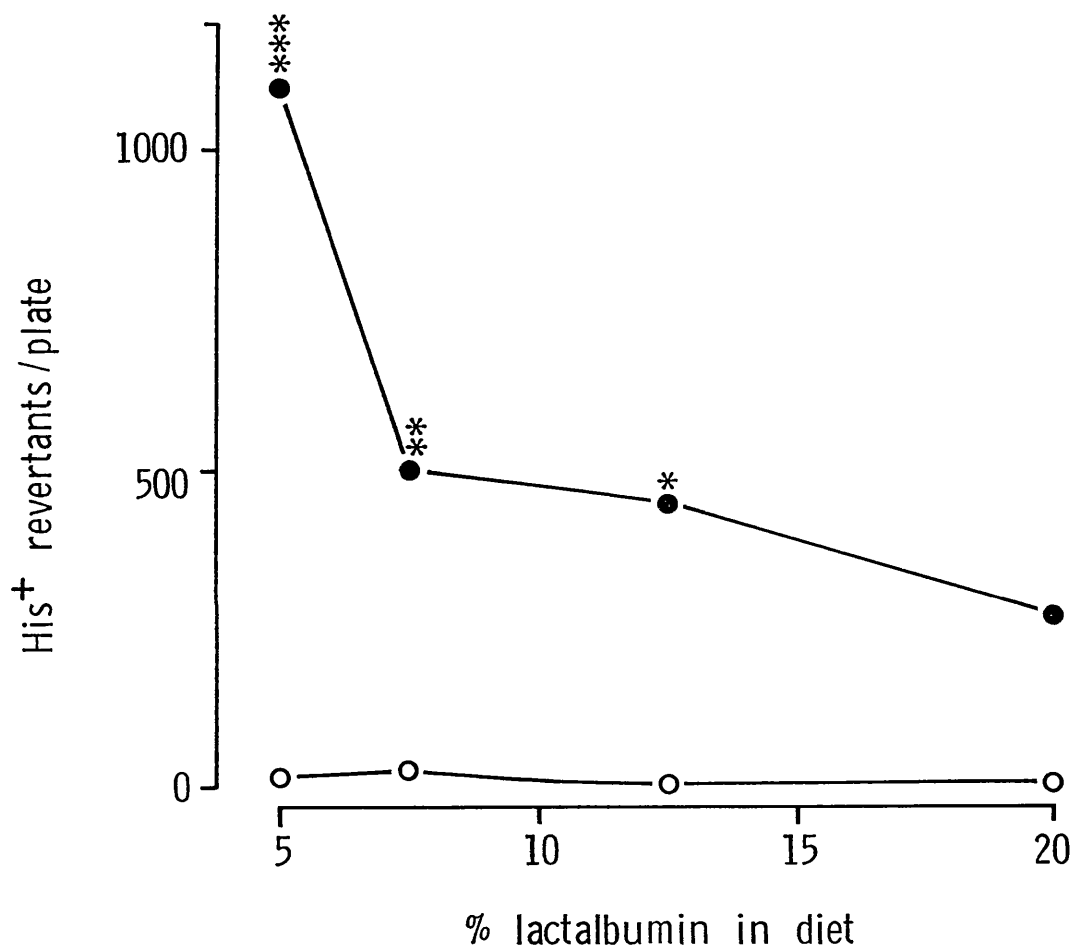


Figure 13 Effect of dietary protein concentration on the *in vivo* mutagenicity of aflatoxin B₁. In the host-mediated assay, with *S. typhimurium* TA98 as indicator (see Methods section 11c) mice were dosed p.o. either with 10mg/kg aflatoxin B₁ (●) or the solvent DMSO (○; Control). The data obtained for aflatoxin B₁ were transformed to log₁₀ values before statistical analysis (Anova). Comparisons between the 20% lactalbumin diet and each of the remaining diets were carried out using the least significant difference criterion, *** p<0.001, ** p<0.01, * p<0.05. Results are given as untransformed means of 6 - 8 mice.

Table 13 The effect of dietary protein concentration on the *in vivo* activity of aflatoxin B₁ (Experiment 2)

| | Diet (% lactalbumin) | |
|---|----------------------|-----------------|
| | 5 | 20 |
| 1. Mutation data: His⁺/plate | | |
| Aflatoxin B ₁ | 356 ± 76*** (6) | 171 ± 50 (7) |
| Controls | 40 ± 12 (4) | 41 ± 8 (4) |
| <i>S. typhimurium</i> TA98 dosing suspension (background reversion): 34 ± 9 | | |
| 2. Radioactivity data | | |
| Blood: dpm (x10 ⁴)/ml | 3.90 ± 1.17* | 2.22 ± 1.10 |
| : % of dose given | 3.81 ± 1.14* | 2.16 ± 1.07 |
| Liver: dpm (x10 ⁴)/liver | 10.21 ± 2.47 | 8.01 ± 3.02 |
| : % of dose given | 8.15 ± 1.61 | 6.52 ± 2.40 |
| Radioactivity given/mouse (dpm x 10 ⁴) | 124.4 ± 9.6 | 123.0 ± 14.2 |

After an iv injection of *S. typhimurium* TA98, mice were given p.o. a dose of [³H]aflatoxin B₁ (10mg;25μCi/kg) in the host-mediated assay (see Methods section 11c and 12a). After 1 hour, mice were sacrificed, the liver and an aliquot of blood removed and treated as described in Methods sections 11c and 12a. The amount of radioactivity and mutagenic activity in the liver and the amount of radioactivity in the blood were determined. Results are given as means ± SD, n in parentheses. Asterisks indicate significant differences from the 20% lactalbumin group; *** p<0.001, * p<0.05 (Anova). Refer to Table 14 for body and liver weights.

Table 14 Body and liver weights of mice fed different concentrations of lactalbumin and subsequently used in host-mediated assays.

| Mutagen | Diet % lactalbumin | n | Body Weight g | Liver Weight g | Relative liver weight ratio ¹ |
|---|-----------------------|---|------------------|-------------------|--|
| 1. <i>Aflatoxin B₁</i> (10mg/kg; Fig. 13) | | | | | |
| | 5 | 7 | 13.57±0.79 | 0.63±0.06 | 0.46±0.05 |
| | 7.5 | 7 | 13.57±0.98 | 0.72±0.06* | 0.53±0.03** |
| | 12.5 | 8 | 13.75±1.39 | 0.65±0.08 | 0.47±0.04 |
| | 20 | 8 | 13.50±1.20 | 0.62±0.09 | 0.46±0.04 |
| 2. <i>Aflatoxin B₁</i> (10mg/kg; Table 13) | | | | | |
| | 5 | 6 | 15.16±1.17 | 0.52±0.06 | 0.34±0.03** |
| | 20 | 7 | 15.00±1.73 | 0.64±0.14 | 0.42±0.05 |
| 3. <i>MeIQ</i> (1.5mg/kg; Fig. 14) | | | | | |
| | 5 | 8 | 12.88±0.99*** | 0.64±0.05*** | 0.50±0.04 |
| | 7.5 | 8 | 12.25±0.89*** | 0.55±0.04*** | 0.45±0.03 |
| | 12.5 | 8 | 13.13±1.25*** | 0.61±0.04*** | 0.46±0.02 |
| | 20 | 8 | 15.25±1.16 | 0.73±0.04 | 0.48±0.06 |
| 4. <i>MeIQ</i> (0.5mg/kg; Fig. 14) | | | | | |
| | 5 | 6 | 16.00±1.26 | 0.71±0.04** | 0.45±0.04** |
| | 7.5 | 6 | 15.50±1.38 | 0.63±0.09 | 0.41±0.03 |
| | 12.5 | 6 | 15.00±0.89 | 0.64±0.06 | 0.43±0.02 |
| | 20 | 7 | 15.14±1.95 | 0.60±0.07 | 0.40±0.01 |
| 5. <i>MeIQ</i> (0.25mg/kg; Fig. 14) | | | | | |
| | 5 | 7 | 15.43±2.64 | 0.66±0.10 | 0.43±0.02 |
| | 7.5 | 7 | 15.43±2.30 | 0.74±0.13 | 0.48±0.03 |
| | 12.5 | 7 | 14.00±1.63 | 0.64±0.09 | 0.45±0.04 |
| | 20 | 7 | 14.43±0.98 | 0.65±0.02 | 0.45±0.04 |
| 6. <i>Trp-P-2</i> (10mg/kg; Fig. 15) | | | | | |
| | 5 | 8 | 12.63±1.06** | 0.57±0.04* | 0.45±0.03 |
| | 7.5 | 8 | 13.13±1.55** | 0.61±0.06 | 0.47±0.04 |
| | 12.5 | 8 | 12.13±1.13*** | 0.63±0.14 | 0.51±0.08 |
| | 20 | 8 | 15.13±1.81 | 0.69±0.14 | 0.46±0.05 |
| 7. <i>Trp-P-2</i> (1mg/kg; Fig. 15) | | | | | |
| | 5 | 7 | 16.00±1.63 | 0.77±0.07 | 0.48±0.02 |
| | 7.5 | 7 | 14.57±1.51 | 0.72±0.06 | 0.50±0.08 |
| | 12.5 | 7 | 16.29±1.89 | 0.70±0.09 | 0.43±0.02 |
| | 20 | 6 | 15.17±1.17 | 0.73±0.05 | 0.48±0.04 |

Results are expressed as means ± SD for each host-mediated assay, (numbered 1-7 in the table above). Table and Figure numbers refer to mutagenicity data. Asterisks indicate significant differences with respect to 20% protein diet. *** p<0.001, ** p<0.01, * p<0.05 (Anova).

¹ The liver weight per 10g body weight.

The most marked effect of changing dietary protein concentration was on the mutagenic activity of aflatoxin B₁ (Fig. 13). As the protein content of the diet increased there was a concomitant decrease in the mutagenicity of aflatoxin, with values falling from 1108 His⁺ revertants/plate in mice fed 5% lactalbumin to 282 revertants in mice fed 20% lactalbumin - an overall decline of 75% (p<0.001; Fig. 13). A considerable reduction was even apparent in mice fed the 7.5% protein diet since the number of revertants recorded at this dietary protein concentration was less than half that induced in animals fed the 5% lactalbumin diet (Fig. 13).

In a second host-mediated assay in which 10mg [³H]aflatoxin B₁/kg body weight was administered to mice fed 5% or 20% lactalbumin, a reduction (of 52%) in the numbers of induced revertants was again observed in those mice fed the high protein diet (Table 13a). However, in comparison to the initial experiment the mutagenic response for both dietary groups was lower, by at least 50%, even though the dose of aflatoxin B₁ was the same (10mg/kg) in both experiments. The body weights of mice were on average 13.5g and 15g for the first and second experiment respectively, with no dietary influence upon these. The liver weights of mice fed 5% lactalbumin in this second experiment were lower (by 19%), resulting in a significantly (p<0.01) lower relative liver weight for these mice compared to those fed 20% lactalbumin (Table 14).

In mice given MeIQ and Trp-P-2 no consistent diet-related change in revertant numbers was observed (Figs. 14 & 15). Mice

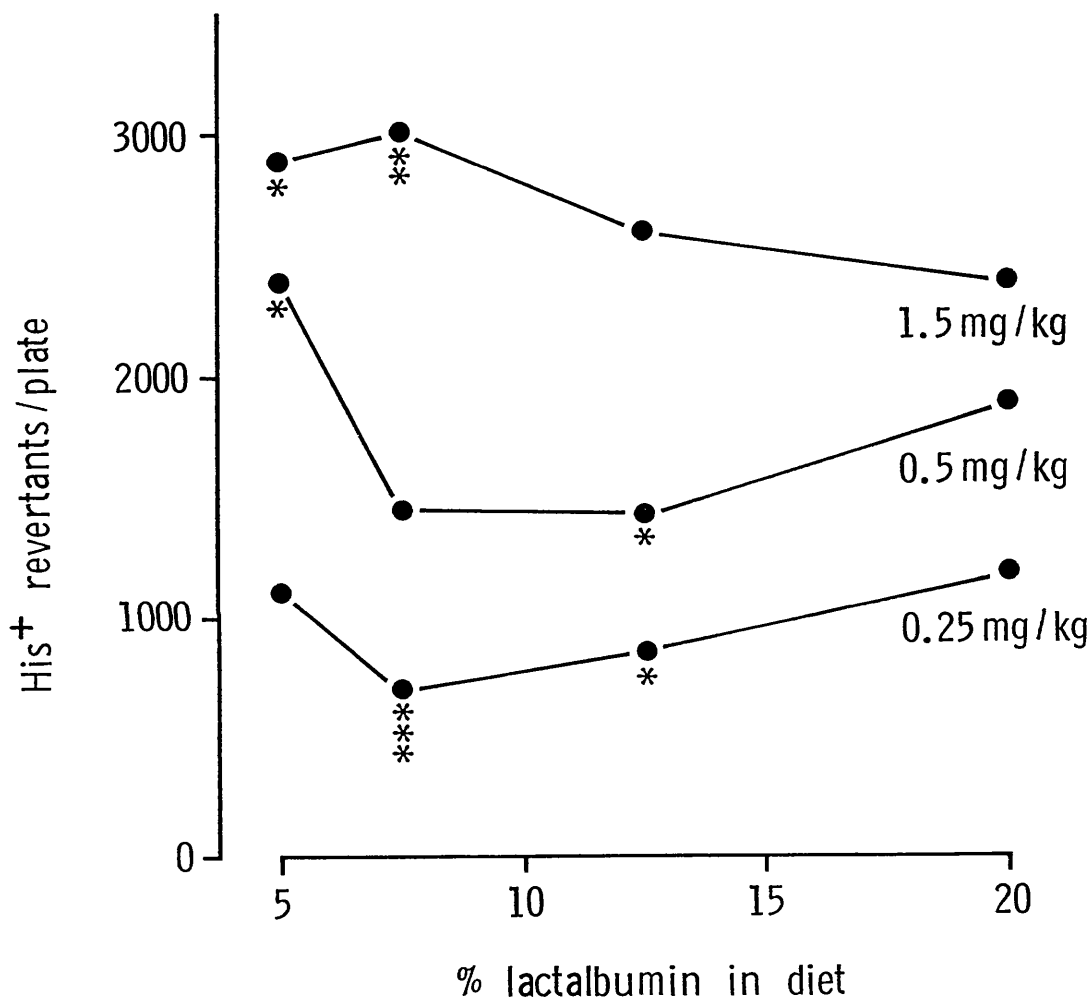


Figure 14 Effect of dietary protein concentration on the *in vivo* mutagenicity of MeIQ. In the host-mediated assay, with *S. typhimurium* TA98 as indicator, (see Methods section 11c), mice were dosed p.o. with different concentrations of MeIQ. Results are given as means of 6 - 8 mice. Asterisks indicate significant differences from those mice fed 20% lactalbumin; *** p<0.001, ** p<0.01, * p<0.05 (Anova).

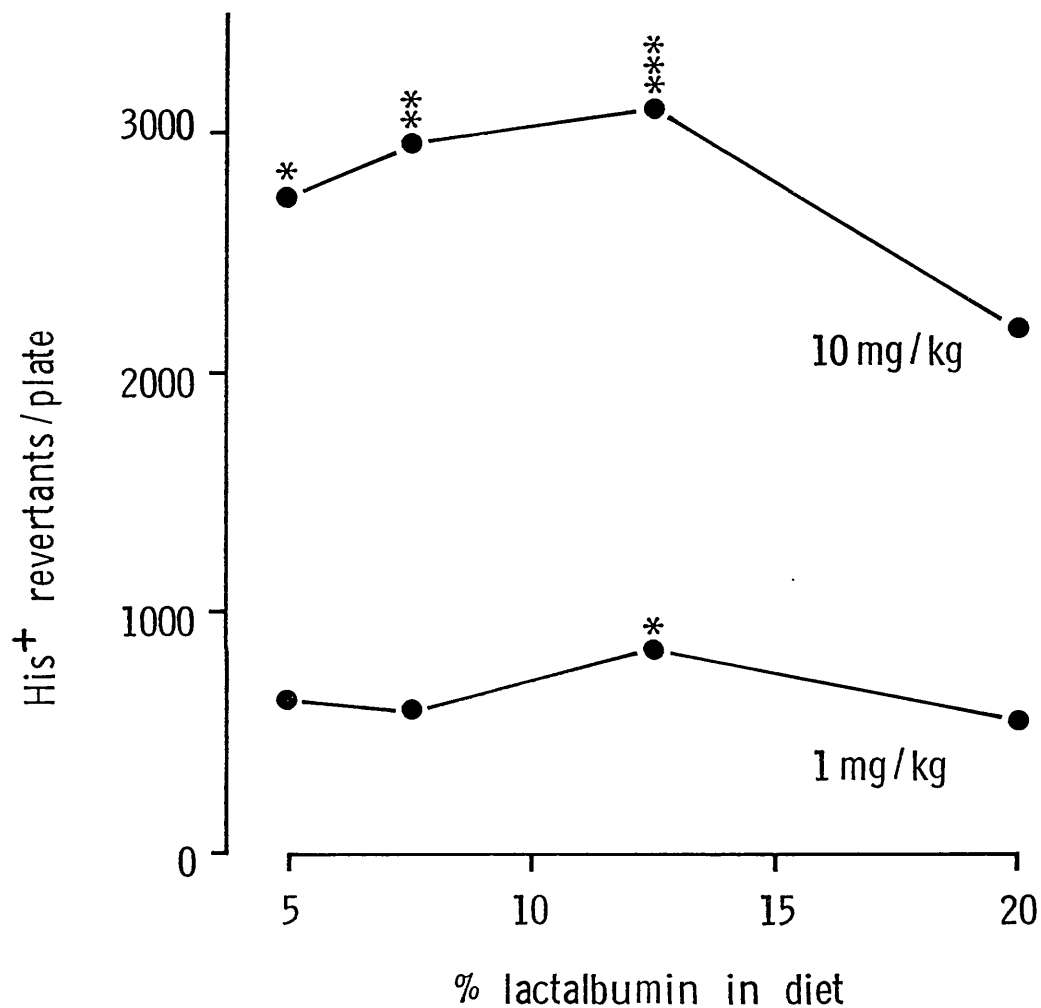


Figure 15 Effect of dietary protein concentration on the *in vivo* mutagenicity of Trp-P-2. In the host-mediated assay with *S. typhimurium* TA98 as indicator (see Methods section 11c), mice were dosed p.o. with 1mg/kg or 10mg/kg Trp-P-2. Results are given as means of 6 - 8 mice. Asterisks indicate significant differences from those mice fed 20% lactalbumin; *** p<0.001, ** p<0.01, * p<0.05 (Anova).

dosed with 1.5mg MeIQ/kg and fed 20% lactalbumin exhibited a reduction in mutagenic activity of 18% (from 2907 to 2383 His⁺/plate) compared to mice fed the 5% lactalbumin diet, whereas animals given 0.25mg and 0.5mg MeIQ/kg expressed an initial fall (of 46% and 36% respectively), followed by a subsequent rise in the mutagenicity of MeIQ as the dietary protein concentration increased (Fig. 14). This resulted in no difference in mutagenic activity between mice fed the two extreme (5% and 20%) protein diets and dosed with 0.25mg MeIQ/kg, and for 0.5mg/kg dose a 20% reduction in induced revertants in animals given the high protein diet (Fig. 14).

The only change in the observed activity of Trp-P-2 (1mg/kg) was in mice fed 12.5% lactalbumin when there was a 1.4 fold increase in the number of revertants compared to mice fed 5%, 7.5% and 20% lactalbumin diets (Fig. 15). Mice administered the higher dose of Trp-P-2 (10mg/kg) exhibited similar levels of mutagenicity of 2900 revertants/plate, at dietary protein concentrations of 5%, 7.5% and 12.5%, but in mice fed the 20% lactalbumin diet there was a 25% reduction in the number of revertants, falling to 2200 revertants/plate (Fig. 15).

The number of *S. typhimurium* TA98 recovered from the livers of mice in all the host-mediated assays, ranged from 7% to 39% of the total bacterial dose injected. A representative sample of these recoveries are shown in Table 15. Consequently, the numbers of bacteria poured onto each Vogel Bonner plate varied between 3×10^7 to 3×10^8 cells which are within the specified limits detailed in Methods section 11c. No consistent diet-related effect was observed in the recovery of bacteria from mice. Nor was there a

Table 15 A representative recovery of *S. typhimurium* TA98 from the livers of mice used in the host-mediated assays of Figures 13 - 15

| Mutagen & dose | Diet % lactalbumin | Mean liver wt(g) | Viable bacteria per | |
|-----------------------|-----------------------|------------------------|--|-------------------|
| | | | 0.1ml resuspended pellet (x10 ⁸) ¹ | liver (% dose) |
| MeIQ 0.5mg/kg | 5.0 | 0.71 | 1.27 ± 0.39 | 21.1 ± 6.0 |
| | 7.5 | 0.63 | 1.66 ± 0.55 | 26.1 ± 8.4 |
| | 12.5 | 0.64 | 1.35 ± 0.39 | 21.4 ± 6.0 |
| | 20.0 | 0.60 | 1.74 ± 0.45 | 26.9 ± 6.0 |
| Controls DMSO only | 5.0 | 0.70 | 1.65 ± 0.27 | 27.9 ± 3.7 |
| | 7.5 | 0.68 | 1.50 ± 0.25 | 27.8 ± 6.4 |
| | 12.5 | 0.67 | 1.09 ± 0.21 | 21.8 ± 5.5 |
| | 20.0 | 0.66 | 1.07 ± 0.27 | 21.4 ± 3.9 |

S. typhimurium TA98 dosed per mouse: 1 x 10¹⁰ cells

Each mouse was injected iv with 0.1ml suspension of *S. typhimurium* TA98 immediately followed by mutagen or the solvent (DMSO) p.o. in the host-mediated assay (Methods Section 11c). After 1 hour, mice were sacrificed, the livers removed and homogenized in Tris/KCl. Bacteria were sedimented by centrifugation and resuspended in 1ml Tris/KCl. Aliquots (0.1ml of appropriate dilutions) of the resuspended bacterial pellets were plated on to nutrient agar and incubated for 48 hrs at 37°C (Methods section 11c). Results are given as means (± SD).

¹ This set of results represents the number of viable bacteria counted on nutrient agar plates and the number of bacteria poured onto each Vogel Bonner plate to determine reversion to His⁺.

significant difference in the recovery of bacteria from mutagen-treated animals compared to the controls (given solvent only; Table 15).

In certain of the host-mediated assays described above the mutagen was radiolabelled (see Table 13 & 16). One hour after administering the radiolabel ($[^{14}\text{C}]\text{MeIQ}$, $[^{14}\text{C}]\text{Trp-P-2}$ or $[^3\text{H}]\text{aflatoxin B}_1$), 1 - 4% of the radioactivity dosed remained in the blood, with no significant difference between the diets apparent for either of the heterocyclic amines (Table 13 & 16). However, for aflatoxin B_1 , blood from mice fed 5% lactalbumin diet contained 1.8 fold more radioactivity ($p < 0.05$) than mice fed the high (20%) protein diet.

In mice dosed with radiolabel^{led} mutagens, it was observed that when $[^{14}\text{C}]\text{MeIQ}$ (0.5mg;7.5 $\mu\text{Ci}/\text{kg}$), Trp-P-2 (1mg;7.5 $\mu\text{Ci}/\text{kg}$) or aflatoxin B_1 (10mg;25 $\mu\text{Ci}/\text{kg}$) were given, the amount of radioactivity in the liver decreased (in total by 32%, 38% and 22% respectively) as the dietary protein concentration increased (Tables 13 & 16). However, this was only statistically significant ($p < 0.05$) for the mutagens MeIQ and Trp-P-2 between the two extreme diets (5% and 20% lactalbumin). No diet-related differences in the amount of radioactivity were apparent in the livers of mice given the lower dose of MeIQ (0.25mg;6.4 $\mu\text{Ci}/\text{kg}$; Table 16).

To ensure that diet-induced changes in weight were not influencing the mutation data, the body and liver weights of all the mice were measured at the time of sacrifice (Table 14). No consistent diet-dependent changes in either body or liver weights

Table 16 Radioactivity in the blood and liver of mice fed a range of protein diets and given [¹⁴C]MeIQ or [¹⁴C]Trp-P-2 in certain host-mediated assays of Figs. 14-15

| Diet % lactalbumin | Radioactivity dosed/mouse (x10 ³) | Total Radioactivity in | | | |
|-----------------------------------|---|---------------------------------|---------------|---------------------------------|----------------|
| | | Blood dpm(x10 ³) | % dose | Liver dpm(x10 ³) | % dose |
| 1. MeIQ (0.5mg;7.5μCi/kg) | | | | | |
| 5 | 263.2 ±20.8 | 9.14 ±2.92 | 3.47 ±1.05 | 21.20* ±5.32 | 7.99* ±1.53 |
| 7.5 | 255.0 ±22.7 | 7.27 ±1.52 | 2.86 ±0.57 | 17.93 ±2.46 | 7.05 ±0.92 |
| 12.5 | 246.8 ±14.7 | 5.25 ±3.18 | 2.10 ±1.18 | 13.04 ±7.53 | 5.21 ±2.76 |
| 20 | 249.1 ±32.1 | 8.52 ±5.48 | 3.31 ±1.72 | 13.79 ±7.27 | 5.40 ±2.18 |
| 2. MeIQ (0.25mg;6.4μCi/kg) | | | | | |
| 5 | 199.6 ±34.1 | 4.99 ±2.10 | 2.44 ±0.72 | 10.09 ±5.76 | 5.10 ±2.73 |
| 7.5 | 199.6 ±29.7 | 4.49 ±1.69 | 2.24 ±0.75 | 10.04 ±3.15 | 5.02 ±1.46 |
| 12.5 | 181.1 ±21.1 | 3.84 ±1.39 | 2.16 ±0.86 | 9.48 ±2.82 | 5.27 ±1.55 |
| 20 | 186.7 ±12.6 | 5.22 ±1.17 | 2.71 ±0.54 | 11.96 ±2.71 | 6.42 ±1.42 |
| 3. Trp-P-2 (1mg;7.5μCi/kg) | | | | | |
| 5 | 272.0 ±27.7 | 6.34 ±1.94 | 2.31 ±0.61 | 17.82* ±4.98 | 6.45* ±1.47 |
| 7.5 | 246.5 ±27.9 | 5.34 ±1.81 | 2.16 ±0.71 | 11.86 ±3.27 | 4.80 ±1.21 |
| 12.5 | 276.9 ±32.1 | 5.93 ±2.95 | 2.24 ±1.27 | 12.44 ±5.00 | 4.67 ±2.20 |
| 20 | 25.8 ±19.9 | 4.20 ±2.51 | 1.59 ±0.89 | 10.60 ±4.74 | 4.02 ±1.63 |

Following a bacterial (iv) injection mice were given p.o. a dose of [¹⁴C] MeIQ or [¹⁴C] Trp-P-2. After 1 hour, the mice were sacrificed, the liver and an aliquot of blood removed and treated as described in Methods section 12a to determine the amount of radioactivity present. Results are given as means ± SD of 6-8 animals. Refer to Table 14 for liver and body weights. Asterisks indicate significant differences from 20% lactalbumin group, * p<0.05 (Anova).

were observed. Furthermore the relative liver weight (weight of liver per 10g body weight) was similar in all mice, irrespective of diet (Table 14).

2. *Salmonella* mutagenicity assay.

Hepatic microsomes were prepared from female BALB/c mice fed 5% or 20% lactalbumin and incubated with each mutagen (MeIQ, Trp-P-2 and aflatoxin B₁) in the *Salmonella* mutagenicity assay (Methods section 10a).

No significant diet-dependent differences in the body or liver weights of mice used for the preparation of hepatic microsomes were apparent with mean values obtained of 15g and 0.73g for the body and liver weights respectively (Table 17). However, the microsomal protein content was found to be 5% greater in mice fed the low (5%) protein diet, although this difference was not statistically significant (Table 17).

In control assays, when the mutagen was replaced with DMSO, an increase from around 24 to 36 revertants was apparent as the microsomal concentration increased reaching a plateau at approximately 3.75 μ l (approximately 45 μ g microsomal protein) of microsomal suspension per assay (Table 18). No obvious diet-related differences in the reversion of *S. typhimurium* TA98 to histidine prototrophy was evident in these controls.

For all three mutagens, the numbers of induced revertants rose as the microsomal concentration increased (Figs. 16 - 18). The rate of increase varied with each mutagen and the dose used,

Table 17 Body and liver weights and protein concentration of hepatic fractions from mice fed 5% and 20% lactalbumin diets and used in *in vitro* assays (Figs. 16 - 18)

| Diet % lactalbumin | Body wt(g) | Liver wt(g) | Protein concentration (mg protein/ml) | | |
|-----------------------|----------------|----------------|--|----------------|---------------------|
| | | | microsomes | cytosol | whole homogenate |
| 5 | 15.18 ±1.37 | 0.736 ±0.07 | 14.07 ±3.60 | 13.86 ±1.38 | 19.76 ±2.27 |
| 20 | 15.04 ±1.27 | 0.738 ±0.05 | 10.43 ±1.27 | 14.44 ±0.51 | 18.92 ±1.27 |

Livers from 5-6 mice were pooled and homogenized in Tris/KCl (at a concentration equivalent to 0.25g liver/ml) with 4 homogenates prepared for each diet. From these whole homogenates microsomal and cytosolic fractions were prepared as described in Methods section 8b. Microsomal pellets were resuspended in Tris buffer to the original liver weight. Results are given as mean ± SD and comparisons between diets were carried out using Anova. No significant differences were apparent.

Table 18 Effect of microsome concentration on the reversion of *S. typhimurium* TA98 to histidine prototrophy in the *Salmonella* mutagenicity assay

| Microsomal concentration (μ l/assay) | His ⁺ revertants/plate | |
|--|-----------------------------------|---------------|
| | Diet (% lactalbumin) | |
| | 5 | 20 |
| 1.25 | 24 \pm 7 | 24 \pm 2 |
| 2.50 | 35 \pm 8*** | 24 \pm 11 |
| 3.75 | 35 \pm 6 | 32 \pm 2*** |
| 5.00 | 33 \pm 5 | 33 \pm 4 |
| 7.50 | 31 \pm 9 | 36 \pm 6 |
| 12.50 | 29 \pm 7 | 32 \pm 8 |

Refer to legend of Fig. 16 and Methods section 10a for details of method. In place of mutagen, 0.1ml DMSO was added to each assay. Results are given as means of triplicate plates. Significant differences from the previous microsomal concentration were assessed using Anova and the LSD criterion; *** represents $p < 0.001$.

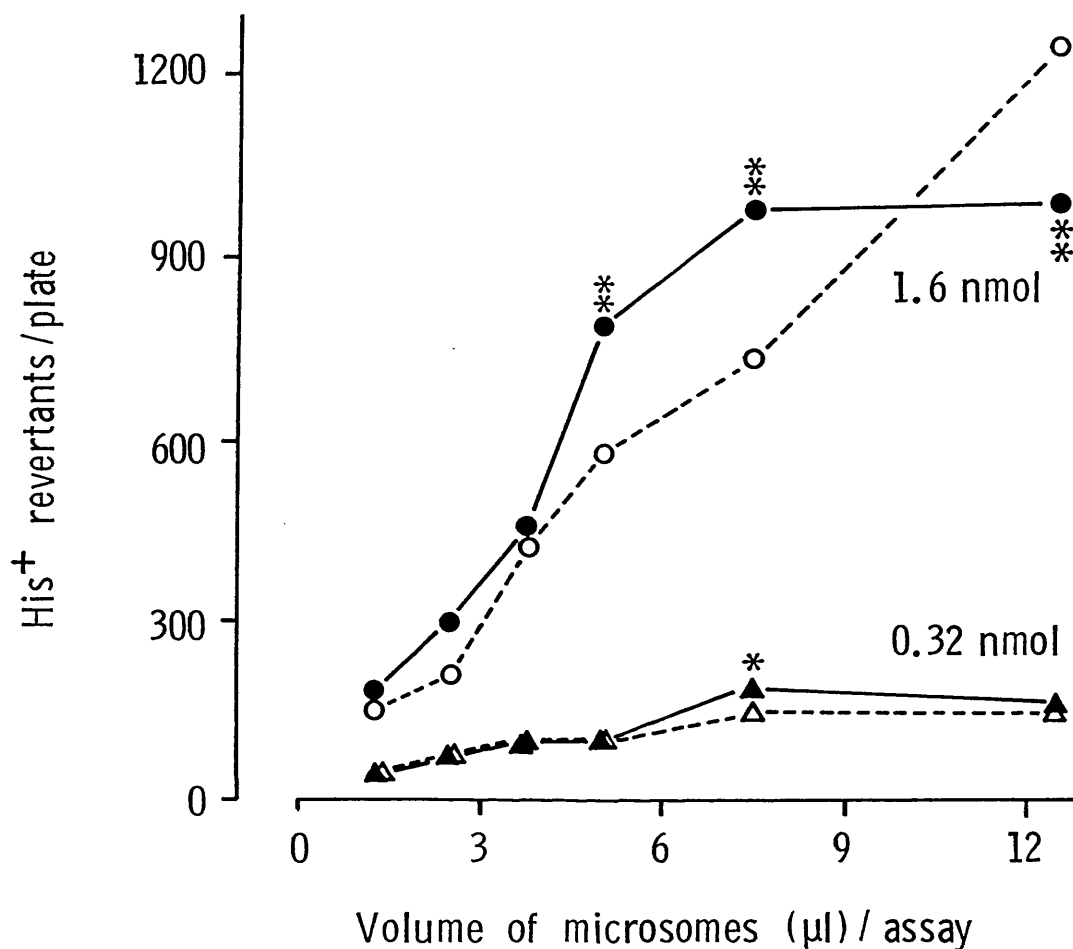


Figure 16. Activation of aflatoxin B₁ by different concentrations of hepatic microsomes from mice fed a 5% or 20% protein diet. Equal volumes of 4 microsomal samples (each prepared from 6 pooled livers at a concentration equivalent to 1g liver/ml) from mice fed either 5% or 20% lactalbumin were mixed to produce a single microsomal suspension per diet. Various concentrations of this pooled microsomal suspension were incubated in a final volume of 0.6ml, with *S. typhimurium* TA98, cofactors, and 0.32nmol or 1.6nmol aflatoxin B₁ for 30 mins at 37°C before being poured, in top agar, on to Vogel Bonner plates (see Methods section 10a). Results are given as means of triplicate plates. Comparison between the two diets was carried out using Anova and the LSD criterion, ** p<0.01, * p<0.05. Diets: (●▲) 5% and (○△) 20% lactalbumin.

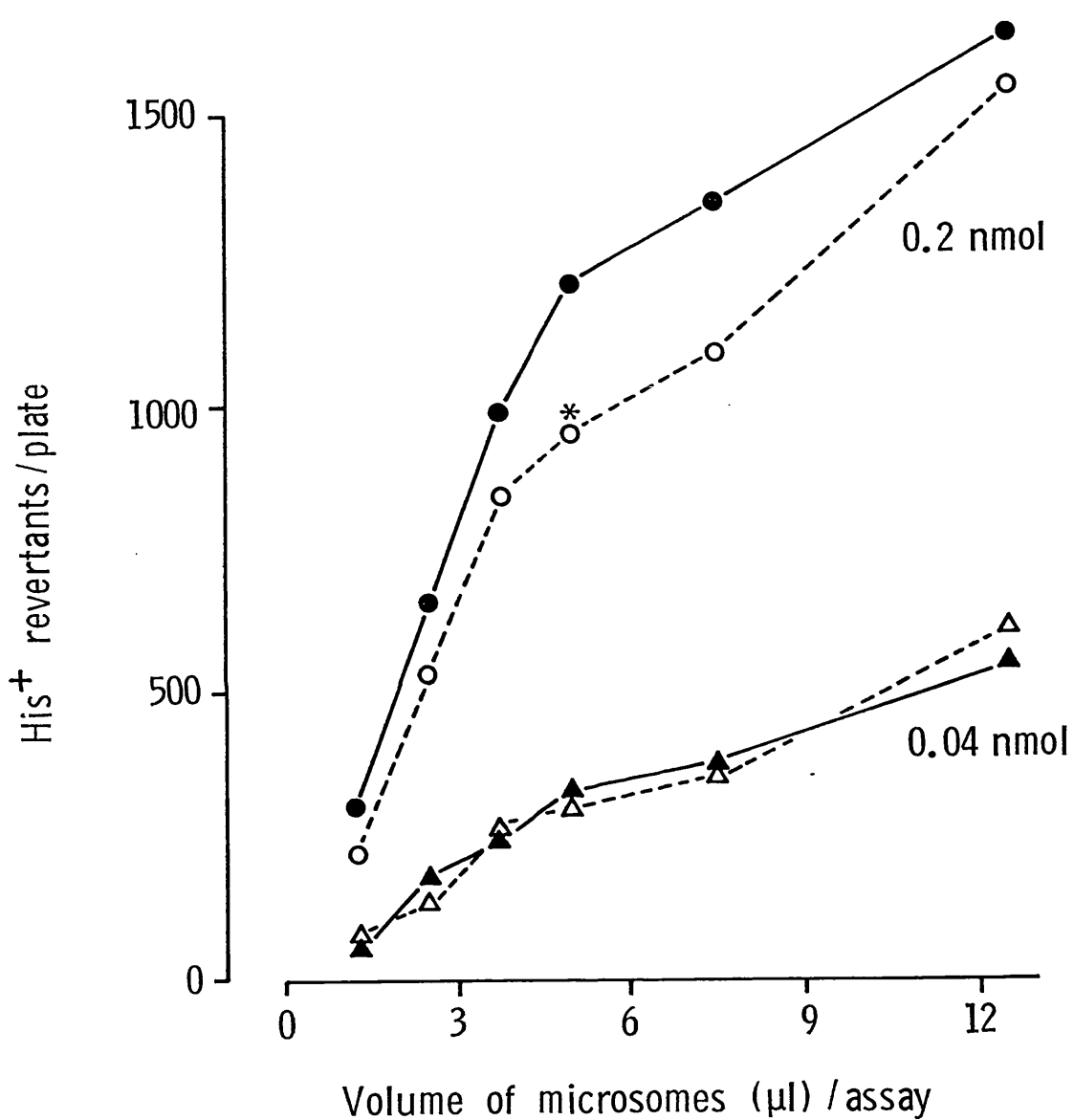


Figure 17. Activation of Trp-P-2 by different concentrations of hepatic microsomes derived from mice fed 5% or 20% protein diets. For details of methods see legend to Figure 16 and Methods section 10a. Results are given as means of triplicate plates. Comparison between the two diets was carried out using Anova and the LSD criterion, * $p < 0.05$. Diets: (●▲) 5% and (○△) 20% lactalbumin.

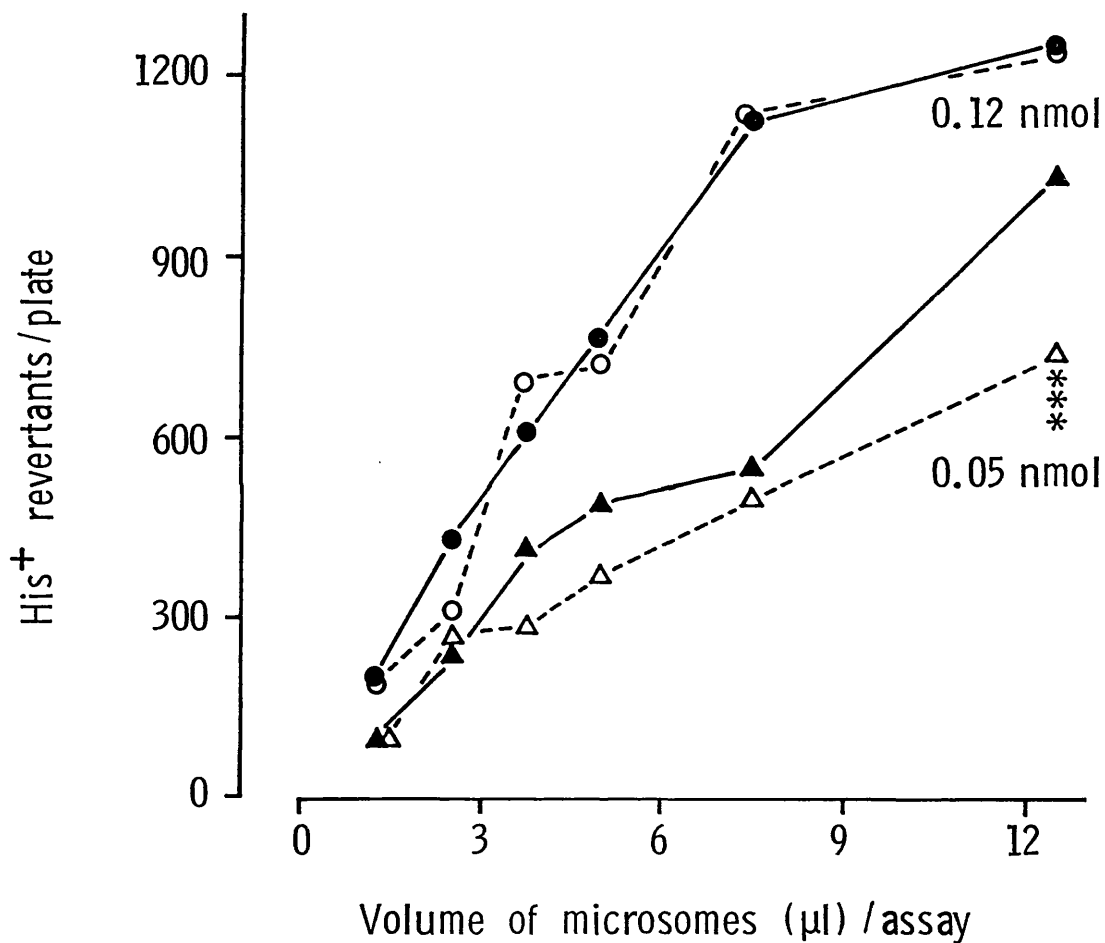


Figure 18. Activation of MeIQ by different concentrations of hepatic microsomes derived from mice fed 5% or 20% protein diets. For details of methods see legend to Figure 16 and Methods section 10a. Results are given as means of triplicate plates. Comparison between the two diets was carried out using Anova and the LSD criterion, *** $p < 0.001$. Diets: (●▲) 5% or (○△) 20% lactalbumin.

although maximum activation was achieved at the highest microsomal concentration (12.5 μ l/0.6ml assay; equivalent to about 150 μ g microsomal protein). For example, when 0.12nmol of MeIQ was included in the incubation, the number of revertants increased from 200 His⁺/plate to 1250 His⁺/plate as the microsomal concentration increased from 1.25 μ l to 12.5 μ l/assay (Fig. 18).

No diet-related differences in the activating capacity of microsomes were observed for MeIQ (0.12nmol/assay; Fig.18) and Trp-P-2 (0.04nmol/assay; Fig. 17). There was a trend for microsomes from mice fed the 5% lactalbumin diet, compared to those from mice fed the 20% lactalbumin diet, to exhibit a greater ability to activate 0.05nmol/assay MeIQ (Fig. 18) and 0.2nmol/assay Trp-P-2 (Fig. 17) but at only one microsomal concentration, 5 μ l for Trp-P-2 and 12.5 μ l for MeIQ (equivalent to approximately 60 and 150 μ g protein respectively) were the differences significant. The activation of aflatoxin B₁ (1.6nmol/assay; Fig. 16) was also higher when microsomes from mice fed the low protein diet were used in the incubation mix, significant at microsomal concentrations of 5 μ l and 7.5 μ l/0.6ml assay (Fig. 16). However, when 12.5 μ l of microsomal suspension was incorporated into the incubation mix containing 1.6nmol aflatoxin B₁, the dietary effect was reversed with microsomes derived from mice fed 20% lactalbumin, inducing the greater number of revertants (Fig. 16). Activation of 0.32nmol/assay aflatoxin B₁ displayed no marked dietary effect, although it should be noted that when 7.5 μ l (approximately 90 μ g protein) of microsomes per assay were used, the activating capacity of microsomes from mice fed the 5% lactalbumin diet was higher ($p < 0.05$) than that of microsomes from mice fed the 20% lactalbumin

diet, with 191 histidine revertants recorded for the 5% lactalbumin diet as compared to 150 for 20% lactalbumin (Fig. 16).

3. Cytochrome P450 content and mixed function oxidase activities.

The influence of dietary protein on hepatic microsomal metabolism was investigated in mice fed the two extreme (5% or 20%) lactalbumin diets. Hepatic microsomes from mice fed the 5% protein diet contained approximately 33% more cytochrome P450 per gram of liver than microsomes from mice fed the 20% protein diet (Table 19). However, when the values were expressed as nmols/mg microsomal protein, to account for the greater microsomal protein content of livers from mice fed 5% lactalbumin (Table 14), the dietary influence upon the amount of hepatic cytochrome P450 was no longer evident since both approximated 0.9 -1.0 nmol cytochrome P450/mg microsomal protein.

A marked reduction in ethoxyresorufin-O-deethylase activity (around 60%) was apparent in microsomes from mice fed 5% lactalbumin compared to the 20% lactalbumin diet irrespective of whether the activity was expressed per gram of liver or per mg microsomal protein (Table 19). No significant differences were apparent in the hepatic activities of benzphetamine demethylase and ethoxycoumarin-O-deethylase of mice fed the the two diets (Table 19), with values of approximately 3.5 μ moles/hr/g liver and 1400 nmoles/hr/g liver respectively, obtained.

4. Glutathione and glutathione conjugates.

The ability of hepatic cytosolic fractions to form aflatoxin B₁-glutathione conjugates was assessed in mice fed 5 and 20%

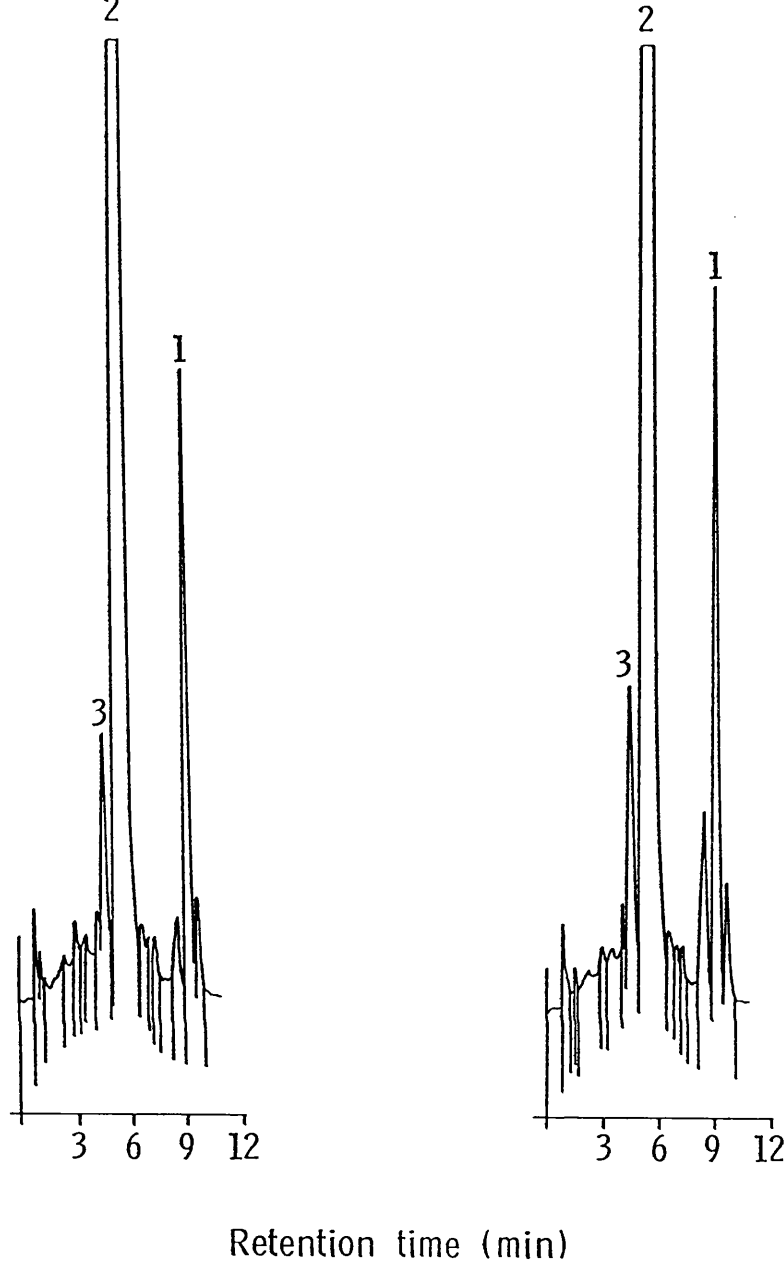
Table 19 Cytochrome P450 and mixed function oxidase activities of microsomal fractions (used in Figs. 16 - 18) from mice fed 5% and 20% protein diets.

| | Diet % lactalbumin | |
|--------------------------------|--------------------|----------------|
| | 5 | 20 |
| Cytochrome P450 | | |
| nmols/g liver | 14.65 ± 1.55* | 10.99 ± 1.37 |
| /mg prot. | 0.92 ± 0.61 | 1.07 ± 0.19 |
| 7-ethoxyresorufin-0-deethylase | | |
| nmoles/hr/g liver | 70.87 ± 21.26** | 163.29 ± 21.60 |
| /mg prot. | 5.48 ± 2.62** | 15.97 ± 3.51 |
| 7-ethoxycoumarin-0-deethylase | | |
| nmoles/hr/g liver | 1444 ± 151 | 1381 ± 188 |
| /mg prot. | 108.6 ± 32.1 | 133.5 ± 20.7 |
| Benzphetamine demethylase | | |
| µmoles/hr/g liver | 3.47 ± 0.53 | 3.82 ± 0.25 |
| /mg prot. | 0.26 ± 0.10 | 0.37 ± 0.05 |

Hepatic microsomes were prepared from mice fed 5% or 20% lactalbumin (Methods section 8b). Cytochrome P450 content and the activities of three mixed function oxidases were determined as described in Methods section 9b. Values are given as means ± SD of 4 samples, each sample containing livers pooled from 5 - 6 mice. Differences between diets were compared using Anova; ** p<0.01, * p<0.05.

lactalbumin diets by the method described in Chapter 2, section 9bii. Quail microsomes were included in the incubation in order to activate *in situ* aflatoxin B₁ to its reactive metabolite, aflatoxin B₁ epoxide. Using reverse phase HPLC the epoxide was detected as aflatoxin B₁-8,9-dihydrodiol in the Tris-diol complex. Figure 19 represents the results of incubations containing hepatic cytosol pooled from 4 samples from mice fed 5% and 20% lactalbumin. However, these experiments have been repeated using individual cytosolic fractions with similar results. At the end of the 15 minute incubation, the amount of the dihydrodiol (identified by co-chromatography) accounted for only 3% of the total metabolites (Fig. 19). The major metabolite was the aflatoxin B₁-glutathione conjugate which accounted for around 90% of the total metabolites, irrespective of the diet of the mice (Fig. 19). At the end of the incubation only 6% of the total metabolites was aflatoxin B₁ (Fig. 19). In these studies excess glutathione was added to the incubation mixture. Since the capacity of the liver to conjugate aflatoxin B₁ or its metabolites to glutathione *in vivo* could be influenced by the concentration of hepatic glutathione, the effect of dietary protein on glutathione concentration in the liver of BALB/c mice was investigated.

Liver homogenates derived from mice fed the 20% lactalbumin diet contained greater amounts, 1.2 and 1.7 fold, of reduced and oxidized glutathione, respectively, than mice fed 5% protein (Table 20). However, this was only statistically significant for the observed differences in oxidized glutathione where values of 2.4 nmoles (2.4 μ M) and 4.08 nmoles oxidised glutathione/g liver (4 μ M) were obtained for the low and high protein diets respectively (Table



| Peak | Metabolite | % of total metabolite | |
|------|---|-----------------------|-------|
| | | Diet (% lactalbumin) | |
| | | 5 | 20 |
| 1 | aflatoxin B ₁ | 5.84 | 6.08 |
| 2 | aflatoxin B ₁ -glutathione conjugate | 89.26 | 87.39 |
| 3 | aflatoxin B ₁ -dihydrodiol | 2.88 | 2.69 |

Figure 19 Reverse phase HPLC chromatograms of aflatoxin B₁ and metabolites produced by incubations of quail microsomes and cytosol prepared from mice fed either 5% (A) or 20% (B) lactalbumin (see Methods section 9bi).

Table 20 Hepatic glutathione content of mice fed 5% and 20% lactalbumin diets.

| Diet % lactalbumin | Glutathione concentration | | | |
|-----------------------|---------------------------|-----------------|--------------------|-----------------|
| | oxidized | | reduced | |
| | nmoles/ g liver | mg protein | nmoles/ g liver | mg protein |
| 5 | 2.388** ±0.38 | 0.044* ±0.01 | 4392 ±744 | 80.29 ±18.89 |
| 20 | 4.080 ±0.84 | 0.071 ±0.02 | 5188 ±660 | 90.02 ±12.32 |

Glutathione content was determined in fresh liver homogenate (at a concentration 0.25g liver/mlTris/KCl) from mice fed 5% or 20% lactalbumin according to the method of Adams *et al* (1983). Results are expressed as means ± SD of 4 samples. Each sample contained livers pooled from 6 mice. Refer to Table 17 for body and liver weights. Asterisks indicate significant differences from 20% lactalbumin group, * p<0.05, ** p<0.01 (Anova).

20). Since the protein content of the liver homogenate was similar in both diet groups (19mg protein/ml homogenate; Table 17), the increased concentrations of glutathione in mice fed 20% protein were still apparent when the amounts of reduced and oxidized glutathione were expressed as per mg protein.

5. Effect of hepatic cytosol on the microsomal activation of food mutagens.

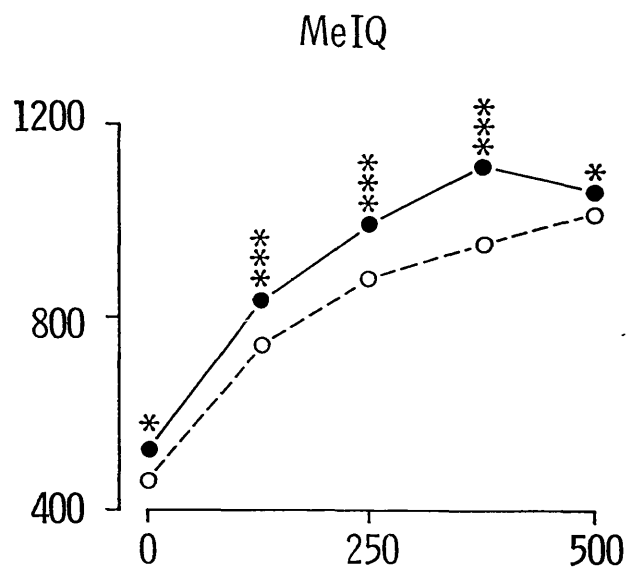
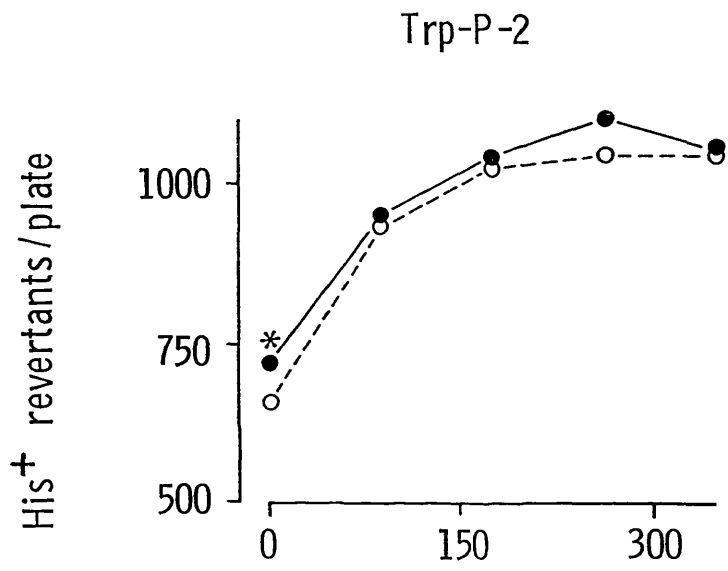
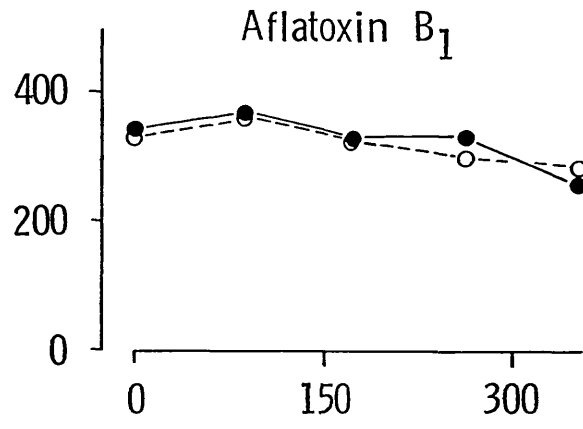
An experiment was carried out to determine whether the addition of cytosol, prepared from mice fed 5 or 20% lactalbumin, would modify the mutagenicity of microsomally-activated aflatoxin B₁, MeIQ and Trp-P-2 in the *Salmonella* mutagenicity assay (Methods section 10a). Hepatic microsomal fractions were derived from mice fed 5 or 20% lactalbumin and were from the same stock as those used for the *Salmonella* mutagenicity assay described in section 2 of this chapter. To those assays containing (in a total volume of 0.6ml) aflatoxin B₁ (1.6nmol/assay) or MeIQ (0.12nmol/assay) 5µl of microsomal suspension was added per assay (equivalent to about 60µg protein) and to samples containing Trp-P-2 (0.2nmol/assay), 3.75µl (45µg protein) of microsomal suspension was added. The concentrations of microsomes and mutagen were chosen from previous experiments, shown in Figures 16 - 18, on the basis that they would induce sufficient numbers of mutants to detect a change in activity and in addition, were positioned on the linear part of the microsomal/activation curve. Different concentrations of hepatic cytosol (0 - 500µg protein) were then added to these assays prior to the 30 min pre-incubation step.

As the concentration of cytosol increased (from 0 - 350µg protein/assay) the activation of aflatoxin B₁ declined from 350 His⁺/plate to 250 His⁺/plate (Fig. 20) although this decrease was not statistically significant. With MeIQ (0.12nmol/assay) and Trp-P-2 (0.2nmol/assay) however, the number of histidine revertants increased concomitant with the increase in cytosol concentration (p<0.001) rising (by 57%) from 700 to 1100 mutants/plate for Trp-P-2 and (by 2.2 fold) 500 to 1100 mutants/plate for MeIQ (0.12nmol/assay; Fig. 20). For the mutagen Trp-P-2, hepatic activation was maximal when cytosol was added to a concentration of 250µg cytosolic protein/assay and between 400 and 500µg of cytosolic protein/assay for MeIQ (Fig. 20).

For aflatoxin B₁ and Trp-P-2 the effect of cytosol on mutagenic activity was not dependent on the diet of the mice from which these hepatic fractions were derived (Fig. 20). However, the activity of MeIQ was significantly lower (by 7 - 14%) when cytosol from mice fed the high protein diet was added to the assay rather than when cytosol from mice fed the low protein diet was used (Fig. 20). Furthermore, in the control groups (in which no cytosol was added) the activation of both MeIQ and Trp-P-2 by microsomes from mice fed the 5% protein diet was significantly greater than those from mice fed the high protein diet (by approximately 10%) - a result that is in contrast to previous results (Fig. 18).

Assays which contained DMSO only in place of the mutagen exhibited an increase in the number of revertants/plate from 12 to 17 and 13 to 22 (using microsomes from mice fed 5% and 20% lactalbumin respectively) as the amount of cytosolic protein

Figure 20. Effect of hepatic cytosol on the microsomal activation of aflatoxin B₁ (1.6nmol/assay), Trp-P-2 (0.2nmol/assay) and MeIQ (0.12nmol/assay). Cytosolic and microsomal fractions were prepared from mice fed a 5 (●) or 20% (○) lactalbumin diet (at concentrations equivalent to 0.25g liver/ml and 1g liver/ml for cytosol and microsomes respectively). These hepatic fractions contained the pooled livers of 6 mice per sample with 4 samples prepared for each diet. The amount of microsomal fraction added per assay was 5ul for aflatoxin B₁ and MeIQ (equivalent to approximately 60µg protein) and 3.75µl (equivalent to approximately 45ug protein) for Trp-P-2 and incubated together with cytosol, *S. typhimurium* TA98, mutagen and cofactors for 30 mins at 37°C before poured, in top agar, on to Vogel Bonner plates (Methods section 10a). Results are given as means of 4 samples with triplicate plates for each sample. Comparisons between diets carried out using Anova and the LSD criterion, *** p<0.001, * p<0.05.



Cytosol concentration (µg protein/assay)

Table 21 Effect of hepatic cytosol on the reversion of *S. typhimurium* TA98 to histidine prototrophy.

| Diet % lactalbumin | His ⁺ revertants/plate | | |
|-----------------------|--|----------------|----------------|
| | Cytosolic concentration (μg protein/assay) | | |
| | 0 | 250 | 500 |
| 5 | 12.3 \pm 4.9 | 16.0 \pm 1.0 | 16.5 \pm 6.0 |
| 20 | 13.2 \pm 3.8 | 17.2 \pm 5.1 | 22.1 \pm 5.6 |

In the *Salmonella* mutagenicity assay control samples contained, per assay, 0.1ml DMSO in place of mutagen and 5 μl of microsomal suspension (see legend to Fig. 20 for further details). Results are given as means \pm SD, with comparisons between diets carried out using Anova.

increased from 0 to 500 μ g (Table 21).

Results presented in this chapter have shown that quantitative changes in dietary protein can markedly influence the *in vivo* mutagenic activity of the fungal toxin, aflatoxin B₁, but has no consistent affect on the activity of cooked food mutagens MeIQ and Trp-P-2. This dietary modification of aflatoxin metabolism would appear not to be related to diet-induced changes in the activities of hepatic enzymes involved in the activation and detoxification of this compound, but may instead, be related to changes in the uptake or disposition of aflatoxin B₁.

CHAPTER 5

*The influence of dietary fibre on metabolism of the gut flora
and on the formation and metabolism
of nitrosodimethylamine.*

The fermentable fibre pectin has been found to modify metabolism ^{of the gut flora.} A preliminary experiment was carried out to determine whether dietary pectin modifies the metabolic activities of the caecal flora of female BALB/c mice. As a result of pectin-induced changes in nitrate reductase activity in the caecal contents of BALB/c mice, the influence of dietary pectin on nitrosation reactions *in vivo* was examined. As a model, the nitrosation of aminopyrine with the production of the carcinogen nitrosodimethylamine was used. Preliminary experiments investigating the effect of dietary pectin on the genotoxicity of nitrosodimethylamine *in vivo* and *in vitro* are also reported.

Although dietary fibre can induce major changes in gut flora metabolism, it would appear from results of the limited study presented in this chapter that there was no apparent change in hepatic metabolism. To determine whether changes to the gut flora can, at their most extreme, alter hepatic metabolism, the *in vivo* mutagenicity of three dietary carcinogens (which require mammalian metabolism to be active) was assessed in germ-free mice and in mice with a conventional flora.

1. Effect of dietary pectin on gut flora metabolism.

The effect of adding 0.5%(w/w) or 5%(w/w) dietary pectin to a

control fibre-free diet on the activities of four caecal enzymes (nitrate reductase, nitroreductase, β -glucuronidase and β -glucosidase), was investigated in female BALB/c mice (refer to Method sections 8 and 9a).

The mice appeared healthy throughout the dietary regime, although at the time of sacrifice mice fed the 5% pectin diet were slightly (8%) smaller in size than mice fed the control diet. The mean body weights at this time were 16.43g, 17.06g and 17.89g for mice fed 5% pectin, 0.5% pectin and the fibre-free diet respectively (Table 22). However, mice fed 5% pectin had enlarged caeca with the mean caecal weight being about twice that of mice fed the control diet ($p < 0.01$; Table 22). The caecal weight of mice fed 0.5% pectin was very similar to that of the controls (0.062g; Table 22).

The addition of 0.5% pectin to the mouse diet had no marked effect on the activities of the four caecal enzymes compared to mice fed the fibre-free diet (Fig. 21). The 5% pectin diet produced significant changes, in particular to the activity of nitrate reductase, which was 6 fold greater (when expressed per gram caecal contents or per 10^{10} bacteria) than that in mice fed the control diet (Fig. 21). This change in the activity of nitrate reductase in pectin-fed mice was increased to 14 fold when the data was expressed in terms of the activity present in the whole animal (activity per caecum) - a calculation which takes into account diet-related differences in caecal weight (Fig. 21).

The 5% pectin diet also produced a 77% reduction in

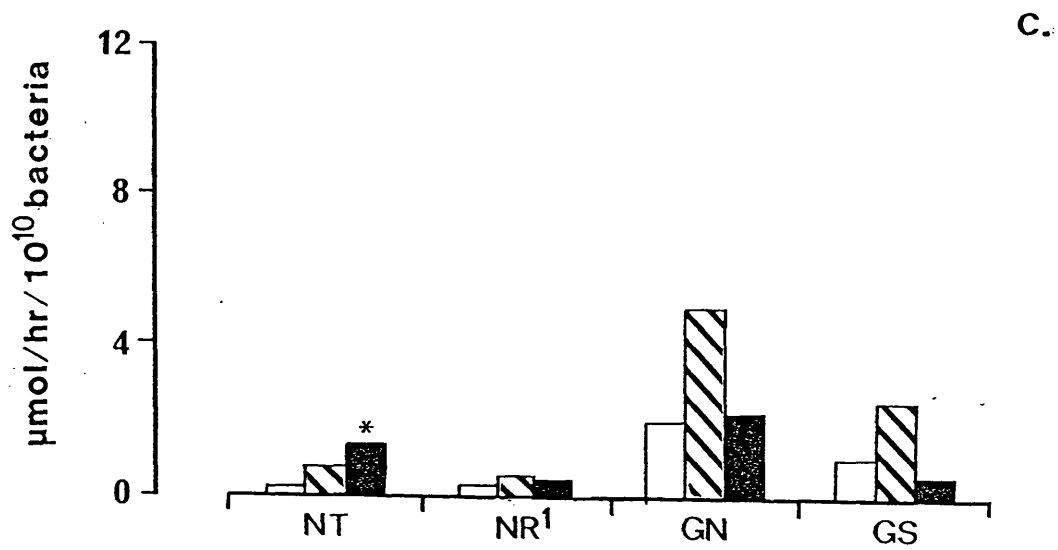
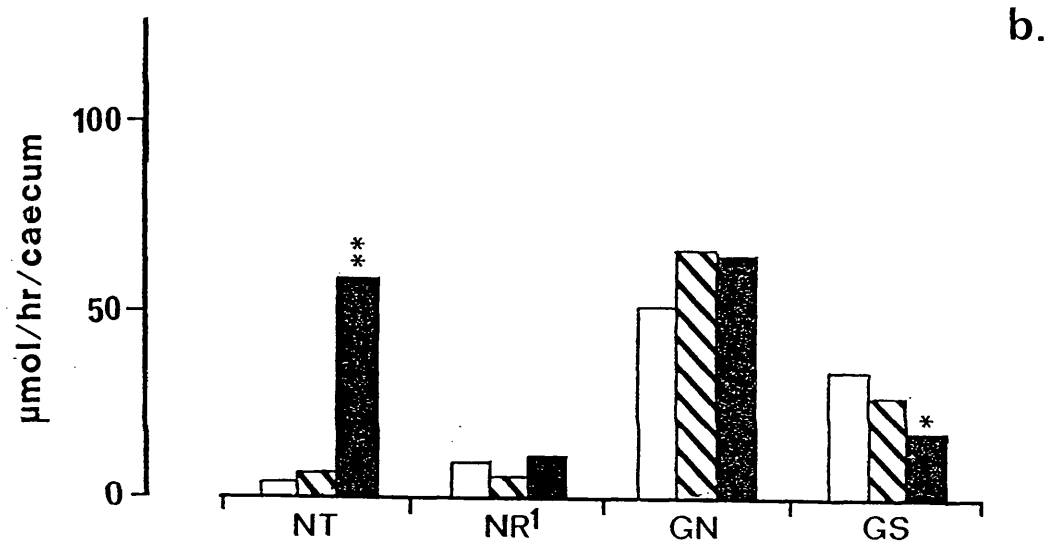
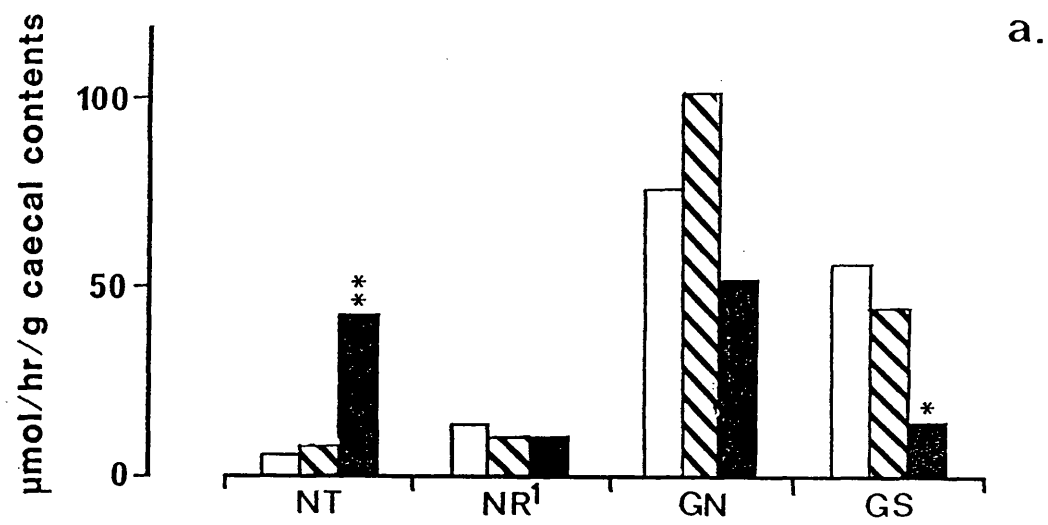
Table 22 Body, liver and caecal weights of mice fed diets containing different amounts of pectin

| | Diet % pectin | | |
|---|---------------|---------------|-----------------|
| | 0 | 0.5 | 5 |
| Body weight (g) | 17.89 ± 1.08 | 17.06 ± 1.17 | 16.43 ± 1.02** |
| Caecal weight (g) | 0.066 ± 0.015 | 0.062 ± 0.006 | 0.126 ± 0.090** |
| Bacteria/g caecal contents (x10 ¹¹) | 7.87 ± 6.32 | 2.27 ± 0.91 | 4.01 ± 2.43 |
| Liver weight (g) | 0.801 ± 0.055 | - | 0.723 ± 0.039 |
| Hepatic S9 protein (mg protein/ml S9) | 44.91 ± 9.78 | - | 42.54 ± 1.89 |
| Hepatic cytochrome P450 (nmol/g liver) | 25.58 ± 2.95 | - | 29.76 ± 5.44 |

Mice were fed different amounts of pectin as described in Methods section 4, Table 1. Caecal contents of 3 mice were pooled and suspended in KH₂PO₄ buffer, with 4 suspensions prepared for each diet (see Methods section 8a). Bacterial enumeration of the caecal contents was carried out by direct microscopic count (Methods section 9a). Hepatic S9 fractions were prepared from mice, with 4 fractions (each containing 3 pooled livers) prepared for each diet (Methods section 8b). Results are given as means ± SD. Asterisks indicate significant differences from the fibre-free (0% pectin) group, ** p<0.05 (Anova).

Figure 21 The effect of dietary pectin on mouse caecal enzyme activities. The caecal contents of mice fed different pectin diets were removed and caecal suspensions prepared (see Methods section 8a). Caecal contents from 3 mice were pooled for each caecal suspension with 4 suspensions prepared for each diet. From these freshly prepared suspensions the activities of nitrate reductase (NT) and nitroreductase (NR) were determined and using frozen suspensions β -glucuronidase (GN) and β -glucosidase (GS) were measured as described in Methods section 9a. Results are given as means and expressed as μmol of product formed: a. per g caecal contents, b. per caecum and c. per 10^{10} bacteria. Diets: fibre-free (\square), 0.5% pectin (\square), 5% pectin (\blacksquare). Asterisks indicate significant differences from the fibre-free diet; *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$.

¹ Values $\times 10$.



β -glucosidase activity and a 35% reduction in the activity of β -glucuronidase (when calculated as per gram caecal contents or as per 10^{10} bacteria) although only the difference in β -glucosidase activity per gram caecal contents was statistically significant ($p < 0.05$). When expressed per caecum (Fig. 21) the size of these differences in enzyme activity was reduced, resulting in approximately similar values (around 5 - 6 $\mu\text{mol/hr/caecum}$) for β -glucuronidase and a 50% reduction (from around 3 to 1.5 $\mu\text{mol/hr/caecum}$) in β -glucosidase activity in pectin-fed mice compared to mice fed a fibre-free diet (Fig. 21).

The rate of nitroreduction was unaffected by either pectin diet (Fig. 21) with approximately 1.1 μmol of p-aminobenzoic acid produced/hr per gram caecal contents, irrespective of diet.

The enhanced nitrate reductase activity in caeca of pectin-fed mice may, as a consequence, cause increased production of nitrite. Nitrite can combine with certain amino groups yielding carcinogenic and mutagenic N-nitrosocompounds. An investigation was carried out to determine whether mice fed pectin exhibit an increased production of these potential carcinogens. As a model for these experiments, the nitrosation of the tertiary amine aminopyrine (a non-mutagen), to the highly potent mutagen, nitrosodimethylamine, was used. This study relied, primarily, on the detection of diet-related differences in mutagenic activity in the livers of mice dosed with aminopyrine. However it was first necessary to establish whether dietary pectin modifies the reaction of aminopyrine with nitrite or if pectin influences nitrosodimethylamine mutagenicity in the liver.

2. Effect of dietary pectin on the mutagenicity of nitrosodimethylamine *in vitro* and *in vivo*.

To investigate the influence of dietary pectin on the *in vitro* activity of nitrosodimethylamine, hepatic S9 fractions were prepared from those mice fed the fibre-free and 5% pectin diets in the previous experiment. The fractions were incubated with various concentrations (0 - 50mM) of nitrosodimethylamine in the *Salmonella* mutagenicity assay with *S. typhimurium* TA100 as the indicator strain (Methods section 10a).

Dietary pectin had no significant effect on the liver weights of mice used in this study (Table 22) or on the amount of protein and cytochrome P450 present in the hepatic fractions (approximately 44 mg protein/ml S9 and 27nmol cytochrome P450/g liver respectively; Table 22).

Hepatic fractions from mice fed either diet were equally capable of activating nitrosodimethylamine to a bacterial mutagen at all but one of the concentrations (25mM) tested (Fig. 22). At this concentration of 25mM the activity of nitrosodimethylamine was 1.17 fold greater ($p < 0.05$) when S9 fractions from mice fed 5% pectin were used than when fractions from mice fed the fibre-free diet were included in the incubation mix. There was no marked diet-dependent difference in the numbers of revertants scored in control samples, containing saline in place of the mutagen, with 34 and 30 His⁺/plate counted for fractions from mice fed the fibre-free and 5% pectin diet respectively (Fig. 22). These control mutation rates also approximated the background reversion frequency of *S. typhimurium* TA100 of 33 His⁺/plate as determined on the day of the experiment.

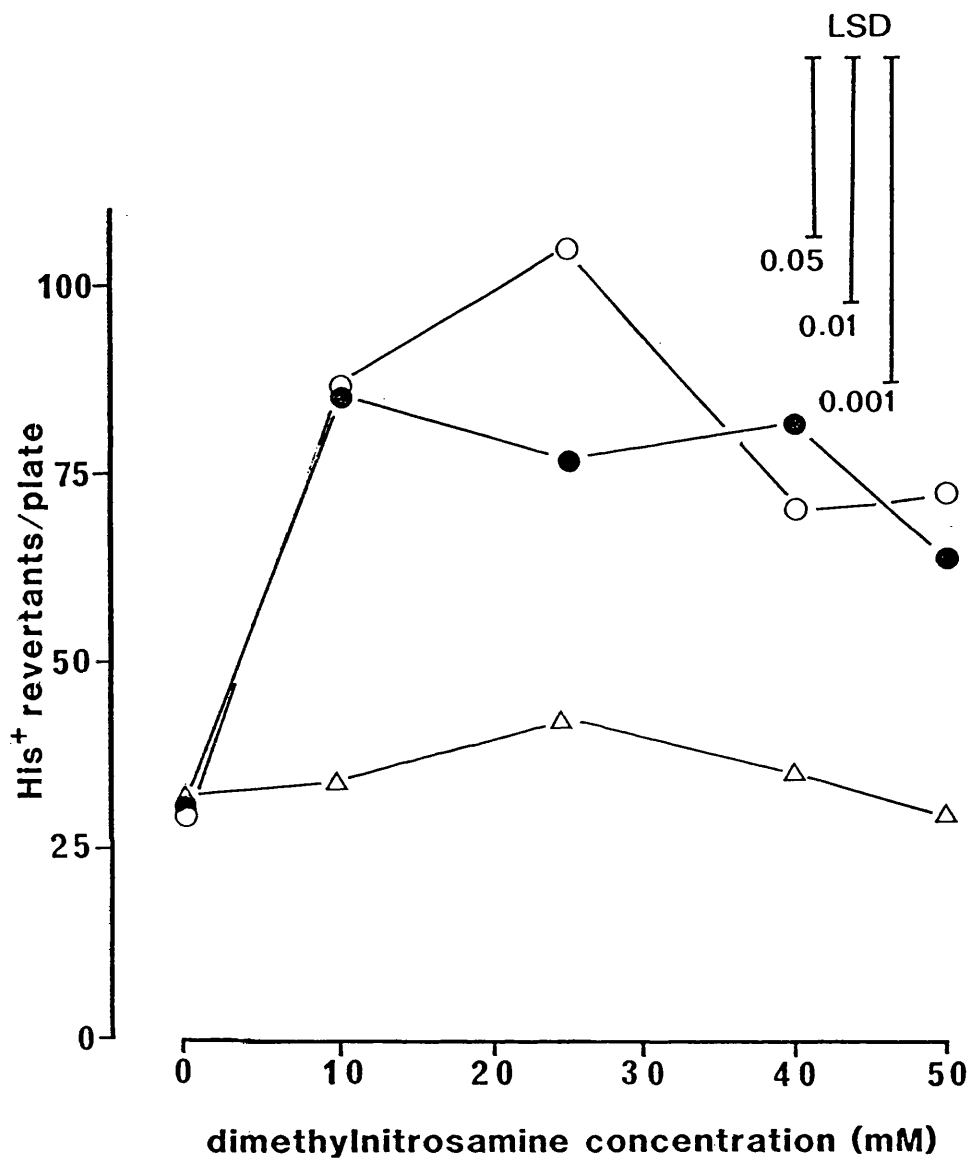


Figure 22 Activation of nitrosodimethylamine by hepatic S9 fractions from mice fed a fibre-free or 5% pectin diet. For each S9 fraction, livers from 3 mice were pooled and 4 S9 fractions (at a concentration of 0.25g liver/ml S9) prepared for each diet. An aliquot (0.06ml) of each hepatic fraction (or saline: Δ) was incubated in a final volume of 0.6ml with *S. typhimurium* TA98, cofactors and different concentrations of nitrosodimethylamine for 30 mins at 37°C before being poured, in top agar, on to Vogel Bonner plates; all assays were carried out in triplicate (Methods section 10a). Results are given as means. Statistical significance was assessed using Anova and the LSD criterion. Diets: Fibre-free (●), 5% pectin (○).

The concentration of nitrosodimethylamine that induced the greatest number of revertants was 25mM, resulting in a 3-fold increase in the number of His⁺ revertants/plate above that of the controls (samples containing saline in place of mutagen). However, this increase in His⁺ revertants was not significantly different from the other tested concentrations of nitrosodimethylamine (10, 40 and 50mM) for which 2 - 2.5 fold increases above the background were observed (Fig. 22).

The *in vivo* mutagenicity of nitrosodimethylamine (2mg[27µmol]/kg) was determined initially in the livers of female BALB/c mice fed either fibre-free, 0.5%, 2% or a 5% pectin diet, using the intrasanguineous host-mediated assay with *E. coli* D494 as the indicator strain (see Methods section 11a and Table 23, Experiment 1). There was no statistically significant diet-related difference in the hepatic mutagenicity of nitrosodimethylamine (2mg/kg) *in vivo* although the mutation rates from mice fed pectin were, on average, lower (by 15 - 26%) than those from mice fed the fibre-free diet. The greatest reduction in mutagenic activity was in mice fed 0.5% pectin (Table 23; Experiment 1.). The numbers of viable bacteria recovered from the livers of these mice was not affected by dietary pectin content as, on average, 80% of the injected dose were recovered in each diet group.

Two further host-mediated assays were carried out using mice fed a fibre-free or 0.5% pectin diet to determine whether the reduced activity of nitrosodimethylamine in mice fed 0.5% pectin was a reproducible and significant effect. In neither experiment was a

Table 23 The effect of dietary pectin on the genotoxicity of nitrosodimethylamine in the host-mediated assay

| Diet % pectin | n | Viable <i>E. coli</i> per | | | Mutation rate (Amp ^R /10 ⁹ viable cells) |
|--|---|---------------------------|------------------|-------------|--|
| | | ml homogenate | | liver | |
| | | Total (x10 ⁸) | Amp ^R | % dose | |
| <i>1. Nitrosodimethylamine (2mg/kg)</i> | | | | | |
| 0 | 4 | 10.45 ± 1.98 | 303 ± 94 | 74 ± 33 | 3085 ± 741 |
| 0.5 | 7 | 10.41 ± 2.56 | 246 ± 38 | 85 ± 18 | 2267 ± 999 |
| 2.5 | 6 | 11.99 ± 6.02 | 272 ± 75 | 89 ± 29 | 2609 ± 1068 |
| 5 | 8 | 9.53 ± 2.30 | 200 ± 61 | 70 ± 19 | 2360 ± 600 |
| <i>E. coli</i> injected/mouse: 2 x 10 ⁹ cells | | | | | |
| <i>2. Nitrosodimethylamine (2mg/kg)</i> | | | | | |
| 0 | 7 | 10.43 ± 3.57 | 180 ± 68 | 32 ± 11 | 1858 ± 1040 |
| 0.5 | 8 | 11.85 ± 5.15 | 218 ± 72 | 36 ± 14 | 1926 ± 652 |
| <i>Controls (saline only)</i> | | | | | |
| 0 | 9 | 16.63 ± 6.91 | 14 ± 7 | 50 ± 21 | 92 ± 33 |
| 0.5 | 5 | 27.19 ± 5.47 | 22 ± 8 | 84 ± 18 | 84 ± 12 |
| <i>E. coli</i> injected/mouse: 6 x 10 ⁹ cells | | | | | |
| <i>3. Nitrosodimethylamine (2mg/kg)</i> | | | | | |
| 0 | 3 | 28.60 ± 4.86 | 1083 ± 254 | 66 ± 10 | 3750 ± 303 |
| 0.5 | 5 | 22.30 ± 4.28 | 764 ± 87 | 50 ± 9 | 3466 ± 435 |
| <i>Controls (saline only)</i> | | | | | |
| 0 | 9 | 20.67 ± 3.74 | 76 ± 13 | 47.1 ± 8.0 | 370 ± 62 |
| 0.5 | 6 | 26.62 ± 7.27 | 94 ± 18 | 47.8 ± 20.2 | 365 ± 59 |
| <i>E. coli</i> injected/mouse: 8 x 10 ⁹ cells | | | | | |

In three separate experiments (1 - 3) a single intravenous injection (0.2ml) containing a combination of bacteria (*E. coli* D494) and nitrosodimethylamine in saline was given to mice (fed different diets; for body and liver weights see Table 24) in the host-mediated assay (Methods section 11a). After 3 hours mice were sacrificed, livers removed, homogenized and aliquots (0.1ml of appropriate dilutions) plated on to non-selective agar and agar selective for ampicillin resistance (Amp^R). Results are expressed as means ± SD from triplicate plates of n samples. Comparisons between mutation rates for mice fed different diets were carried out using Anova.

marked diet-related change in mutation rate observed, with mean values from both diet groups approximating, in the first experiment, 1900 Amp^R mutants/10⁹ viable bacteria and in the second, 3600 Amp^R mutants/10⁹ viable bacteria. From these above results it can be seen that the induction of ampicillin resistance, by nitrosodimethylamine, from one experiment to another can vary considerably, even though the dose of nitrosodimethylamine is unchanged (2mg/kg). This variation would seem to be unrelated to differences in mouse size since the mean body weights of mice used in these experiments were similar (approximately 16g) with the relative liver weight averaging 0.47 (Table 24). The experiments were performed in a an eight week period and used the same stock culture of *E. coli* D494 stored in DMSO at -80°C (Methods section 7a).

In control mice (given saline in place of mutagen) fed various concentrations of pectin, the *in vivo* mutation of *E. coli* to Amp^R was not affected by diet, although the mean mutation rate varied on a daily basis from 80 to 400 Amp^R mutants/10⁹ survivors (Table 23).

Nitrosodimethylamine can be metabolized to a reactive intermediate in organs other than the liver. During my studies an investigation comparing the *in vivo* mutagenic activity of nitrosodimethylamine (5mg[67µmol]/kg) in a number of organs (liver, spleen, kidney and lung) of female BALB/c mice was carried out. The greatest mutagenic activity was observed in the lung where the mean mutation rate was 3174 Amp^R mutants/10⁹ survivors (Table 25). However, there were large variations in the mutation rates obtained in this tissue, as indicated by the standard deviation value of 1820

Table 24 Body and liver weights of mice fed different amounts of pectin and used in the experiments presented in Table 23

| Expt | Diet % pectin | n | Body weight g | Liver weight g | Relative liver weight ¹ |
|--|---------------|---|---------------|----------------|------------------------------------|
| <i>1. Nitrosodimethylamine treated</i> | | | | | |
| | 0 | 4 | 16.15 ± 0.36 | 0.74 ± 0.04 | 0.46 ± 0.02 |
| | 0.5 | 7 | 17.32 ± 0.56 | 0.84 ± 0.07 | 0.48 ± 0.04 |
| | 2 | 6 | 15.45 ± 1.11 | 0.71 ± 0.04 | 0.46 ± 0.46 |
| | 5 | 8 | 16.49 ± 0.63 | 0.81 ± 0.09 | 0.49 ± 0.05 |
| <i>2. Nitrosodimethylamine treated</i> | | | | | |
| | 0 | 7 | 15.43 ± 1.63 | 0.75 ± 0.13 | 0.48 ± 0.05 |
| | 0.5 | 8 | 16.46 ± 1.63 | 0.77 ± 0.10 | 0.48 ± 0.02 |
| <i>Controls (given saline in place of mutagen)</i> | | | | | |
| | 0 | 9 | 15.11 ± 1.50 | 0.72 ± 0.14 | 0.47 ± 0.04 |
| | 0.5 | 5 | 16.68 ± 0.71 | 0.78 ± 0.08 | 0.46 ± 0.03 |
| <i>3. Nitrosodimethylamine treated</i> | | | | | |
| | 0 | 3 | 16.58 ± 1.02 | 0.79 ± 0.06 | 0.47 ± 0.01 |
| | 0.5 | 5 | 16.33 ± 1.12 | 0.73 ± 0.05 | 0.45 ± 0.04 |
| <i>Controls</i> | | | | | |
| | 0 | 9 | 16.69 ± 0.83 | 0.76 ± 0.06 | 0.45 ± 0.03 |
| | 0.5 | 6 | 17.10 ± 0.95 | 0.77 ± 0.04 | 0.45 ± 0.03 |

Results are expressed as means ± SD for each experiment (1 - 3).

¹ The liver weight per 10g body weight

Table 25 The *in vivo* mutagenicity of nitrosodimethylamine in different mouse organs

| Organ | Organ weight (g) | Viable <i>E. coli</i> per | | | Mutation Rate (Amp ^R /10 ⁹ viable cells) |
|-------|------------------|---------------------------|------------------|----------|--|
| | | ml homogenate | | organ | |
| | | Total (x10 ⁸) | Amp ^R | (% dose) | |

1. Nitrosodimethylamine (5mg/kg)

| | | | | | |
|--------|-----------|------------|------------|-----------|-----------|
| Liver | 0.96±0.09 | 10.22±2.89 | 162.9±84.6 | 56.6±12.7 | 2121± 845 |
| Lung | 0.14±0.02 | 2.58±1.87 | 69.7±36.3 | 3.0±2.1 | 3174±1820 |
| Spleen | 0.10±0.02 | 3.83±2.82 | 42.6±35.5 | 3.9±3.1 | 1149± 194 |
| Kidney | 0.26±0.03 | 0.20±0.08 | 2.7±2.4 | 0.3±0.2 | 1190± 734 |

2. Controls (saline in place of mutagen)

| | | | | | |
|--------|-----------|---------|----------|-----------|--------|
| Liver | 0.90±0.12 | 7.1±3.0 | 7.5±9.3 | 34.6±23.6 | 19±18 |
| Lung | 0.15±0.02 | 7.3±1.2 | 10.0±9.3 | 2.1±2.8 | 60±15 |
| Spleen | 0.10±0.02 | 1.2±0.5 | 13.8±7.4 | 2.4±1.1 | 125±64 |
| Kidney | 0.25±0.04 | 0.2±0.1 | ND | 0.3±0.3 | ND |

E. coli injected/mouse: 3.5 x 10⁹ cells

A single i.v. injection (0.2ml) containing a combination of nitrosodimethylamine and/or bacteria (*E. coli* D494) in saline was given to 4 female BALB/c mice in the host-mediated assay (Methods section 11a). After 3 hours mice were sacrificed, the appropriate organs removed, homogenized and aliquots (0.1ml of appropriate dilutions) plated on to non-selective agar and agar selective for ampicillin resistance. Results are expressed as means ± SD.

ND - below the level of detection.

Amp^R mutants/10⁹ survivors. *E. coli* D494 also exhibited a high mutation rate in the livers of nitrosodimethylamine-treated mice (2121 Amp^R mutants/10⁹ survivors) with the least mutagenic activity observed in the spleen and kidney with values, in both tissues, approximating 1150 Amp^R mutants/10⁹ survivors (Table 25).

In control mice (given saline in place of mutagen) the mutation rates, of *E. coli* to Amp^R, were dependent on the tissue from which the bacteria were recovered. For instance the greatest background activity was in the spleen in which 160 Amp^R mutants per 10⁹ bacteria were recorded whereas in the kidney the mutation rate was below the detection level for this assay (Table 25). The recovery of bacteria was similar in both control mice and those mice treated with nitrosodimethylamine. As expected, the greatest number of cells were located in the liver from which 40 - 50% of the injected bacteria were, on average, recovered. In the lung and spleen 2 - 3% of the injected cells were found and in the kidney the recovery rate fell to 0.3% (Table 25).

3. The effect of dietary pectin on the activity of aminopyrine and sodium nitrite in the host-mediated assay.

The previous studies indicate that the genotoxicity of nitrosodimethylamine in mouse liver (*in vivo* and *in vitro*) is not greatly influenced by dietary pectin. A further study was carried out to determine whether dietary pectin affects the reaction of aminopyrine and sodium nitrite *in vivo*, by measuring hepatic mutagenicity in mice dosed with these compounds. In this experiment different concentrations of aminopyrine (15 - 60mg/kg) were administered *per os* with sodium nitrite in the host-mediated

assay using *E. coli* D494 (Methods section 11a). The reaction of aminopyrine with sodium nitrite is such that 2 moles of sodium nitrite react per mole of aminopyrine, with the production of 1 mole of nitrosodimethylamine. In order to provide sufficient nitrite for optimal activation of aminopyrine to occur, equivalent amounts, by weight, of aminopyrine and nitrite were dosed which provided approximately 4 moles of sodium nitrite (MW: 69.0) for each mole of aminopyrine (MW: 231.3).

From Fig. 23 and Table 26 it can be seen that a 5% pectin diet had no significant effect on the *in vivo* mutagenicity of various doses (15, 22, 30 or 60 mg/kg body weight) of aminopyrine and sodium nitrite. A further study (Table 26; Experiment 2), similar to that described above but used only a single dose of aminopyrine and nitrite (30mg/kg of each compound), also failed to detect any influence of dietary pectin on the observed mutagenic activity present in the liver, since a mutation rate of 1860 ± 1310 Amp^R mutants/10⁹ viable bacteria (mean \pm SD) was recorded for mice fed the control fibre-free diet and 2150 ± 1453 mutants for pectin-fed animals (Table 26).

The greatest mutagenic response recorded in mice dosed with different amounts of aminopyrine and sodium nitrite (Table 26) was for the 22mg/kg dose which gave rise to a mutation rate of around 2300 Amp^R mutants/10⁹ bacteria. At the higher concentrations, 30 and 60mg aminopyrine and nitrite/kg, mutagenic activity declined by a maximum of 15 and 52% respectively compared to those mice given the 22mg/kg dose (Fig. 23, Table 26). Similar dose response curves were obtained in two earlier experiments in mice fed only the

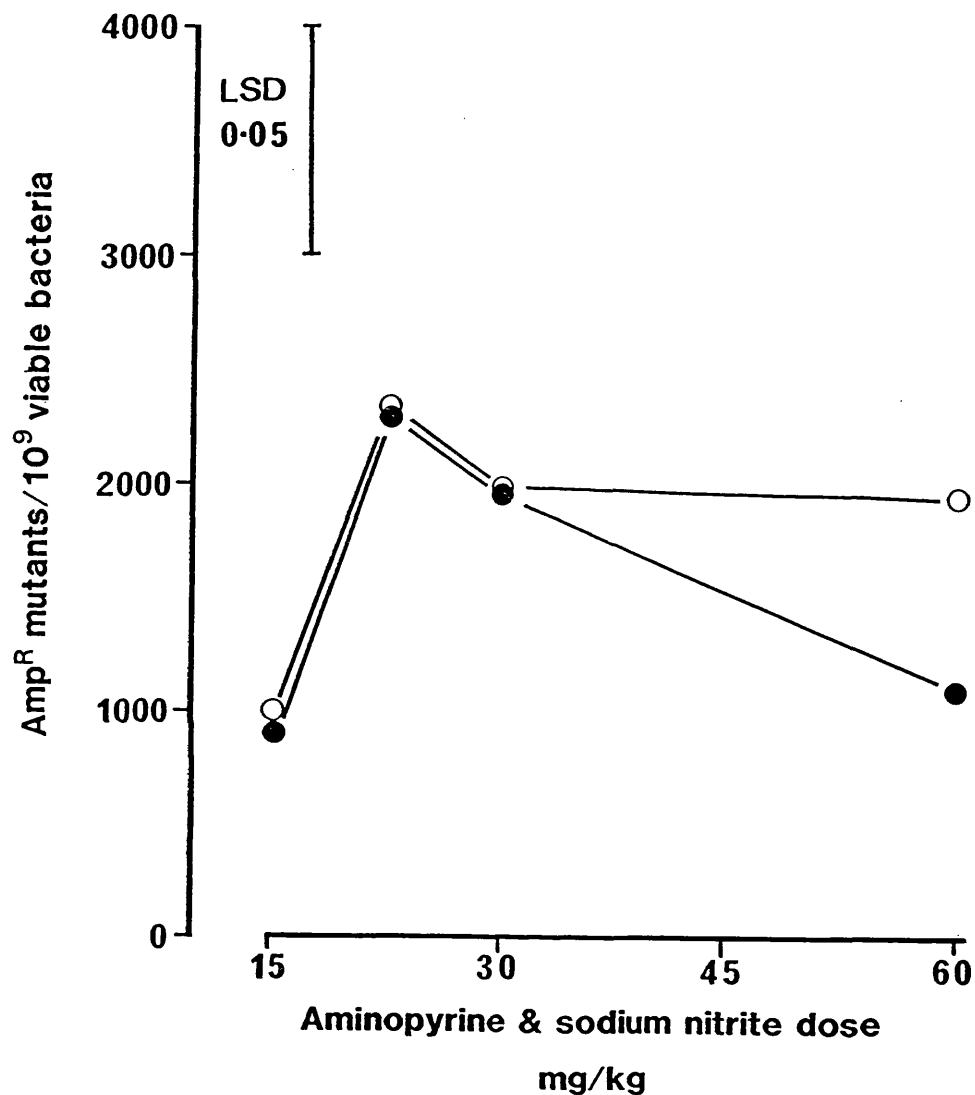


Figure 23 The influence of dietary pectin on the nitrosation of aminopyrine to a bacterial mutagen. Mice fed either a fibre-free or 5% pectin diet were injected iv with *E. coli* D494 and orally with different concentrations of aminopyrine and sodium nitrite in the host-mediated assay. For further details refer to Table 26 and Methods section 11a. Results are given as means from 4 mice. Comparisons between diets were carried out using Anova and the LSD criterion. Diets: Fibre-free (●); 5% pectin (O).

Table 26 Effect of dietary pectin on the activity of aminopyrine (AP) and sodium nitrite (NO₂) in the host-mediated assay

| Expt | Diet | Dose mg/kg | | n | Viable <i>E. coli</i> per | | Mutation rate (Amp ^R /10 ⁹ viable cells) | |
|------|------------|------------|-----------------|---|---|--------|--|-----------|
| | | AP | NO ₂ | | ml homogenate | liver | | |
| | | AP | NO ₂ | | Total(x10 ⁸) Amp ^R | % dose | | |
| 1. | Fibre-free | 15 | 15 | 4 | 10.7±2.3 | 78±48 | 64±15 | 906±258 |
| | 5% pectin | 15 | 15 | 4 | 7.6±2.9 | 85±52 | 45±17 | 991±470 |
| | Fibre-free | 22 | 22 | 4 | 7.1±1.2 | 142±35 | 41±9 | 2316±1006 |
| | 5% pectin | 22 | 22 | 4 | 5.7±1.6 | 155±74 | 34±9 | 2354±969 |
| | Fibre-free | 30 | 30 | 4 | 6.6±2.9 | 111±45 | 38±18 | 1980±955 |
| | 5% pectin | 30 | 30 | 4 | 8.3±2.1 | 148±16 | 46±13 | 2013±383 |
| | Fibre-free | 60 | 60 | 4 | 6.3±1.2 | 68±33 | 37±7 | 1115±648 |
| | 5% pectin | 60 | 60 | 4 | 8.3±1.3 | 162±46 | 49±7 | 1984±607 |
| 2. | Fibre-free | 0 | 30 | 9 | 23.4±6.3 | 18±11 | 75±31 | 79±53 |
| | 5% pectin | 0 | 30 | 8 | 31.4±9.7 | 35±12 | 93±42 | 98±51 |
| | Fibre-free | 30 | 30 | 8 | 29.8±8.2 | 109±45 | 85±37 | 364±151 |
| | 5% pectin | 30 | 30 | 8 | 27.2±7.5 | 136±65 | 83±29 | 416±298 |

E. coli injected/mouse: 2.8 x 10⁹ cells

In 2 experiments (1 & 2) mice were injected i.v. with a suspension (0.2ml) of *E. coli* D494 immediately prior to an injection p.o. of aminopyrine and sodium nitrite in saline in the host-mediated assay (Methods section 11a). After 3 hours mice were sacrificed, livers removed, homogenized and aliquots (0.1ml of appropriate dilutions) plated on to non-selective agar and agar selective for ampicillin resistance (Amp^R). Results are given as means ± SD, from triplicate plates of n samples (see Fig. 23). Comparisons between mutation rates for mice fed different diets were carried out using Anova.

Table 27 Dose response of aminopyrine (AP) and sodium nitrite (NO₂) in the host-mediated assay

| Expt | Dose mg/kg | | n | Viable <i>E. coli</i> per | | | Mutation rate (Amp ^R /10 ⁹ viable cells) |
|------|----------------|-----------------|---|---------------------------|------------------|---------|--|
| | AP | NO ₂ | | ml homogenate | | liver | |
| | | | | Total(x10 ⁸) | Amp ^R | % dose | |
| 1. | 0 ¹ | 0 ¹ | 4 | 10.5 ± 3.2 | 47 ± 9 | 52 ± 17 | 472 ± 88 |
| | 58 | 58 | 5 | 7.5 ± 1.6 | 192 ± 44 | 38 ± 7 | 2624 ± 657 |
| | 115 | 115 | 5 | 8.8 ± 2.0 | 73 ± 26 | 43 ± 11 | 831 ± 306 |
| 2 | 0 ¹ | 0 ¹ | 4 | 11.3 ± 1.1 | 87 ± 16 | 54 ± 5 | 751 ± 59 |
| | 15 | 15 | 4 | 13.0 ± 3.6 | 174 ± 78 | 58 ± 12 | 1491 ± 1000 |
| | 30 | 30 | 4 | 11.6 ± 4.6 | 207 ± 41 | 54 ± 18 | 1868 ± 550 |
| | 60 | 60 | 4 | 12.4 ± 5.3 | 133 ± 22 | 59 ± 22 | 1219 ± 523 |
| 3 | 0 ¹ | 0 ¹ | 3 | 14.2 ± 4.6 | 46 ± 9 | 56 ± 2 | 326 ± 36 |
| | 0 ¹ | 50 | 3 | 10.7 ± 3.3 | 48 ± 15 | 42 ± 13 | 468 ± 147 |
| | 12 | 12 | 3 | 13.6 ± 2.8 | 48 ± 32 | 56 ± 16 | 406 ± 169 |
| | 25 | 25 | 3 | 12.1 ± 1.2 | 63 ± 26 | 48 ± 23 | 540 ± 198 |
| | 50(pH3) | 50 | 4 | 11.7 ± 2.6 | 60 ± 32 | 45 ± 6 | 623 ± 434 |

E. coli injected/mouse: 3 - 4 x 10⁹ cells

In 3 experiments (1 - 3) mice were injected i.v. with a suspension (0.2ml) of *E. coli* D494 immediately prior to an injection p.o. of aminopyrine and sodium nitrite in saline in the host-mediated assay (Methods section 11a). After 3 hours mice were sacrificed, livers removed, homogenized and aliquots (0.1ml of appropriate dilutions) plated on to non-selective agar and agar selective for ampicillin resistance (Amp^R). Results are given as means ± SD, from triplicate plates of n samples. Comparisons between mutation rates for mice fed different diets were carried out using Anova.

¹ Mice given saline in place of the aminopyrine or nitrite.

fibre-free diet. In the first, 2 doses of aminopyrine and nitrite (58mg/kg and 115mg/kg) were administered, giving rise to mean mutation rates of 2624 and 831 Amp^R mutants/10⁹ bacteria respectively (Table 27, experiment 1) whereas for the controls (mice given saline in place of aminopyrine and nitrite) a mutation rate of 472 Amp^R mutants/10⁹ survivors was obtained. In the second experiment, lower doses of aminopyrine and nitrite were used (15, 30 and 60 mg/kg; Table 27, experiment 2). Maximum mutagenicity occurred when a dose of 30mg of aminopyrine and nitrite/kg was administered to mice with a mutation rate of 1865 Amp^R mutants/10⁹ bacteria recorded. As before, the mutation rate of the 60mg/kg dose was lower, by 35%, than that for the 30mg/kg dose (Table 27). In mice given an oral dose containing nitrite (50mg/kg) only, the mutation rate of *E. coli* D494 was 1.4 fold greater than that of mice given saline only (Table 27, experiment 3) although this was not statistically significant.

To investigate the decline in the *in vivo* activity of aminopyrine and nitrite at doses greater than 30mg/kg, a further experiment was carried out. In this experiment the pH of a 50mg aminopyrine/kg dose was reduced from its normal pH value of 7.5 to pH 3.0 (using 1M HCl) and administered to mice *per os* immediately followed by an oral dose of sodium nitrite (50mg/kg). The results showed that the mutation rate obtained for mice dosed with 50mg aminopyrine was not less (as had been observed previously), but 15% greater, than that for the 25mg/kg dose of aminopyrine and nitrite (Table 27, experiment 3). However, a control group comprising mice administered with 50mg aminopyrine (pH 7.5) and nitrite/kg was not included in the experiment, so this result can only indicate

that the reduction in hepatic mutagenicity at high aminopyrine concentrations is associated with an increased stomach pH.

It would appear from results presented in this chapter that dietary pectin has no marked influence on the nitrosation of aminopyrine to a bacterial mutagen in the presence of excess sodium nitrite. Investigations then continued to determine the effect of dietary pectin on aminopyrine nitrosation *in vivo* when the availability of nitrite is limited. In a preliminary host-mediated assay aminopyrine (30mg/kg) was administered orally to mice (fed a fibre-free or 5% pectin diet) with 30 or 15mg sodium nitrite/kg (Table 28). It was found that, under these experimental conditions, the 50% reduction in the concentration of nitrite (from 30 to 15 mg/kg) still provided sufficient nitrite for maximal nitrosation of aminopyrine to occur as a mean mutation rate of around 1000 Amp^R mutants/10⁹ survivors was obtained for both groups of mice, irrespective of diet (Table 28, Experiment 1). However in mice dosed with aminopyrine only (30mg/kg) the mutation rate was similar to the background mutation rate of the dosing suspension (4 Amp^R mutants/10⁹ viable bacteria).

In a second experiment mice were dosed with 22mg aminopyrine /kg together with 22, 10 or 5 mg sodium nitrite/kg (Fig. 24, Table 28 Experiment 2). Concomitant with the decline in nitrite concentration (from 20mg/kg to 10 and 5mg/kg) there was a decline in the mutation rate, of 30 and 73% respectively, with values falling from 1200 Amp^R mutants/10⁹ viable bacteria in mice dosed with 22mg aminopyrine and nitrite/kg to about 300 in those mice dosed with 5mg sodium nitrite/kg. In neither of the above experiments (Table 28)

Table 28 Effect of altering nitrite (NO₂) concentration on the nitrosation of aminopyrine (AP) to a bacterial mutagen in the host-mediated assay

| Expt | Dose mg/kg | | Diet | n | Viable <i>E. coli</i> ml homogenate liver | | | Mutation rate (Amp ^R /10 ⁹ viable cells) | |
|--|---|-----------------|------------|------------|--|------------------|--------|--|----------|
| | AP | NO ₂ | | | Total(x10 ⁸) | Amp ^R | % dose | | |
| 1. | 30 | 30 | Fibre-free | 3 | 20.3±7.7 | 132±22 | 73±15 | 822±246 | |
| | 30 | 30 | 5% pectin | 4 | 16.6±4.8 | 163±41 | 60±19 | 1094±294 | |
| | 30 | 15 | Fibre-free | 6 | 8.8±3.0 | 97±32 | 32±11 | 1147±314 | |
| | 30 | 15 | 5% pectin | 8 | 13.6±3.2 | 137±17 | 52±12 | 1015±385 | |
| | 30 | 0 | Fibre-free | 6 | 23.1±9.2 | 1.2±1.6 | 64±26 | 3.8±4.4 | |
| | 30 | 0 | 5% pectin | 5 | 18.1±3.8 | 1.4±1.5 | 65±11 | 8.6±8.6 | |
| | <i>E. coli</i> injected/mouse: 4.88 x 10 ⁹ cells | | | | | | | | |
| | 2. | 22 | 22 | Fibre-free | 3 | 15.0±3.4 | 190±77 | 83±19 | 1225±333 |
| 22 | | 22 | 5% pectin | 6 | 12.5±4.5 | 141±34 | 65±23 | 1251±426 | |
| 22 | | 10 | Fibre-free | 4 | 10.2±1.6 | 83±29 | 48± 8 | 936±177 | |
| 22 | | 10 | 5% pectin | 6 | 8.2±3.5 | 70±49 | 38±17 | 821±313 | |
| 22 | | 5 | Fibre-free | 5 | 9.9±4.3 | 31±16 | 47±22 | 373±263 | |
| 22 | | 5 | 5% pectin | 5 | 8.4±1.5 | 17±10 | 36± 5 | 284± 54 | |
| <i>E. coli</i> injected/mouse: 3.4 x 10 ⁹ | | | | | | | | | |

In 2 experiments (1 & 2) mice were injected i.v. with a suspension (0.2ml) of *E. coli* D494 immediately prior to an injection p.o. of aminopyrine and sodium nitrite in saline in the host-mediated assay (Methods section 11a). After 3 hours mice were sacrificed, livers removed, homogenized and aliquots (0.1ml of appropriate dilutions) plated on to non-selective agar and agar selective for ampicillin resistance (Amp^R). Results are given as means ± SD, from triplicate plates of n samples. Comparisons between mutation rates for mice fed different diets were carried out using Anova.

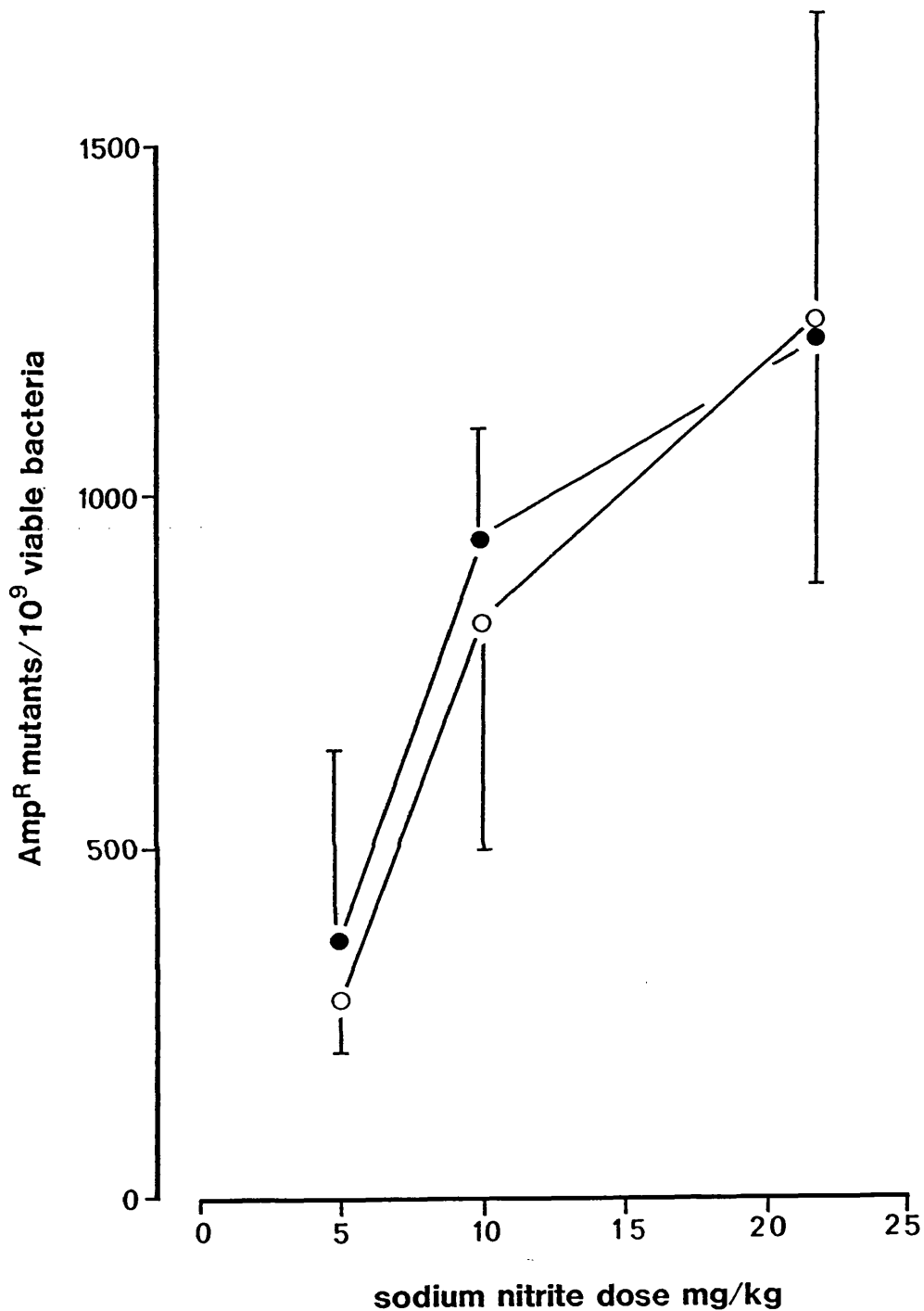


Figure 24 Effect of changes in nitrite concentration on the nitrosation of aminopyrine to a bacterial mutagen. Mice fed either a fibre-free or 5% pectin diet were injected iv with *E. coli* D494 and orally with 22 mg aminopyrine/kg body weight together with different concentrations of sodium nitrite in the host-mediated assay. For further details refer to Table 28 and Methods section 11a. Results are given as means of 3-6 mice. Comparisons between diets were carried out using Anova and the LSD criterion. Diets: Fibre-free (●); 5% pectin (○).

was dietary pectin (5%) found, at any time, to affect the mutation rate to ampicillin resistance, despite nitrite being limiting in certain groups (Fig. 24, Table 28).

4. Effect of dietary pectin on the activity of aminopyrine and sodium nitrate in the host-mediated assay.

The fibre-free and 5% pectin diets were devoid of nitrite and nitrate (Table 1) so the only sources of nitrite available to mice fed these diets were either from the drinking water or from endogenous production. Since the mutation rates from mice dosed with aminopyrine only, in the host-mediated assay, were similar to that of mice dosed with saline (Table 28) it would suggest that the amount of nitrite available for reaction with aminopyrine was insufficient to be detected (as a mutagenic response) in this *in vivo* assay. As the caecal flora was able to reduce nitrate to nitrite via the enzyme nitrate reductase (the activity of which was diet-dependent; see section 1 of this chapter), a further study was carried out in which mice, fed a fibre-free or 5% pectin diet, were given sodium nitrate in the form of an oral dose 75 mins prior to an oral dose of aminopyrine and sodium nitrite. The dose of nitrite (10mg sodium nitrite/kg) was insufficient to allow maximum nitrosation of aminopyrine (22mg/kg) to a bacterial mutagen to occur (see previous section & Table 28) and the dose of sodium nitrate, 1.25g/kg, was that determined by other members of the department at BIBRA to be the maximum dose to be tolerated by mice over 24 hours. The resulting mutation rates, for this experiment, exhibited marked variations from mouse to mouse as reflected by the large standard deviations (Table 29). Consequently no apparent, significant, differences in mutation rates were detected between mice given

Table 29 Effect of an acute dose of sodium nitrate on the nitrosation of aminopyrine (AP) to a bacterial mutagen

| Diet | n | Stomach pH | Viable <i>E. coli</i> per | | Mutation rate | |
|---|---|------------|---------------------------|------------------|--|---------|
| | | | ml homogenate | liver | (Amp ^R /10 ⁹ viable cells) | |
| | | | Total(x10 ⁸) | Amp ^R | % dose | |
| 1. Sodium nitrate-treated | | | | | | |
| Fibre-free | 3 | 4.8±0.8 | 20.9±7.4 | 19± 9 | 86±30 | 142± 68 |
| 5% pectin | 5 | 4.9±1.4 | 28.9±5.4 | 60±52 | 91±34 | 203±210 |
| 2. Controls (saline in place of nitrate) | | | | | | |
| Fibre-free | 5 | 4.6±0.5 | 20.6±10.4 | 60±50 | 77±39 | 410±292 |
| 5% pectin | 5 | 3.5±1.5 | 19.3± 7.9 | 82±81 | 82±32 | 378±345 |
| <i>E. coli</i> dosed/mouse: 4 x 10 ⁹ cells | | | | | | |

Mice were given an oral dose of sodium nitrate (1.25g/kg), 75mins prior to an oral dose of aminopyrine (22mg/kg) and sodium nitrite (10mg/kg) and an iv injection of *E. coli* D494 in the host-mediated assay (Methods section 11a). After 3 hrs, mice were sacrificed, livers removed, homogenised and aliquots (0.1ml of appropriate dilutions) plated on to non-selective agar and agar selective for ampicillin resistance (Amp^R). Results are given as means ± SD, from triplicate plates for n samples. Stomach pH was assessed by placing pH indicator paper in to stomach contents.

Table 30 Effect of chronic dosing of nitrate on the nitrosation of aminopyrine *in vivo*.

| NaNO ₃ concn. (%) | Dose (mg/kg) | | Viable <i>E. coli</i> / ml homogenate (x10 ⁷) | | Relative survival (%) |
|------------------------------------|--------------|-----------------|--|-------------|-----------------------------|
| | AP | NO ₂ | 343/765 | 343/753 | |
| 0 | 0 | 0 | 50.4 ± 48.7 | 55.2 ± 58.9 | 103 ± 16 |
| 0 | 22 | 0 | 51.7 ± 21.4 | 49.0 ± 22.0 | 93 ± 11 |
| 0.5 | 0 | 0 | 63.9 ± 18.3 | 68.9 ± 19.8 | 105 ± 19 |
| 0.5 | 22 | 0 | 47.1 ± 8.9 | 56.0 ± 13.7 | 108 ± 26 |
| 2.0 | 0 | 0 | 56.4 ± 23.3 | 59.8 ± 25.4 | 105 ± 34 |
| 2.0 | 22 | 0 | 71.4 ± 13.2 | 72.0 ± 19.2 | 103 ± 10 |
| 0 | 22 | 22 | 75.9 ± 10.5 | 21.4 ± 10.6 | 28 ± 9 |

E. coli injected per mouse : 1 x 10⁸ per strain

Mice were given sodium nitrate (NaNO₃) in the drinking water (sterile) for 5 days at a concentration of either 0.5 or 2% (w/w). At the end of 5 days, the mice (and mice given no nitrate) were injected iv with bacteria (*E. coli* 343/765 and 343/753) immediately followed by an oral dose of aminopyrine (AP) or aminopyrine and sodium nitrite (NO₂). After 90 mins mice were killed, livers removed and homogenized in PBS-S. To assess genotoxic activity aliquots of appropriate dilutions of homogenate were plated on to NRS-S plates and incubated for 24hr at 37°C. The numbers of colonies of the two bacterial strains were then counted and survival of the repair deficient strain (343/753) relative to the repair proficient strain (343/765) was calculated and expressed as a percentage. Results are given as means ± SD from 5 mice. For further details see Methods section 11b. Results are given as means ± SD of 5 mice.

sodium nitrate and the control mice (given saline in place of the sodium nitrate) or between animals fed the 5% pectin and fibre-free diets (Table 29).

In an earlier experiment, which was designed to elevate chronically the *in vivo* concentration of nitrite, mice were given *ad libitum*, for a 5 day period, sterile distilled water or sterile solutions of 2% or 0.5% sodium nitrate (concentrations of nitrate known to induce methaemoglobinaemia in rats - personal communication I.R. Rowland). On the fifth day of providing sodium nitrate in the drinking water, mice were dosed, *per os*, with either saline or 22mg aminopyrine/kg, in the host-mediated assay using the paired indicator strains *E. coli* 343/765 and 343/753. A positive control, mice dosed with 22mg aminopyrine and sodium nitrite/kg, was also included in the experiment. The oral dose of sodium nitrate had no apparent affect on the *in vivo* mutagenicity of aminopyrine. In fact, the only detectable increase in genotoxic activity in this experiment, as measured by a reduction in the relative survival of *E. coli* 343/753 was in the positive control (Table 30) - mice dosed with aminopyrine and nitrite (22mg/kg).

5. The sensitivity of the DNA-repair host-mediated assay to detect changes in the nitrosation of aminopyrine.

The results presented in the above sections suggest that feeding pectin to mice has no apparent affect on the amount of nitrite available for reaction with aminopyrine (as measured by the host-mediated assay). An experiment was carried out to determine what degree of change in nitrite concentration would be required to detect a change in genotoxic activity in mice dosed with

aminopyrine. Mice fed a fibre-free diet were given an oral dose of 22mg aminopyrine/kg together with 6, 7, 8 or 9mg sodium nitrite/kg. In mice dosed with 6 or 7mg/kg sodium nitrite, a 30% reduction in the relative survival of *E. coli* 343/753 was observed compared to the ratio of the two strains in the bacterial dosing suspension (Fig. 25, Table 31). At the two higher doses of sodium nitrite, 8 and 9mg/kg, further decreases in the relative survival of the repair deficient strain (of 7 and 10%) were apparent although these differences in relative survival were not statistically significant (Fig. 25, Table 31). Under these experimental conditions, it can be seen that the minimum change in nitrite concentration that produces a detectable change in genotoxic activity in the host-mediated assay is equivalent to a 2mg/kg oral dose of sodium nitrite (which, in this experiment, corresponds to approximately 0.5 μ mol of sodium nitrite or a decrease of 20 - 30% in the amount of nitrite). These results indicate that the DNA-repair host-mediated assay is not a sensitive method to detect small differences in nitrite concentration.

6. Effect of a nitrosation catalyst on the genotoxic activity of aminopyrine and nitrite in the host-mediated assay.

Thiocyanate can catalyse nitrosation reactions. Consequently, the influence of potassium thiocyanate on the *in vivo* reaction of aminopyrine and nitrite was assessed using the host-mediated assay with *E. coli* 343/765 and 343/753 as indicator. The results show (Table 32) that an oral dose of potassium thiocyanate, at a concentration of 120mg of potassium thiocyanate/kg, is not itself genotoxic since the relative survival of *E. coli* 343/753 in this group of mice was similar to that of the controls (mice given saline

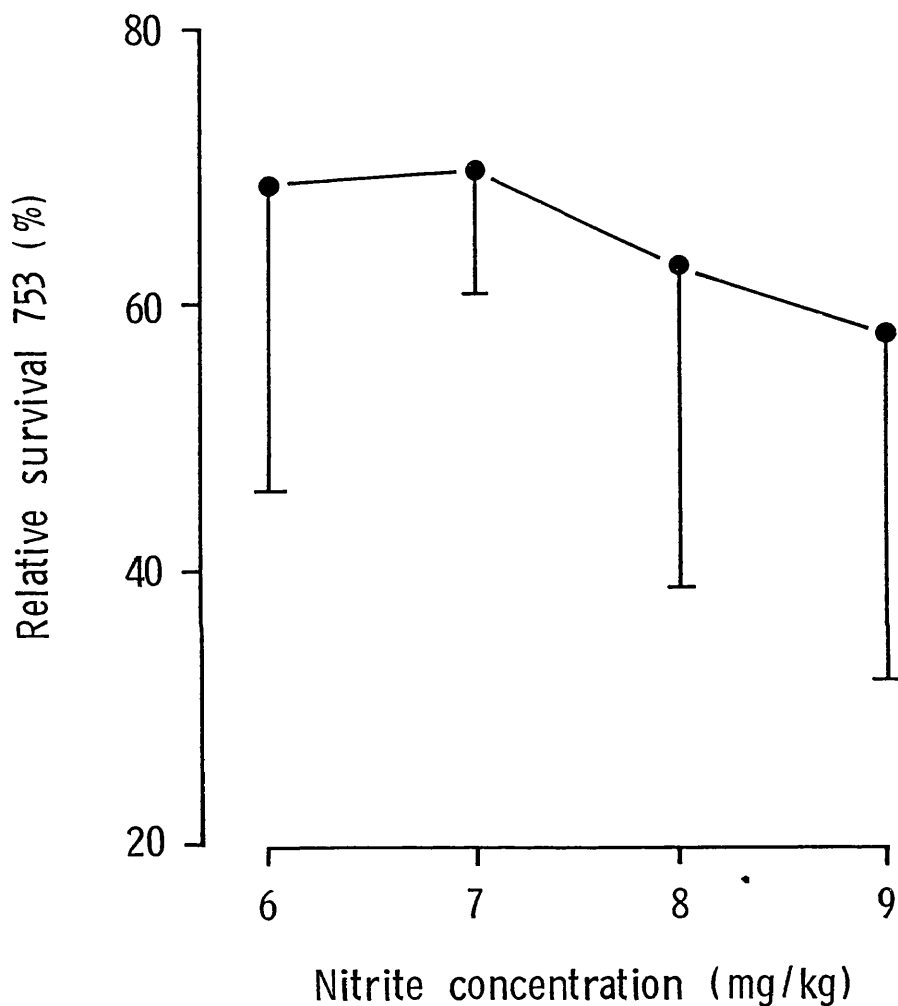


Figure 25 The sensitivity of the DNA-repair host-mediated assay in detecting changes in the nitrosation of aminopyrine to a genotoxin. Mice were given an iv injection of *E. coli* 343/765 and 343/753 and an oral injection of aminopyrine with different concentrations of sodium nitrite in the host-mediated assay. For further details refer to Table 31 and Methods section 11b. Results are given as means \pm SD of 5 mice. The ratio of *E. coli* 343/753 to 343/765 in the dosing suspension was equivalent to 100%.

Table 31 Ability of the DNA-repair host-mediated assay to detect changes in the nitrosation of aminopyrine to a genotoxin.

| Sodium nitrite concentration (mg/kg) | Viable <i>E. coli</i> / ml homogenate (x10 ⁷) | | Relative survival (%) |
|--------------------------------------|---|------------|-----------------------|
| | 343/765 | 343/753 | |
| 6 | 15.1 ± 9.9 | 7.72 ± 7.0 | 58.8 ± 26.5 |
| 7 | 17.4 ± 5.1 | 10.2 ± 3.7 | 63.2 ± 23.9 |
| 8 | 16.6 ± 5.0 | 10.2 ± 4.0 | 70.0 ± 9.6 |
| 9 | 19.0 ± 3.4 | 11.7 ± 4.1 | 68.7 ± 20.3 |

E. coli injected per mouse: 1 x 10⁹ of each strain

Mice were given an iv injection of bacteria (*E. coli* 343/765 and 343/753) immediately followed by an oral dose of aminopyrine (22mg/kg) together with different concentrations of sodium nitrite. After 90 mins the mice were killed, livers removed and homogenized in PBS-S. To assess genotoxic activity, aliquots of appropriate dilutions of each homogenate were plated on to NRS-S agar and incubated at 37°C for 24hr. The numbers of colonies of each strain were then counted and the survival of the repair deficient strain (343/753) relative to the repair proficient strain (343/765) was calculated and expressed as a percentage. For further details see Methods section 11b and Fig. 25. Results are given as means ± SD of 5 mice.

Table 32 Effect of thiocyanate on the nitrosation of aminopyrine *in vivo*

| Dose (mg/kg) | | Relative survival 343/753 (%) ¹ | |
|--------------|-----------------|--|--------------|
| AP | NO ₂ | Potassium thiocyanate (mg/kg) | |
| | | 0 | 120 |
| 22 | 0 | 104.3 ± 11.5 | 109.6 ± 13.4 |
| 22 | 7 | 38.2 ± 19.7 | 33.8 ± 11.2 |
| 22 | 8 | 30.3 ± 10.9 | 26.6 ± 19.7 |

Mice were given an iv injection of bacteria (*E. coli* 343/765 and 343/753) immediately followed by an oral dose containing potassium thiocyanate and/or aminopyrine (AP) or aminopyrine and sodium nitrite (NO₂). After 90 mins, mice were killed the livers removed and homogenized in PBS-S. Aliquots of appropriate dilutions were then plated on to NRS-S agar. For further details of method, refer to Methods section 11b).

¹ Survival of the repair deficient strain (343/753) relative to the repair proficient strain (343/765) and expressed as a percentage.

only *per os*). In those mice dosed with aminopyrine (22mg/kg) and nitrite (7 or 8mg/kg) the relative survival decreased to approximately 36% and 29%, for the 7 and 8mg/kg doses of sodium nitrite respectively, with thiocyanate having no statistically significant effect on these values (Table 32).

7. Effect of germ-free status on the *in vivo* mutagenicity of food mutagens.

The role of the gut flora on the mutagenicity of the carcinogens aflatoxin B₁ (5mg/kg), MeIQ (0.5mg/kg and 10mg/kg) and Trp-P-2 (10mg/kg) in the livers of mice was assessed using germ-free and conventional flora female BALB/c mice (see Methods section 2) in the host-mediated assay with *S. typhimurium* TA98 as indicator (Methods section 11c).

Body and liver weights of the germ-free animals were found to be smaller, by 16% and 21% respectively ($p < 0.001$) than of those with a conventional flora (Table 33), although the relative liver weight was similar (approximately 0.47) in both groups.

In the control mice (given DMSO in place of mutagen) the presence of a gut flora had no effect on the reversion to histidine prototrophy of *S. typhimurium* TA98. However, in mutagen-treated animals the number of revertants/plate was consistently lower for the germ-free animals than for mice with a conventional flora (Table 33). This reduction was particularly marked for the fungal metabolite aflatoxin B₁ ($p < 0.001$) as the mutagenicity of this compound declined, by 52%, from an average of 142 His⁺/plate for conventional flora animals to 68 His⁺/plate in the germ-free group

Table 33 Body and liver weights and genotoxicity of aflatoxin B₁, MeIQ and Trp-P-2 in conventional and germ-free mice in the host-mediated assay

| Mutagen (dose) | His ⁺ revertants/plate | |
|--------------------------------------|-----------------------------------|-----------------------|
| | Conventional | Germ-free |
| Aflatoxin B ₁ (5mg/kg) | 142 ± 59 (5) | 68 ± 36*** (5) |
| MeIQ (0.5mg/kg) | 298 ± 233 (5) | 156 ± 111* (5) |
| MeIQ (10mg/kg) | 2019 ± 212 (4) | 1216 ± 351 *** (5) |
| Trp-P-2 (10mg/kg) | 804 ± 175 (3) | 558 ± 271** (5) |
| Controls (DMSO only) | 23 ± 6 (5) | 26 ± 6 (5) |
| Body weight g | 20.30 ± 1.17 | 17.06 ± 2.36*** |
| Liver weight g | 0.99 ± 0.10 | 0.78 ± 0.13*** |
| Relative liver weight ¹ | 0.48 ± 0.06 | 0.46 ± 0.06 |

Results are expressed as means ± SD, n in parenthesis. Asterisks indicate a significant difference from mice with a conventional flora, *** represents p<0.001, ** p<0.01, * p<0.05; Anova.

¹ The liver weight per 10g body weight.

Table 34 Recovery of *S. typhimurium* TA98 from the livers of germ-free and conventional flora mice in the host-mediated assay of Table 33

| Mutagen (dose) | Viable bacteria per | | | |
|--------------------------|--|-----------------|-----------------|-----------------|
| | 0.1ml resuspended pellet ($\times 10^7$) ¹ | | Liver (% dose) | |
| | CV | GF | CV | GF |
| Aflatoxin B1 (5mg/kg) | 4.40 \pm 3.82 | 2.84 \pm 4.31 | 62.1 \pm 56.5 | 36.4 \pm 58.0 |
| MeIQ (0.5mg/kg) | 1.60 \pm 1.55 | 1.03 \pm 1.11 | 19.6 \pm 22.0 | 13.6 \pm 15.7 |
| MeIQ (10mg/kg) | 3.25 \pm 1.78 | 2.98 \pm 1.52 | 23.7 \pm 19.4 | 15.4 \pm 12.3 |
| Trp-P-2 (10mg/kg) | 2.44 \pm 2.28 | 2.13 \pm 1.42 | 32.6 \pm 27.8 | 24.8 \pm 19.8 |
| Controls (DMSO only) | 1.43 \pm 0.96 | 0.39 \pm 0.52 | 20.0 \pm 13.3 | 4.1 \pm 5.5 |

Bacterial suspension: 1.43×10^9 cells/ml

Germ-free mice (GF) or mice with a conventional flora (CV) were injected iv with 0.1ml bacterial suspension immediately followed by mutagen or the solvent, DMSO, (Controls) as in the host-mediated assay described in Methods section 11c. One hour after the bacterial injection, mice were sacrificed, the liver removed, homogenized and the bacteria isolated by centrifugation of the homogenate. The bacterial pellet was resuspended in 1ml Tris/KCl and aliquots (0.1ml of appropriate dilutions) plated on to nutrient agar and incubated at 37°C (see Methods section 11c).

¹ This set of results represents the number of viable bacteria counted on nutrient agar and the number of bacteria poured on to each Vogel Bonner plate.

(Table 33). A highly significant decrease was also observed in the mutagenicity of MeIQ (10mg/kg) in germ-free compared to conventional flora mice, as a decline of 40% was apparent in the numbers of revertants, falling from 2019 to 1216 His⁺/plate. Similar percentage decreases were observed in germ-free mice dosed with 0.5mg MeIQ/kg and for 10mg Trp-P-2/kg. Owing to the greater inter-group variance (of between 175 and 351 His⁺/plate) of these two sets of data, the statistical significance was not as great as observed before but was still greater than the 95% confidence level.

The recovery of *S. typhimurium* TA98 from the livers of germ-free mice was, on average, lower (by 20 - 80%) than that for mice with a conventional gut flora (Table 34). However, the large variation in the recovery of bacteria (from less than 4% to greater than 90% of the administered dose) resulted in there being little or no statistical significance in the bacterial recovery from mice of different microbial status.

In brief, the results of experiments presented in this chapter show that dietary pectin can modify gut flora metabolism, in particular nitrate reductase activity, in female BALB/c mice. However, dietary pectin has no apparent influence on the metabolism of nitrosodimethylamine, or on its formation from aminopyrine and nitrite. However, marked changes in the amount of nitrite available for reaction with aminopyrine would be required before changes in genotoxicity would have been observed. Changes to the gut flora, even if extreme (ie. the use of germ-free animals) can result in changes to hepatic mutagen metabolism.

CHAPTER 6

Developmental changes in hepatic and gut flora metabolism

The first section of this chapter is devoted to experiments investigating the hepatic genotoxicity of dietary carcinogens, *in vivo*, in 4 and 24-week-old mice using the host-mediated assay. These results are then compared to the *in vitro* activation of these compounds by using hepatic fractions prepared from mice aged 2 - 24 weeks. Results for the heterocyclic amines MeIQ and Trp-P-2 and the fungal metabolite aflatoxin B₁ are presented. Complementary results on age-related changes in cytochrome P450 content and function and glutathione-transferase activities are also described.

In the developing mammal major changes have been found to occur in the bacterial composition of the gut flora. It seems likely therefore, that concomitant changes occur in the metabolic activity of the microflora. In the second section of this chapter developmental changes in three bacterial enzymes associated with the caecal flora of mice (over the period from 2 to 24 weeks after birth) are reported. Since the developing gut flora is in a state of flux, it is possible that it is sensitive to modification by diet. Consequently, the sensitivity of the gut flora of mice at different ages to modification by pectin has been studied.

1. Age-related changes in hepatic metabolism *in vivo* and *in vitro*.

a. Host-mediated assays.

The influence of age on the *in vivo* activity of the dietary mutagens, aflatoxin B₁ (10mg[32μmol]/kg), MeIQ (2.5mg[12μmol]/kg) and Trp-P-2 (10mg[40μmol]/kg) was investigated using 4 and 24 week old mice in the intrasanguineous host-mediated assay with *S. typhimurium* TA98 as indicator (see Methods section 11c).

The body weights of mice used in these experiments averaged 24g for the 24 week old mice and 12g for the 4 week old animals (Table 35). The change in liver weight with age was not so marked, with values for the older hosts being 1.25 fold greater than that of mice 4 weeks of age (Table 35). Consequently, the relative liver weight was greater in the 4 week old mice, 0.58 compared to 0.36 in mice 24 weeks of age.

In mice given DMSO in place of mutagen (Controls), age had no apparent effect upon the reversion of *S. typhimurium* TA98 to histidine prototrophy since the mean number of revertants recorded per plate was 8 for both age groups (Table 36). This control value was similar to the background reversion frequency of the original bacterial dosing suspension (6 ± 5 His⁺/plate; Table 36).

For those mice treated with mutagen the numbers of induced revertants were greater in 4 week old mice compared to mice 24 weeks of age by 121%, 49% and 28% for Trp-P-2, MeIQ and aflatoxin B₁ respectively ($p < 0.001$; Table 36).

There was considerable variation in the number of bacteria recovered from the livers of mice since values ranging from 4% to

Table 35 Body and Liver weights of female
BALB/c mice of different ages

| Age weeks | No. of mice | Body weight g | Liver weight g |
|--|-------------|---------------|----------------|
| <i>a. Host-mediated assays.</i> | | | |
| 4 | 26 | 12.04 ± 1.45 | 0.70 ± 0.08 |
| 24 | 28 | 24.14 ± 2.76 | 0.88 ± 0.09 |
| <i>b. Salmonella mutagenicity assay.</i> | | | |
| 2 | 29 | 5.89 ± 0.91 | 0.24 ± 0.04 |
| 4 | 24 | 9.43 ± 1.49 | 0.50 ± 0.08 |
| 6 | 23 | 14.04 ± 1.70 | 0.76 ± 0.07 |
| 8 | 14 | 15.41 ± 1.54 | 0.86 ± 0.19 |
| 12 | 15 | 18.02 ± 1.29 | 0.85 ± 0.03 |
| 24 | 18 | 19.58 ± 1.66 | 0.88 ± 0.12 |
| <i>c. Hepatic enzyme activity and function</i> | | | |
| 4 | 36 | 15.39 ± 1.15 | 0.76 ± 0.08 |
| 24 | 30 | 25.45 ± 2.73 | 0.84 ± 0.04 |

Mice were fed a purified fibre-free diet from weaning (see Methods section 4) with body and liver weights determined at sacrifice. Results are shown as mean ± SD. Mutagenicity experiments using these mice are reported for a. in Table 36 and for b. in Figures 26 - 32. Measurements of hepatic enzyme activity and function are given in Tables 38, 40 & 41.

Table 36 Effect of age on the activity of food mutagens in the host-mediated assay

| Age weeks | His ⁺ revertants/plate | | | |
|-----------|-----------------------------------|--------------------|----------------------|---------------------|
| | Controls DMSO only | Aflatoxin 10mg/kg | MeIQ 2.5mg/kg | Trp-P-2 10mg/kg |
| 4 | 8 ± 4 (4) | 230 ± 47 (8) | 2950 ± 521 (7) | 1753 ± 238 (6) |
| 24 | 8 ± 3 (4) | 179 ± 54*** (8) | 1974 ± 869*** (8) | 793 ± 253*** (8) |

S. typhimurium TA98 dosing suspension: 6 ± 5

Female BALB/c mice of 4 and 24 weeks of age, fed a purified fibre-free diet, were exposed to mutagens and *S. typhimurium* TA98 in the host-mediated assay (Methods section 11c). Values are given as mean of n liver samples ± SD, n in parentheses. Asterisks indicate values for 24 week old mice differing significantly from the 4 week age group, *** p<0.001 (Anova).

Table 37 Recovery of *S. typhimurium* TA98 from the livers of mice in the host-mediated assays of Table 36

| Mutagen | Age weeks | Mean Liver wt(g) | Viable bacteria per | |
|-------------------------------------|-----------|------------------|---|----------------|
| | | | 0.1ml resuspended pellet (x10 ⁶) ¹ | liver (% dose) |
| Aflatoxin B ₁ 10mg/kg | 4 | 0.60 | 82 ± 43 | 23.5 ± 12.2 |
| | 24 | 0.86 | 60 ± 29 | 20.4 ± 10.6 |
| MeIQ 2.5mg/kg | 4 | 0.73 | 42 ± 23 | 13.2 ± 7.1 |
| | 24 | 0.85 | 81 ± 50 | 27.7 ± 17.8 |
| Trp-P-2 10mg/kg | 4 | 0.74 | 131 ± 40 | 41.4 ± 12.6 |
| | 24 | 0.95 | 77 ± 41 | 23.4 ± 14.1 |
| Controls DMSO only | 4 | 0.77 | 34 ± 25 | 11.0 ± 8.1 |
| | 24 | 0.86 | 104 ± 52 | 35.0 ± 18.0 |

S. typhimurium TA98 dosed per mouse: 5.5 x 10⁹ cells

Mice were injected iv with 0.1ml suspension of *S. typhimurium* TA98 immediately followed by mutagen or the solvent (DMSO) p.o. in the host-mediated assay (Methods section 11c). After 1 hour, mice were sacrificed, the livers removed and homogenized in Tris/KCl. Bacteria were sedimented by centrifugation and resuspended in 1ml Tris/KCl. To determine the numbers of bacteria present in the liver, aliquots (0.1ml of appropriate dilutions) of the resuspended bacterial pellets were plated on to nutrient agar and incubated for 48 hours at 37°C (see Methods section 11c). Results are given as means (± SD)

¹ This column of data represents the number of viable bacteria counted on nutrient agar plates and the number of bacteria poured on to each Vogel Bonner plate in order to determine the reversion to His⁺.

52% of the administered dose were obtained (Table 37). The number of bacteria plated on to Vogel Bonner agar was estimated to range between 1×10^7 - 2×10^8 cells (Table 37).

b. *Salmonella mutagenicity assay.*

To compare the *in vivo* activity of the three mutagens with their activation *in vitro*, hepatic S9 fractions prepared from mice aged 2, 4, 6, 8, 12 and 24 weeks of age were incubated with the appropriate mutagen in the *Salmonella* mutagenicity assay, again using *S. typhimurium* TA98 as the indicator strain (see Methods section 10a).

The liver weights of the mice used for the preparation of hepatic S9, increased rapidly over the first six weeks of life rising from 0.24 ± 0.04 g (mean \pm SD) for the 2 week old mice to 0.76 ± 0.08 g for 6 week old mice - an increase of more than 60% during this 4 week period (Table 35). This was concomitant with changes in body weight (5.89 ± 0.91 g and 14.04 ± 1.7 g for the preweanlings and 6 week old mice respectively; Table 35). Subsequently, the rate of increase in liver and body weight declined resulting in only a 13% increase in liver weight and a 21% increase in body weight over the next 12 weeks (Table 35).

The amount of S9 protein present in the hepatic post-mitochondrial supernatant (S9) was lowest for preparations from the 2 week old mice at 77 ± 5 mg protein/ml S9, and increased with increasing donor age until week 8 post-partum when 118 ± 14 mg of S9 protein/g liver were measured (Table 38). The concentration of cytochrome P450 per gram of liver was fairly constant, at about

Table 38 Protein and Cytochrome P450 content of hepatic S9 fractions from mice of different ages

| Age weeks | n | S9 Protein mg/g liver | Cytochrome P450 nmol/g liver | Cytochrome P450 nmol/mg S9 protein |
|-----------|---|-----------------------|------------------------------|------------------------------------|
| 2 | 4 | 77 ± 5 | 16.3 ± 2.0 | 0.21 ± 0.03 |
| 4 | 4 | 87 ± 8 | 16.6 ± 1.6 | 0.19 ± 0.01 |
| 6 | 4 | 90 ± 7 | 17.1 ± 1.7 | 0.19 ± 0.01 |
| 8 | 4 | 118 ± 14*** | 12.6 ± 3.5 | 0.11 ± 0.03*** |
| 12 | 4 | 99 ± 4* | 13.8 ± 2.4 | 0.14 ± 0.02 |
| 24 | 6 | 116 ± 10 | 17.0 ± 4.8 | 0.14 ± 0.03 |

Hepatic S9 protein and cytochrome P450 content were determined from female BALB/c mice of different ages (Method sections 8c & 9b). Each S9 sample contained livers pooled from 4 mice (7 in the case of the 2 week old mice). Values given as means of n S9 samples ± SD. Asterisks indicate values significantly different from the previous age group, *** p<0.001, * p<0.05 (Anova).

16nmol/g liver in all mice although the specific content of haemoprotein per mg S9 protein exhibited a significant age-related decrease between week 6 and 8 (Table 38).

Initially a series of preliminary experiments were carried out in which each mutagen (at a concentration of 0.64nmol/assay for aflatoxin B₁, 0.05nmol/assay for MeIQ or 0.12nmol/assay for Trp-P-2) was incubated with various amounts of hepatic S9 fraction from mice aged 4, 8 and 24 weeks of age in the *Salmonella* mutagenicity assay (Fig. 26).

The level of S9 in the incubation mixture required to produce the greatest activation of 0.05nmol MeIQ to a bacterial mutagen, was when the volume of added S9 was 10% of the final incubation volume (equivalent to 2 - 3mg S9 protein/ml incubation mix) irrespective of the age of the mice (Fig. 26). For example, at this concentration (10%), S9 from 24 week old mice induced almost 1200 His⁺ revertants, as compared to 975 or 1060 revertants per plate when the S9 concentration was 5% or 20%. For Trp-P-2 and aflatoxin B₁, the optimal concentration of S9 was 5% (v/v; equivalent to 1 - 1.5mg S9 protein/ml incubation mix). Again the optimal concentration of S9 was similar for all age groups used (Fig. 26). These optimal S9 concentrations were used for each substrate in subsequent experiments.

No consistent changes in the background mutation frequency of *S. typhimurium* TA98 were seen in the presence of various concentrations of S9 fractions, or S9 fractions from mice of different ages, with the recorded mean values ranging between 4 and

Table 39 Effect of S9 concentration on the background reversion of *S. typhimurium* TA98 to histidine prototrophy.

| S9 concentration (% of assay;v/v) | His ⁺ revertants/plate | | |
|--------------------------------------|-----------------------------------|--------|--------|
| | Host age (weeks) | | |
| | 4 | 8 | 24 |
| 1.0 | 11 ± 3 | 19 ± 1 | 7 ± 4 |
| 2.5 | 11 ± 4 | 12 ± 2 | 10 ± 5 |
| 5.0 | 14 ± 11 | 11 ± 4 | 9 ± 5 |
| 10.0 | 9 ± 3 | 14 ± 2 | 12 ± 3 |
| 20.0 | 14 ± 6 | 13 ± 5 | 4 ± 3 |

In the *Salmonella* mutagenicity assay control samples contained, per assay, 0.1ml DMSO in place of mutagen. Refer to legend of Fig. 26 for further details. Results are given as means ± SD of triplicate plates.

19 His⁺ revertants per plate (Table 39).

With all three mutagens there was a trend for the numbers of induced mutants to decrease with increasing age (Fig. 26). This effect occurred at all S9 concentrations for the heterocyclic amines (Trp-P-2 and MeIQ), but for aflatoxin B₁ marked differences between the 4 and 24 week old mice occurred only at S9 concentrations of 1% and 10% of the incubation mix (Fig. 26).

Further experiments were carried out to investigate this age-related change in the activation of three chemicals to bacterial mutagens. At first, two concentrations of each mutagen were incubated with hepatic S9 fractions (at the optimal concentration) from mice aged 2, 4, 6, 12 and 24 weeks (Figs. 27, 29, 31). In a second experiment ^a range of mutagen concentrations were incubated with hepatic S9 fractions from mice aged 4 and 24 weeks of age (at the optimal S9 concentration; Figs. 28, 30, 32).

It may be seen in Fig. 27 that hepatic activation of MeIQ, at concentrations of 0.01 nmol and 0.07 nmol/assay, was maximal (giving approximately 1200 and 2700 His⁺/plate respectively) when S9 from 2 or 4 week old mice was used with a general decline (of 30% in total) in the activation of MeIQ to a bacterial mutagen as the age of the animals increased.

This greater activation of MeIQ by hepatic S9 from young mice (4 weeks) was apparent over a wide range of mutagen concentrations (0.025 - 0.25nmol MeIQ/assay; Fig. 28), with an approximate doubling in the numbers of revertants generated when compared with

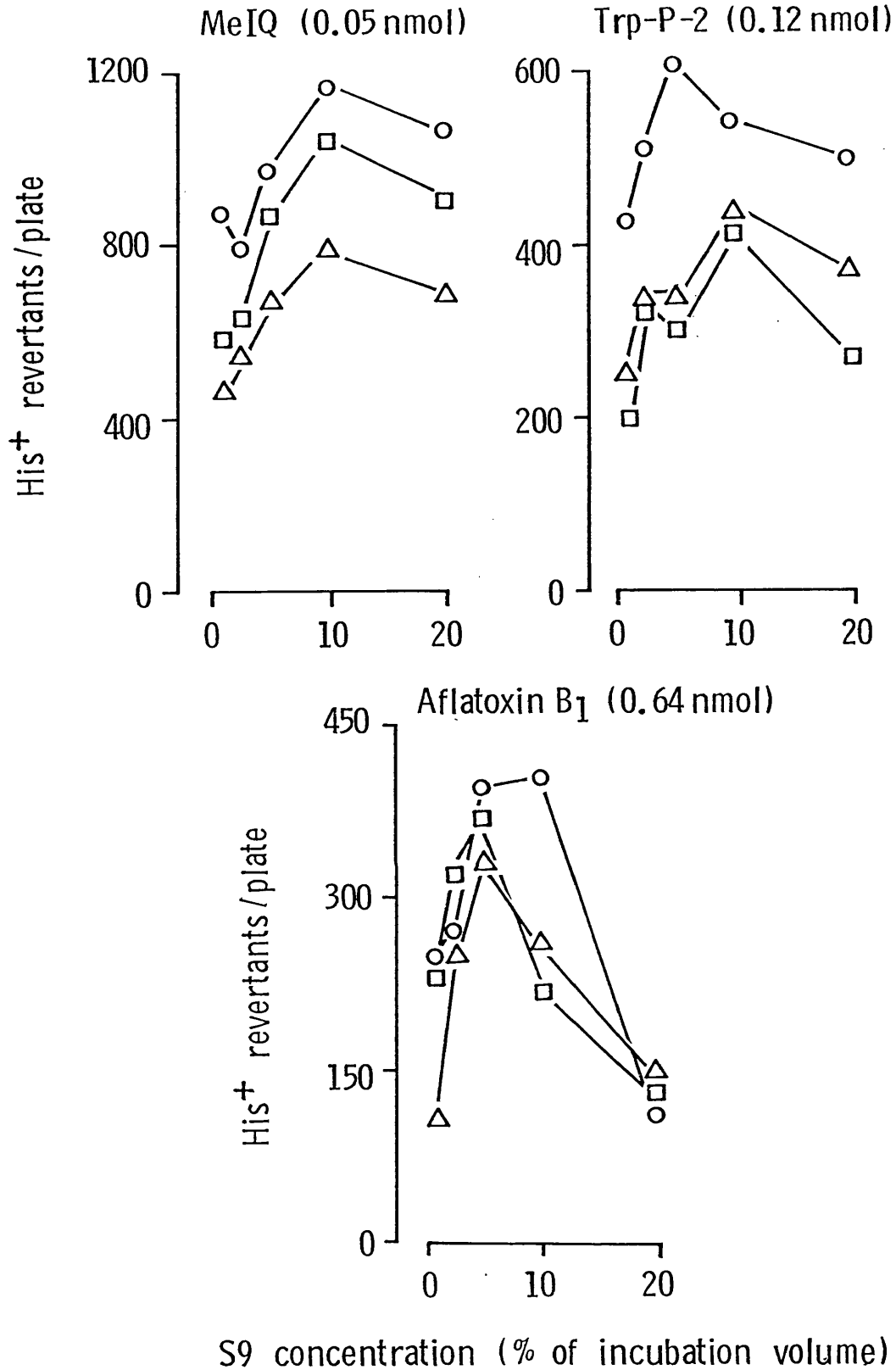


Figure 26 Determination of optimal S9 concentration for mutagen activation *in vitro*. Different concentrations of hepatic S9 (expressed as % of incubation volume [0.8ml]) from mice aged 4 weeks (O), 8 weeks (□) or 24 weeks (Δ) were incubated with the appropriate mutagen, *S. typhimurium* TA98 and cofactors, for 30 mins at 37°C before being poured in top agar, on to Vogel Bonner plates and incubated for 48 hr at 37°C. Each incubation was performed in triplicate. Results are given as means for 3 pooled S9 preparations.

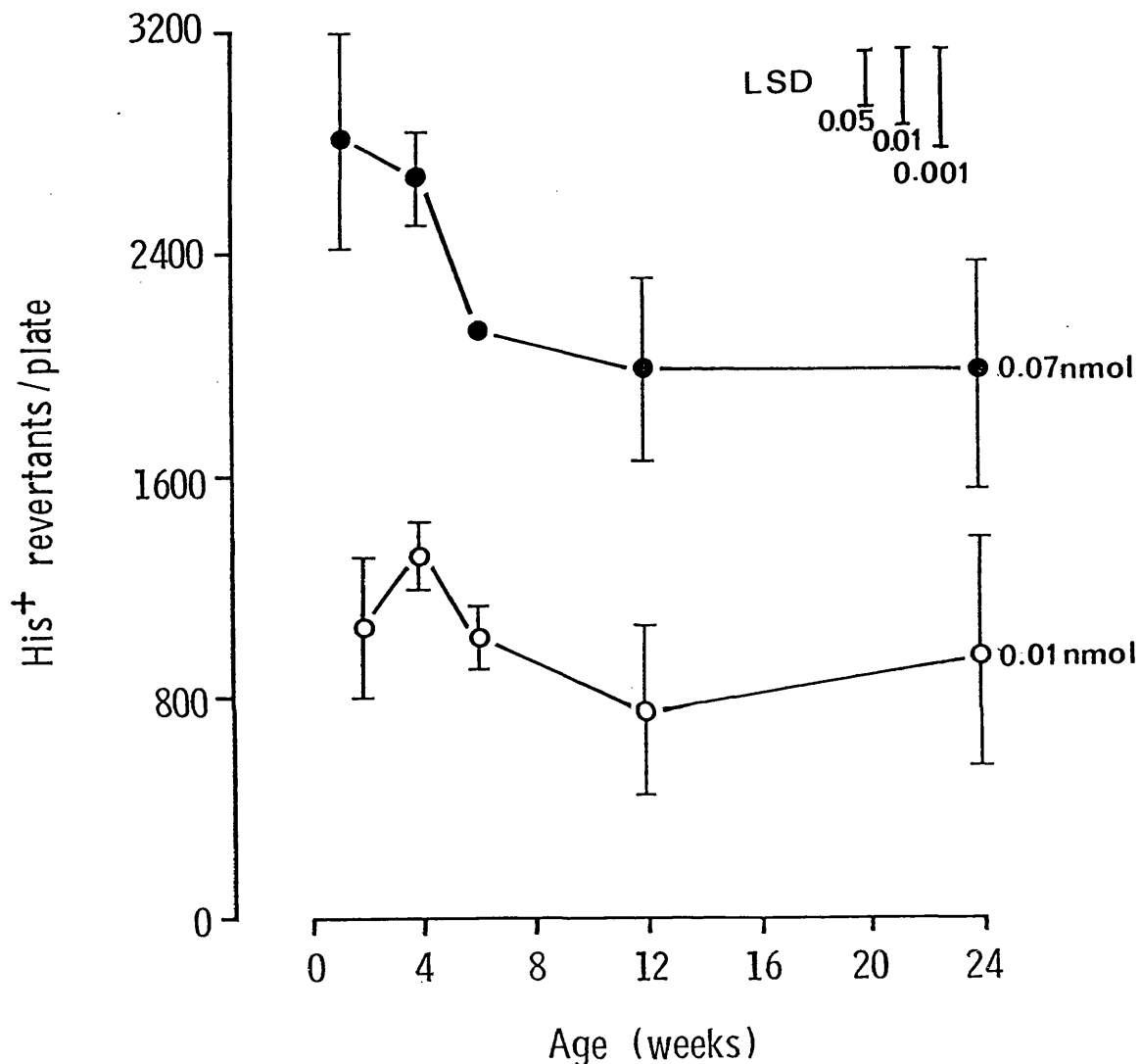


Figure 27. Activation of MeIQ by hepatic S9 fractions from mice of different ages. Mutagen, cofactors, *S. typhimurium* TA98, and S9 fractions were incubated for 30 min at 37° before being poured in top agar onto Vogel Bonner plates and incubated for 48 hours at 37°C (see Methods 10a). The concentration of S9 fractions in the incubation mixture was 10% (v/v) of the incubation volume (0.8ml). Each incubation was carried out in triplicate. Results are expressed as means (bars:SD) for four S9 preparations with statistical significance assessed using Anova and the LSD criterion.

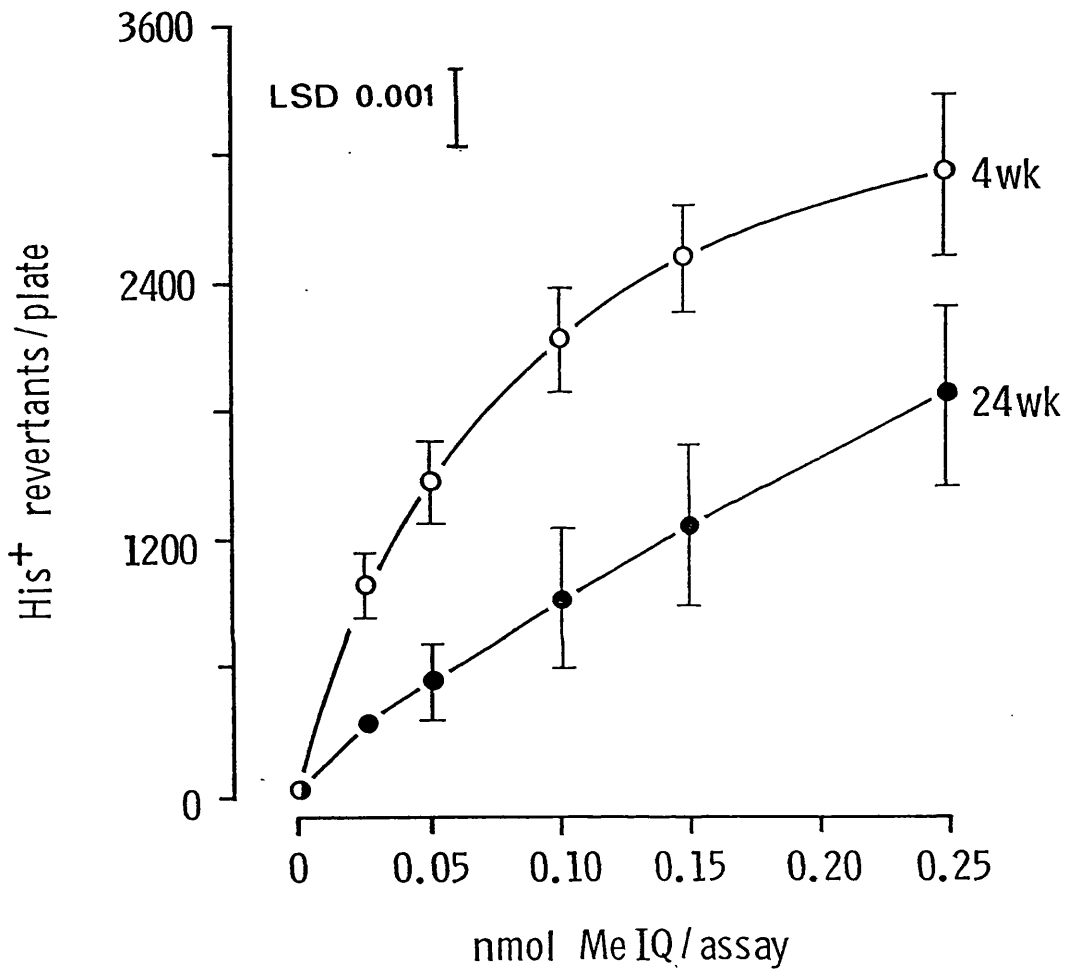


Figure 28. Activation of different concentrations of MeIQ by hepatic S9 fractions from mice aged 4 and 24 weeks. For details refer to legend to Figure 27.

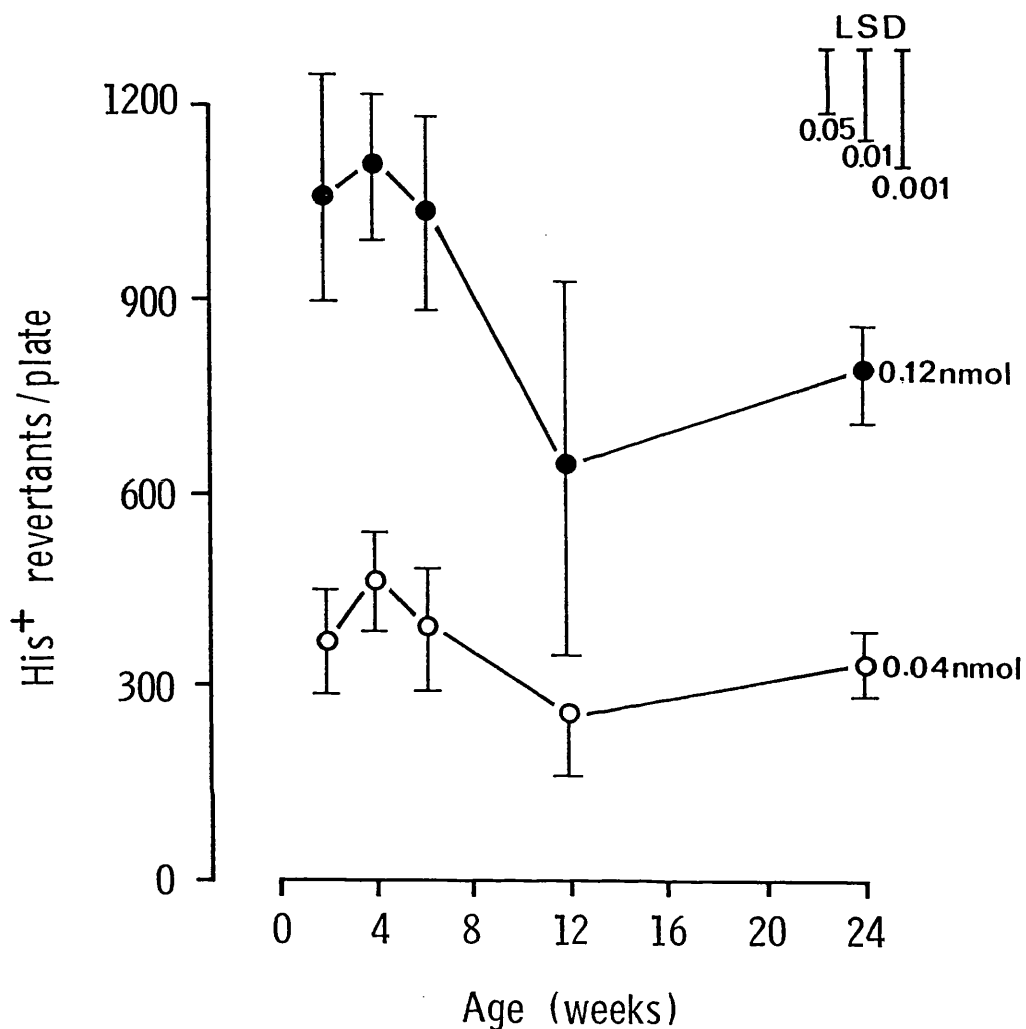


Figure 29 Activation of Trp-P-2 by hepatic S9 fractions from mice of different ages. Mutagen, cofactors, *S. typhimurium* TA98 and S9 fractions were incubated for 30 min at 37°C before being poured in top agar on to Vogel Bonner plates and incubated for 48 hr at 37°C (Methods section 10a). The concentration of S9 fraction in the incubation was 5% (v/v) of the total incubation volume (0.8ml). Each incubation was carried out in triplicate. Results are expressed as mean (bars:SD) for four S9 preparations with statistical significance assessed using Anova and the LSD criterion.

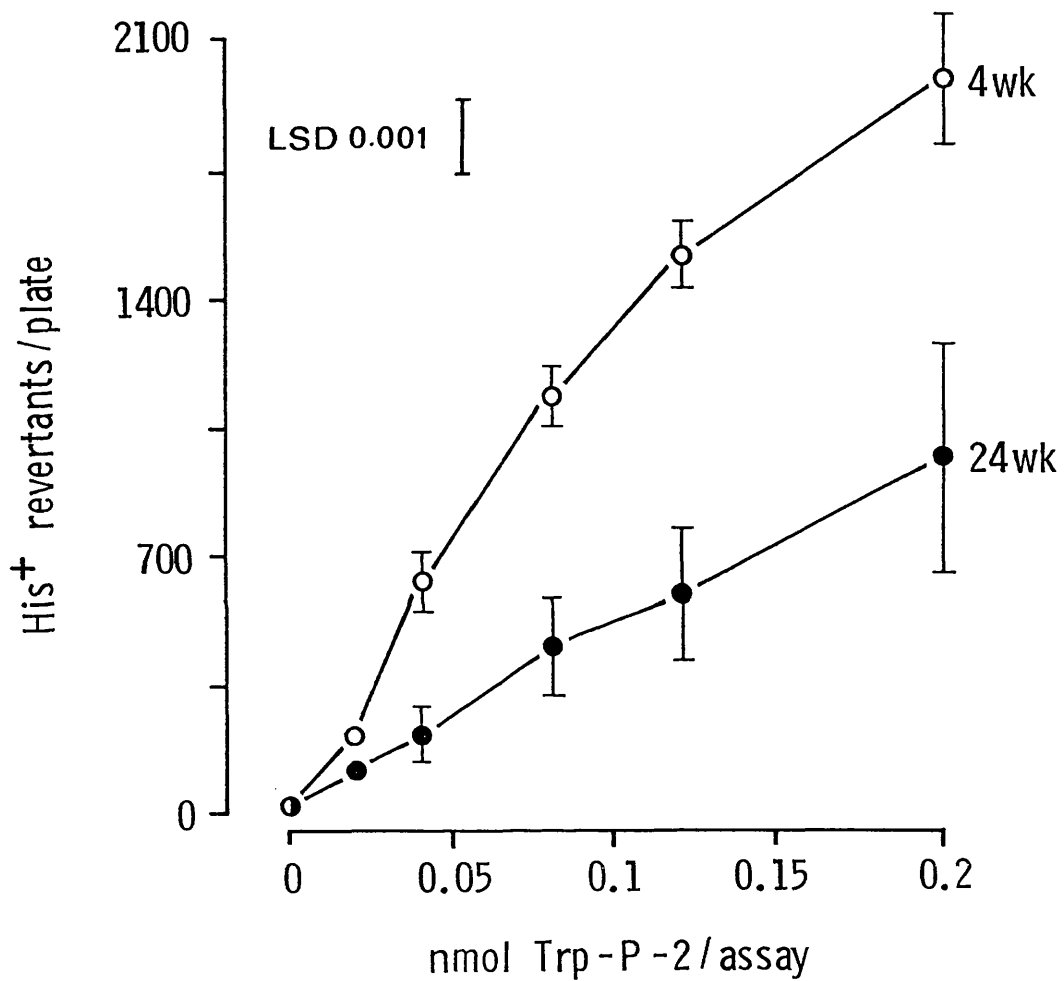


Figure 30 Activation of different concentrations of Trp-P-2 by hepatic S9 fractions from mice aged 4 and 24 weeks. For further details refer to legend to Figure 29.

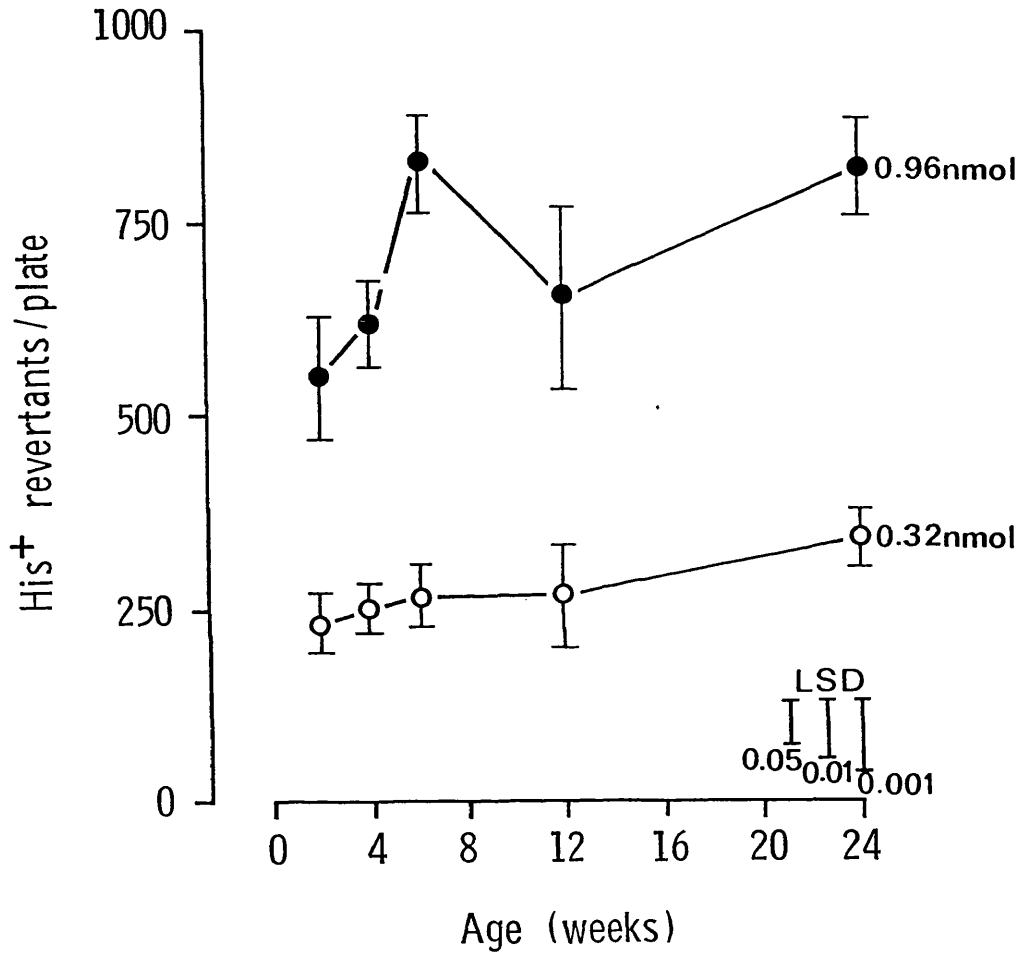


Figure 31 Activation of aflatoxin B₁ by hepatic S9 fractions from mice of different ages. Mutagen, cofactors, *S. typhimurium* TA98 and S9 fractions were incubated for 30 min at 37°C before being poured in top agar on to Vogel Bonner plates and incubated for 48 hr at 37°C (Methods section 10a). The concentration of S9 fraction in the incubation mixture was 5% (v/v) of the incubation volume (0.8ml). Each incubation was carried out in triplicate. Results are expressed as means (bars:SD) for four S9 preparations with statistical significance assessed using Anova and the LSD criterion.

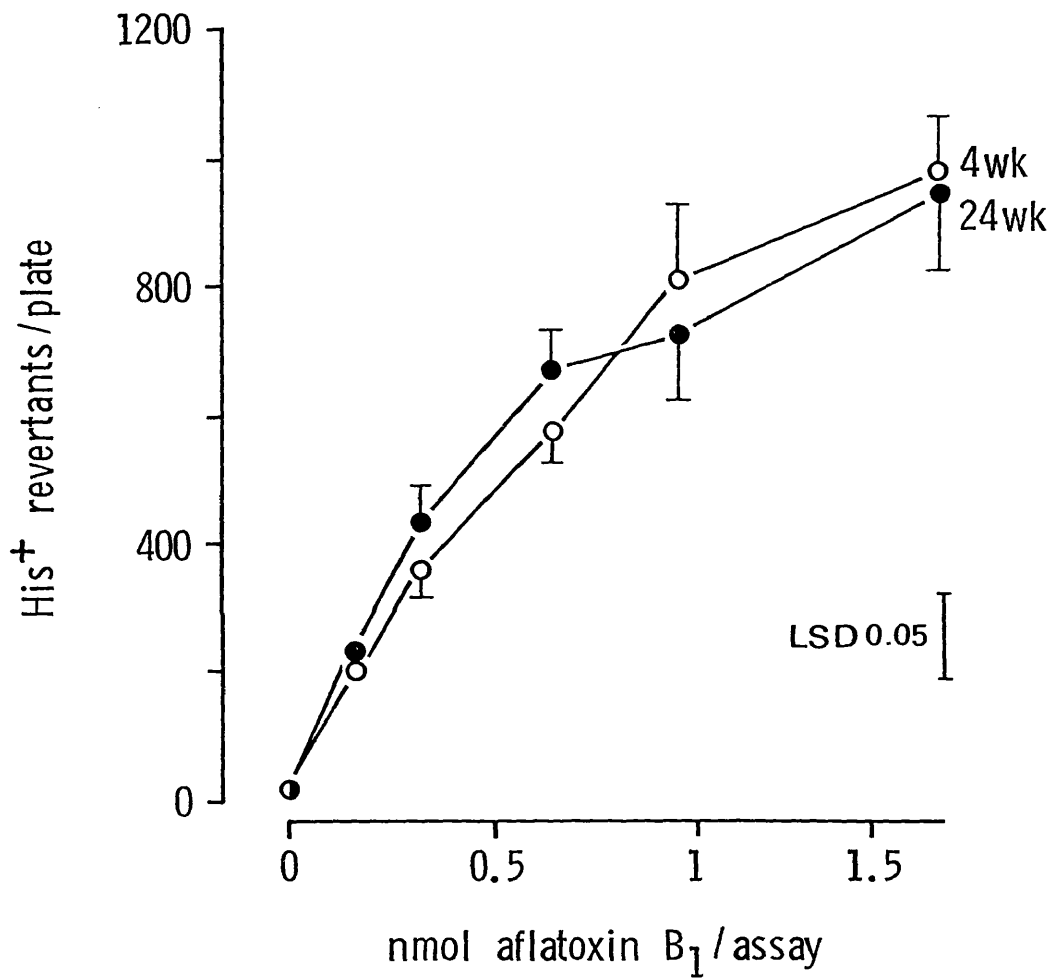


Figure 32 Activation of different concentrations of aflatoxin B₁ by hepatic S9 fractions from mice aged 4 and 24 weeks. For further details refer to legend to Figure 31.

incubations containing S9 from 24 week old mice (Fig. 28). For example, at a concentration of 0.15nmol MeIQ/assay, 1251 His⁺ revertants were induced, on average, by S9 fractions from mice 24 weeks of age as compared to 2530 His⁺/plate for fractions from mice aged 4 weeks.

A similar age-related response was obtained for Trp-P-2 (0.04nmol and 0.12nmol/assay; Fig. 29) although in this case, the mutagenicity in the presence of S9 from 2, 4 and 6-week-old mice remained unchanged (at around 1050 His⁺/plate at the higher mutagen concentration) followed by a marked fall of approximately 40% in activating capacity between 6 and 12 weeks (Fig. 29). When a range of concentrations of Trp-P-2 were incubated with liver S9 fractions from 4 and 24-week-old mice a greater mutagenic response was observed, as before, in the older animals over the entire dose range tested (0.02 - 0.20nmol Trp-P-2/assay; Fig. 30).

In contrast, aflatoxin B₁, at all concentrations studied (0.16 - 1.6nmol aflatoxin B₁/assay) was equally well metabolized to a mutagen by mouse hepatic fractions irrespective of donor age (Fig. 31 & 32). For instance, when 0.32nmol aflatoxin/assay was incubated with S9 fractions from mice age 2, 4, 6, 12 and 24 weeks the numbers of revertants approximated 250 per plate.

c. Mixed function oxidase activities.

Levels of cytochrome P450 and the activities of three mixed function oxidases and cytochrome c reductase were measured in hepatic microsomal fractions prepared from mice of 4 and 24 weeks of age (Table 40). The body and liver weights of these mice are given

Table 40 Cytochrome P450, Cytochrome C reductase and mixed function oxidase activities of microsomal tissue from mice aged 4 and 24 weeks.

| | Age | |
|--|--------------|----------------|
| | 4 weeks | 24 weeks |
| Protein content mg protein/g liver | 21.46 ± 1.89 | 25.97 ± 3.89 |
| Cytochrome P450 nmoles/mg prot | 0.63 ± 0.12 | 0.70 ± 0.07 |
| nmoles/g liver | 13.41 ± 2.20 | 18.65 ± 3.60** |
| Cytochrome c reductase nmoles/min/mg prot. | 79.42 ± 5.60 | 71.76 ± 9.12 |
| nmoles/min/g liver | 1705 ± 189 | 1806 ± 125 |
| Ethoxycoumarin-O-deethylase nmoles/hr/mg prot. | 104.2 ± 11.5 | 121.7 ± 15.5 |
| nmoles/hr/g liver | 2235 ± 278 | 3228 ± 390*** |
| Ethoxyresorufin-O-deethylase nmoles/hr/mg prot. | 28.52 ± 3.31 | 27.21 ± 6.10 |
| nmoles/hr/g liver | 614 ± 99 | 708 ± 99 |
| Benzphetamine demethylation µmoles/hr/mg prot. | 0.26 ± 0.09 | 0.26 ± 0.07 |
| µmoles/hr/g liver | 5.46 ± 1.83 | 5.72 ± 0.74 |

The protein and cytochrome P450 content and the activities of cytochrome c reductase and three mixed function oxidases were determined in hepatic microsomal fractions of female BALB/c mice of 4 and 24 weeks of age (see Methods sections 8c & 9b). Values are given as mean ± SD of 6 samples; each sample containing livers pooled from 5 - 6 mice. Asterisks indicate significant differences from values for the 4 week age group, *** p<0.001; ** p<0.01 (Anova).

in Table 35. The age-related increase in body and liver size was not as marked as observed previously, with increases of 65% and 11% (for body and liver weights respectively) recorded.

The protein content of the microsomal suspensions was, on average, 21% greater in fractions prepared from the 24 week old mice than from those animals 4 weeks of age (Table 40).

No significant difference was observed in either the cytochrome P450 content (per mg microsomal protein) or the specific activity of cytochrome c reductase in the hepatic microsomal fractions from mice aged 4 and 24 weeks. However, when results were expressed per g liver, the older mice showed a 40% increase in total cytochrome P450 content, rising from 13.41 nmol/g liver in 4 week old mice to 18.65 nmol/g liver in the older animals (Table 40). The three mixed function oxidases exhibited no significant age related change in activity when expressed as per mg S9 protein (Table 40), with values approximating 110, 28 and 260nmol/hr/mg microsomal protein at both mouse ages for ethoxycoumarin-O-deethylase, ethoxyresorufin-O-deethylase and benzphetamine demethylase respectively. As a result of the greater protein content of microsomes prepared from 24 week old mice than from mice 4 weeks of age (see Table 40) the activity of ethoxycoumarin-O-deethylase, when calculated per gram of liver, was significantly higher (by 1.5 fold). However, the greater activity of ethoxyresorufin-O-deethylase or benzphetamine demethylase per gram liver in older mice was not significant^{different} from the value for fractions from mice 4 weeks of age.

Table 41 Determination of glutathione content and glutathione transferase activities in the livers of mice aged 4 and 24 weeks.

| | Age | |
|---|--------------|-----------------|
| | 4 weeks | 24 weeks |
| Protein content: | | |
| a. Hepatic cytosol mg protein/g liver | 105.4 ± 1.9 | 110.5 ± 5.4 |
| b. Whole liver homogenate mg protein/g liver | 210.5 ± 14.6 | 233.9 ± 30.2 |
| Total glutathione nmol/mg protein | 36.5 ± 5.6 | 32.4 ± 9.1 |
| nmol/g liver | 7659 ± 1110 | 7384 ± 1216 |
| Oxidized glutathione nmol/mg protein | 0.04 ± 0.01 | 0.03 ± 0.01 |
| nmol/g liver | 9.02 ± 2.08 | 6.88 ± 0.80 |
| DCNB | | |
| µmol/min/mg protein | 0.05 ± 0.02 | 0.06 ± 0.01 |
| µmol/min/g liver | 5.26 ± 1.92 | 6.72 ± 1.81 |
| CDNB | | |
| µmol/min/mg protein | 1.57 ± 0.26 | 2.18 ± 0.35** |
| µmol/min/g liver | 164.8 ± 26.2 | 240.6 ± 34.5*** |
| ENPP | | |
| µmol/min/mg protein | 0.23 ± 0.05 | 0.16 ± 0.05* |
| µmol/min/g liver | 23.88 ± 5.32 | 17.89 ± 4.91 |

Measurements of total and oxidized glutathione content in the whole liver homogenate and the activities of glutathione transferases in hepatic cytosolic fractions towards the substrates 1,2-dichloro-4-nitrobenzene (DNCB), 1-chloro-2,4-dinitrobenzene (CDNB) and 1,2-epoxy-3-(p-nitrophenoxy)propane (ENPP) were determined in female BALB/c mice of 4 and 24 weeks of age (see Methods section 9b). Values are given as mean ± SD of 6 samples; each sample containing livers pooled from 6 mice. Asterisks indicate values significantly different from values in the 4 week age group, *** p<0.001; ** p<0.05; * p<0.01 (Anova).

d. Glutathione and glutathione transferase activities.

No marked age-related difference was found in the total glutathione content of whole liver homogenate from mice aged 4 and 24 weeks, with values approximating 7450 nmol/g liver (Table 41). However, the concentration of oxidized glutathione was lower in the older hosts, falling from 9.02nmol/g liver in 4 week old mice to 6.88nmol/g liver in mice 24 weeks of age, although this difference was not statistically significant. Similar glutathione transferase activities were recorded for the two age groups when dichloronitrobenzene and epoxynitrophenoxypropane were used as substrates, with activities of 6 and 20nmol/hr/g liver measured respectively. However, using chlorodinitrobenzene, glutathione transferase activity was 1.5 fold greater in the older hosts ($p < 0.001$; Table 41).

2. Effect of host age on gut flora metabolism.

For reasons of economy and practical convenience, the same batch of mice were used for both this study and the previous study (investigating the effect of host age on hepatic metabolism) described above, but additional mice of the same ages were purchased and fed the 5% pectin diet.

The activity of nitrate reductase in mice fed the fibre-free diet was unaffected by age, remaining around 5 - 10 μ mol of nitrite produced per hour per gram caecal contents (μ mol/hr/g) and similar to the activity measured in preweanling mice (2 weeks; Fig. 33, Table 42). In contrast, animals fed the 5% pectin diet, exhibited at 4 weeks of age a 12 fold increase in the caecal activity of nitrate reductase compared to that of mice fed the fibre-free diet

Figure 33. The effect of age and diet on caecal nitrate reductase activity in mice. Caecal contents from 3 - 5 mice of similar age and fed either fibre-free or 5% pectin diet were pooled and a crude suspension prepared. For each age and diet group 4 - 6 suspensions were prepared. Nitrate reductase activity was determined using sodium nitrate as substrate (Methods section 9a). Results are presented as means and expressed as either a. μmol of nitrite formed/hr/g caecal contents, b. $\mu\text{mol/hr/caecum}$ or c. $\mu\text{mol/hr}/10^{10}$ bacteria. Asterisks indicate significant differences from the value for 4 week old mice, *** $p < 0.001$; ** $p < 0.01$ (Anova).
Fibre-free diet (●); 5% pectin diet (○).

Nitrate reductase activity

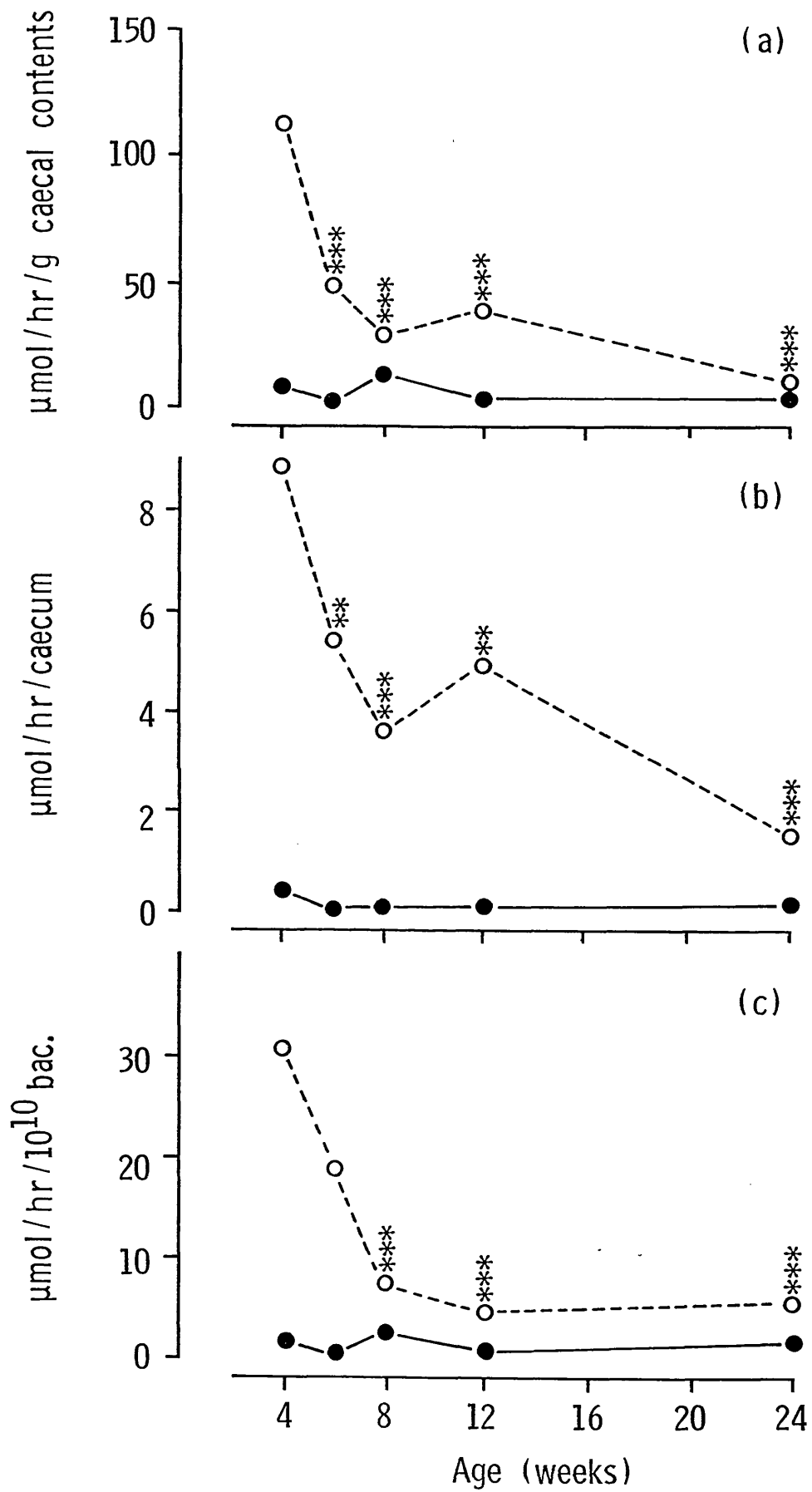


Figure 34. The effect of age and diet on caecal nitroreductase activity in mice. Caecal contents from 3 - 5 mice of similar age and fed either fibre-free or 5% pectin diet were pooled and a crude suspension prepared. For each age and diet group 4 - 6 suspensions were prepared. Nitroreductase activity was determined using p-nitrobenzoic acid as substrate (Methods section 9a). Results are presented as means and expressed as either μmol of p-aminobenzoic acid formed/hr/g caecal contents, b. $\mu\text{mol/hr/caecum}$ or c. $\mu\text{mol/hr}/10^{10}$ bacteria. Asterisks indicate significant differences from the value for 4 week old mice, *** $p < 0.001$; ** $p < 0.01$ (Anova). Fibre-free diet (●); 5% pectin diet (○).

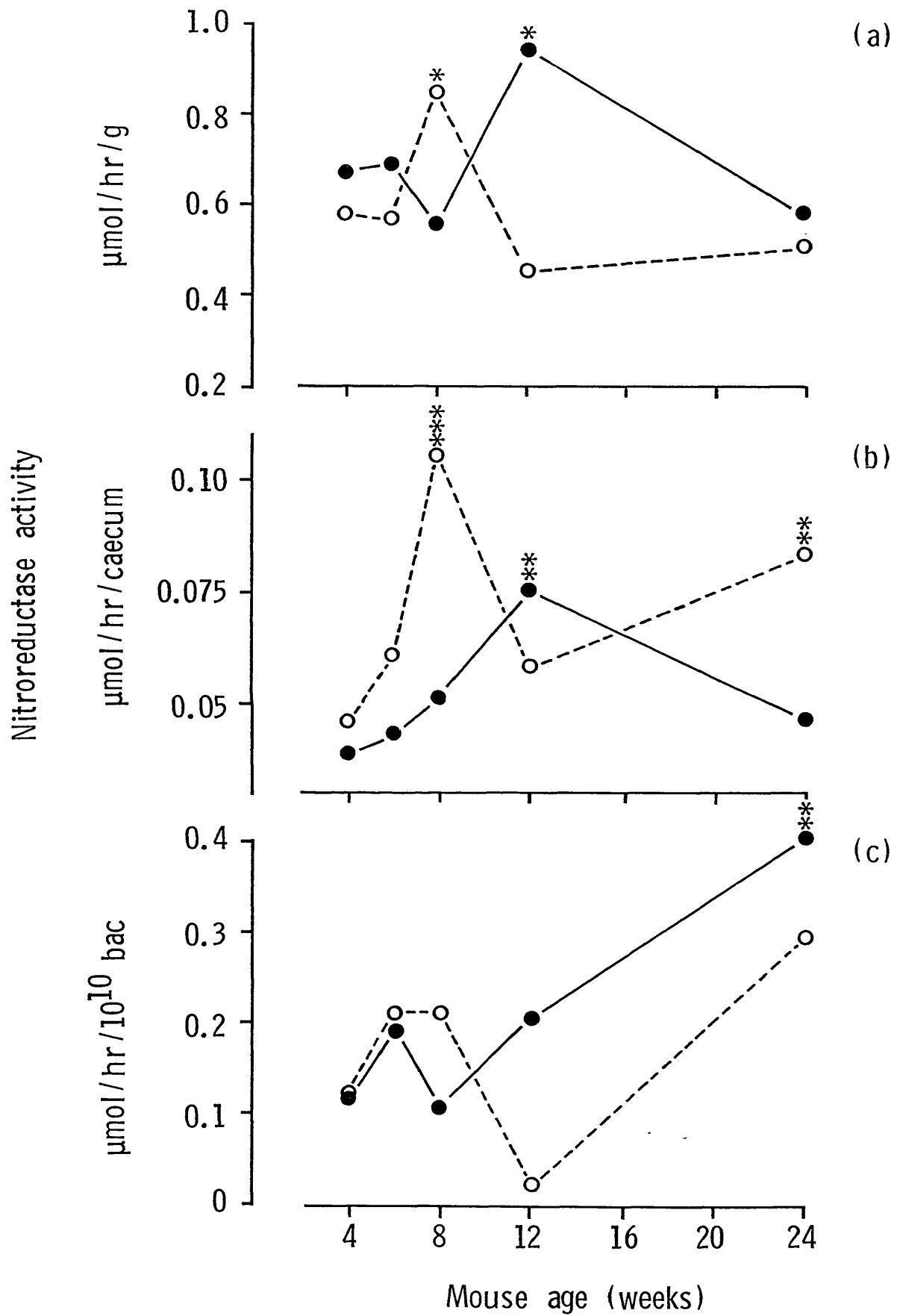


Figure 35. The effect of age and diet on caecal β -glucuronidase activity in mice. Caecal contents from 3 - 5 mice of similar age and fed either fibre-free or 5% pectin diet were pooled and a crude suspension prepared. For each age and diet group 4 - 6 suspensions were prepared. β -glucuronidase activity was determined using these fresh prepared suspensions and p-nitrophenol- β -D-glucuronide as substrate (Methods section 9a). Results are presented as means and expressed as either a. μmol of p-nitrophenol formed/hr/g caecal contents; b. $\mu\text{mol/hr/caecum}$ or c. $\mu\text{mol/hr}/10^{10}$ bacteria. Asterisks indicate significant differences from the value for 4 week old mice, *** $p < 0.001$; ** $p < 0.01$ (Anova). Fibre-free diet (\bullet); 5% pectin (\circ).

β -glucuronidase activity

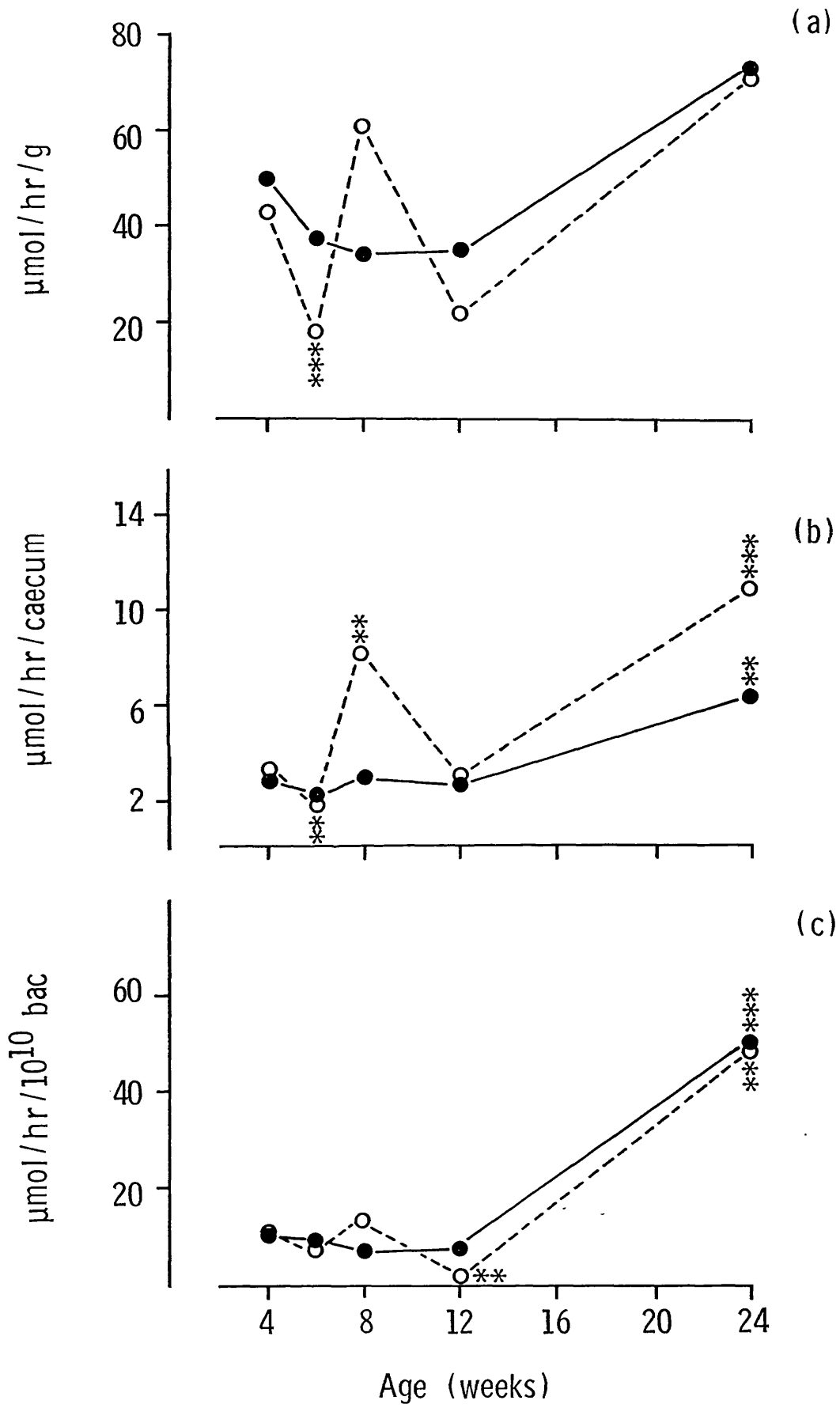


Table 42 Caecal enzyme activities of 2 week old preweanling mice and their mothers.

| Enzyme | Enzyme activity | |
|-------------------------------------|---------------------|-------------------|
| | Preweanling mice(4) | Mothers(3) |
| Nitrate reductase | | |
| $\mu\text{mol/hr/g}$ | 14.2 \pm 19.2 | 0.30 \pm 0.32 |
| $\mu\text{mol/hr/caecum}$ | 0.21 \pm 0.32 | 0.25 \pm 0.28 |
| $\mu\text{mol/hr}/10^{10}$ bacteria | 2.91 \pm 4.41 | 0.08 \pm 0.09 |
| Nitroreductase | | |
| $\mu\text{mol/hr/g}$ | 0.049 \pm 0.060* | 0.194 \pm 0.039 |
| $\mu\text{mol/hr/caecum}$ | 0.001 \pm 0.001* | 0.151 \pm 0.040 |
| $\mu\text{mol/hr}/10^{10}$ bacteria | 0.011 \pm 0.013* | 0.045 \pm 0.016 |
| β -glucuronidase | | |
| $\mu\text{mol/hr/g}$ | 17.37 \pm 9.96 | 32.08 \pm 8.91 |
| $\mu\text{mol/hr/caecum}$ | 0.22 \pm 0.12* | 25.25 \pm 5.91 |
| $\mu\text{mol/hr}/10^{10}$ bacteria | 3.25 \pm 1.90* | 7.30 \pm 2.26 |

Caecal suspensions were prepared (Methods section 8a) from the pooled caecal contents of 7 preweanling mice or 3 adult (mother) mice with the number of prepared suspensions given in parenthesis. Caecal weights and bacterial counts are given in Table 43. From the suspensions, activities of 3 enzymes were determined as described in Methods section 9a. Results are given as means \pm SD of the amount of product formed. Asterisks indicate significant differences between the 2 groups of mice. * $p < 0.05$ (Anova).

(Fig. 33). Subsequently, the activity declined although at all ages nitrate reductase was significantly ($p < 0.001$) greater (by at least a factor of 2) than that of mice of a comparable age and fed the fibre-free diet.

Enzyme activities of mouse caecal contents were also expressed as total activity per caecum and activity per unit number (10^{10}) of bacteria. The total activity per caecum takes into account changes in caecal size which occur with both age and diet and so provides information relevant to the host animal. Expressing data per 10^{10} bacteria indicates whether the overall activity of the organisms are affected. For nitrate reductase activity, similar profiles with increasing host age were obtained when the activity was calculated as either the amount of nitrite formed per caecum or as the amount of nitrite formed per 10^{10} caecal bacteria (Fig. 33).

For the enzyme nitroreductase, no consistent age- or diet-related change in the activity was apparent when expressed as either per gram of caecal contents (fluctuating between 0.4 - $1 \mu\text{mol/hr/ caecum}$: Fig. 34) or per 10^{10} bacteria (Fig. 34), although it should be noted that nitroreductase activity in mice aged 4 - 24 weeks and fed either diet was always greater (by at least 2 fold but usually by a factor greater than 10) than the activity measured in preweanling mice (2 weeks old; Fig. 34, Table 42).

When the values of enzyme activity were adjusted to account for differences in caecal weight, which are noted in Table 43, nitroreductase initially exhibited an age-related increase in activity with increasing age (Fig. 34). Approximately a 2 fold

Table 43 Body weight, Caecal weight and Caecal bacterial numbers of mice of different ages fed a fibre-free or 5% pectin diet.

| Age weeks | Body weight g | | Caecal weight g | | Bacteria/g caecal contents (x 10 ¹⁰) | |
|----------------------|----------------|----------------|-----------------|-----------------|--|----------------|
| | fibre free | 5% pectin | fibre free | 5% pectin | fibre free | 5% pectin |
| 4 | 9.43 ±1.49 | 8.78 ±1.58 | 0.056 ±0.020 | 0.081 ±0.010 | 5.95 ±3.08 | 5.22 ±3.30 |
| 6 | 14.04 ±1.70 | 14.11 ±1.23 | 0.061 ±0.010 | 0.110 ±0.030 | 4.11 ±1.21 | 3.01 ±1.35 |
| 8 | 15.41 ±1.54 | 15.46 ±1.65 | 0.094 ±0.030 | 0.128 ±0.019 | 5.67 ±3.13 | 4.81 ±1.73 |
| 12 | 18.02 ±1.29 | 17.69 ±1.24 | 0.079 ±0.010 | 0.130 ±0.004 | 4.84 ±1.52 | 10.82 ±4.14 |
| 24 | 19.58 ±1.66 | 20.58 ±1.45 | 0.081 ±0.020 | 0.161 ±0.020 | 2.27 ±2.02 | 2.47 ±1.71 |
| 2 ¹ | 5.89 ± 0.91 | | 0.012 ± 0.003 | | 8.34 ± 9.93 | |
| Mothers ¹ | 32.17 ± 4.08 | | 0.769 ± 0.070 | | 4.49 ± 1.15 | |

Mice were fed either a fibre-free or 5% pectin diet from weaning at 3 weeks of age.

¹ Values for preweanling mice (2 weeks old) and their mothers (fed on fibre-free diet) are also given. Results are expressed as means ± SD.

rise in nitroreductase activity was observed in mice fed the fibre-free diet between 4 and 12 weeks of age or between 4 and 8 weeks of age in mice fed the 5% pectin diet (Fig. 34). Subsequent to this enzyme activity declined by 39% and 45% for the fibre-free and 5% pectin diet respectively, followed by a second significant increase in activity of 0.25 μ mol/hr/g in 24 week old mice fed the fibre-free diet (Fig. 34).

β -glucuronidase activity remained relatively constant in caecal contents of mice fed the fibre-free diet and aged 4 - 12 weeks (Fig. 35) although this activity was markedly greater (by 11-fold) than that of preweanling mice (Table 42). However, between 12 and 24 weeks of age, the total activity of this hydrolytic enzyme in mice fed the fibre-free diet increased by 2.4 fold (Fig. 35), although when expressed per 10^{10} bacteria, a 7 fold increase in β -glucuronidase activity was observed between these two age groups. For mice fed the 5% pectin diet, β -glucuronidase activity fluctuated between 35 and 70 μ mol/hr/g caecal contents showing no consistent age-related effect. When the values were adjusted to equivalent bacterial numbers, the profile of β -glucuronidase activity in pectin fed mice of different ages was similar to that of mice fed the fibre-free diet (Fig. 35).

The activities of the three enzymes, nitrate reductase, nitroreductase and β -glucuronidase were also determined in the mothers (fed on a fibre-free diet) of the preweanling mice (Table 42). Nitrate reductase activity in the mothers, when expressed as the total activity in the caecal sac or per 10^{10} bacteria, was similar to that of the preweanling mice (Table 42). When the values of

this reductive enzyme were recalculated to estimate the activity per gram caecal contents, the activity was almost 50 times greater in the maternal mice compared to their young, indicative of the considerable discrepancy in the amount of caecal contents between the two groups (see Table 43). In contrast, the activities of nitroreductase and β -glucuronidase (per gram of caecal contents or per 10^{10} bacteria) were 4 and 1.8 fold greater respectively in the older mice compared to the preweanlings. The larger caecal sacs of the parent mice (approximately 65 fold greater) than those of their young (Table 43), resulted in preweanling mice having only 0.5% of the total caecal nitroreductase activity, and 0.9% of β -glucuronidase activity per caecum than that present in their mothers (Table 42).

The body weights of mice used in this study increased from around 5.9g in the 2-week-old mice to 20g in 24-week-old mice, irrespective of diet (Table 43). An increase in caecal weight was observed of 46% for mice fed fibre-free diet and 99% for mice fed 5% pectin, from 4 to 24 weeks. Furthermore, mice fed 5% pectin diet had consistently larger caecal weights than mice fed the fibre-free diet varying from a 1.4 fold difference (0.056g versus 0.081g respectively) in 4 week old mice to a 2 fold difference (0.08g versus 0.16g) in 24 week old mice. The proportion of caecal weight to body weight was 0.5% in mice fed the fibre-free diet and a significantly larger 0.8% in mice fed 5% pectin, irrespective of age.

A large variation was observed in the numbers of bacteria per g caecal contents in any one age-group as indicated by the large

standard deviations (Table 43), resulting in no obvious diet or age-related changes in bacterial concentration in the caecal contents.

In this study investigating the effect of age and diet on certain gut flora enzyme activities, the diet (either fibre-free or 5% pectin) was fed to mice from weaning. To confirm that the data obtained was related to increasing age and not an adaptation to the diet, a second experiment was carried out in which the activities of nitrate reductase, nitroreductase and β -glucuronidase were determined in mice aged 14 weeks and fed on 5% pectin for either a duration of 11 or 2 weeks. It can be seen from Table 44 that, when expressed as per gram of caecal contents, no significant difference existed in any of the three enzyme activities between these 2 groups of mice. For example the rate of nitrite production was $27.5\mu\text{mol/hr/g}$ in mice fed pectin for 11 weeks and $26.7\mu\text{mol/hr/g}$ for mice fed pectin for 2 weeks. Nor were any significant differences in enzyme activity apparent between mice fed pectin for 2 or 11 weeks when values were adjusted to 10^{10} bacteria (to account for the 1.45 fold greater bacterial concentration of mice fed pectin for 2 weeks; Table 44). Table 43 shows that the caeca of mice fed 5% pectin for 2 weeks were on average 25% smaller than those of mice fed pectin for 11 weeks (0.131g compared to 0.174g respectively). As a result, the activities of nitrate reductase, nitroreductase and β -glucuronidase were 36%, 30% and 5% lower respectively in mice fed pectin for a 2 week duration.

In summary, the results indicate that the *in vitro* and/or *in vivo* mutagenicity of certain dietary genotoxins can be markedly

Table 44 Body weights, caecal weights and caecal enzyme activities of 14 week old mice fed 5% pectin for either 2 or 11 weeks.

| | Mice fed a 5% pectin diet for | |
|--|-------------------------------|---------------|
| | 11 weeks | 2 weeks |
| Body weight g | 19.25 ± 1.48 | 19.23 ± 1.59 |
| Caecal weight g | 0.174 ± 0.02 | 0.131 ± 0.02 |
| Bacteria/g caecal contents(x10 ¹⁰) | 9.93 ± 3.63 | 14.42 ± 6.16 |
| Nitrate reductase | | |
| µmol/hr/g | 27.75 ± 9.47 | 26.69 ± 4.36 |
| µmol/hr/caecum | 4.71 ± 1.12 | 3.46 ± 0.48 |
| µmol/hr/10 ¹⁰ bacteria | 31.07 ± 17.00 | 22.18 ± 11.60 |
| Nitroreductase | | |
| µmol/hr/g | 0.575 ± 0.086 | 0.548 ± 0.152 |
| µmol/hr/caecum | 0.099 ± 0.007 | 0.076 ± 0.013 |
| µmol/hr/10 ¹⁰ bacteria | 0.661 ± 0.307 | 0.430 ± 0.180 |
| β-glucuronidase | | |
| µmol/hr/g | 28.00 ± 4.54 | 32.49 ± 12.25 |
| µmol/hr/caecum | 4.84 ± 0.84 | 4.54 ± 1.65 |
| µmol/hr/10 ¹⁰ bacteria | 30.68 ± 10.52 | 26.41 ± 15.13 |

Suspensions were prepared from caecal contents of mice fed pectin for 2 or 11 weeks (Methods section 8a). Caecal contents from 3 mice were pooled for each caecal suspension with 4 suspensions prepared for each diet. Bacterial enumeration and enzyme activities were determined as described in Methods section 9a. Results are given as means ± SD. Comparisons between the 2 groups of mice were carried out using Anova.

influenced by the age of the animal. Developmental changes in the enzymes involved in the activation and detoxification of these compounds appear to be responsible for the observed changes in genotoxic activity.

In contrast, no consistent age-related change in gut-flora metabolism was apparent. Dietary pectin, however, greatly increased nitrate reductase activity in the caecal contents, irrespective of host age, although pectin had no marked effect on either nitroreductase or β -glucuronidase activities.

Modifications to the DNA-repair host-mediated assay

In the DNA-repair host-mediated assay of Mohn (1984), repairable DNA damage is determined using a pair of streptomycin-dependent *E. coli* K-12 derivatives which differ in DNA-repair capacity and in their ability to ferment lactose. During my studies various modifications to the DNA-repair host-mediated assay were tested to try to improve the sensitivity of this assay. These modifications included the use of :

1. *E. coli* strains deficient in cell layer lipopolysaccharide (LPS^{def}).
2. Bacteria in logarithmic growth in place of bacteria in stationary growth.
3. Treating the bacteria with EDTA to increase the permeability of the cell wall.

The main advantage of the intrasanguineous host-mediated assay, as a bacterial mutagenicity test, is that it combines the indicator microorganisms with mammalian metabolism. As shown by experiments presented in this thesis, a large proportion of an intravenous injection of bacteria (*E. coli* or *S. typhimurium* TA98) are recovered from the liver. Because the liver is an important site of xenobiotic metabolism, the host-mediated assay is able to provide close contact between indicator bacteria and reactive metabolites. However, it is thought that bacteria, such as *E. coli*, are unable to penetrate hepatocytes - the main site of metabolic activation. An investigation was carried out to determine the location of bacteria

within the liver of female BALB/c mice, after intravenous injection of bacteria, as in the host-mediated assay described in Methods section 11a.

The DNA-repair host-mediated assay can also be used to measure genotoxicity in the gastro-intestinal tract. Preliminary experiments are reported investigating the survival and transit time of the paired *E. coli* strains 343/765 and 343/753 through the mouse gastro-intestinal tract.

1. *E. coli* strains deficient in cell layer lipopolysaccharide

In the DNA-repair host-mediated assay the bacterial strains *E. coli* 343/765 (*uvr*⁺, *rec*⁺, Lac⁻) and *E. coli* 343/753 (*uvrB*, *recA*, Lac⁺) are used (see Methods section 11b). Derivatives of both strains, which contain an incomplete lipopolysaccharide cell layer, were developed by Mohn (1984) through mutation and isolated by their resistance to phage T7. These LPS-deficient (LPS^{def}) strains, *E. coli* 343/801 (*uvr*⁺, *rec*⁺, Lac⁻) and *E. coli* 343/772 (*uvrB*, *recA*, Lac⁺) were compared to the parent strains (*E. coli* 343/765 and 343/753 respectively) in their ability to detect the genotoxic anti-schistosomal drug, hycanthone. This compound was chosen as it was hoped to be used as a model chemical in future studies at the laboratory at University College to investigate dietary modification of glucuronide hydrolysis with the production of genotoxic agents.

Prior to using these LPS^{def} *E. coli* strains *in vivo* they were tested initially in the *in vitro* genotoxicity assay as described in Methods section 10b. For the *in vitro* genotoxicity assay, cultures of each strain were grown up overnight yielding final viable

populations (per ml) of 2.47×10^9 *E. coli* 343/765, 1.62×10^8 343/753, 4.24×10^8 343/801 and 3.1×10^7 343/772. It can be seen that the repair-deficient and the LPS^{def} cultures grew to smaller final populations than their proficient counterparts. From the above results the viability of *E. coli* 343/753, 343/801 and 343/772 was calculated as 6.6%, 17.2% and 1.3% respectively, of that of *E. coli* 343/765.

Each overnight culture of *E. coli* bacteria was sedimented and resuspended in PBS-S to give an optical density of 5. Aliquots (0.2ml) of these bacterial suspensions were incubated with 22 or 220nmol hycanthone or saline (in place of hycanthone) in a total volume of 1ml.

From Table 45, it can be seen that adjusting each bacterial suspension to the same optical density reading, still produced marked differences in the numbers of viable bacteria per unit volume. For instance, approximately 9×10^7 *E. coli* 343/772 (LPS^{def}, *recA*, *uvrB*) were added to incubation tubes compared to 9×10^8 of the sister (repair-proficient) strain *E. coli* 343/801. Similarly, there were greater numbers (by a factor of 6.2) of the repair proficient and LPS⁺ strain (*E. coli* 343/765) than its paired, repair-deficient strain (*E. coli* 343/753; Table 45)

In control samples, in which saline was added to the incubation in place of hycanthone, there was no loss of viability of either LPS⁺ strain (Table 45). However, for the LPS^{def} bacteria, there was a 64% reduction in viable cell numbers for both strains during the preincubation period (Table 45). Since the genotoxicity data

Table 45 Survival of *E. coli* 343/765 and its repair deficient and LPS-deficient derivatives treated with hycanthone in the *in vitro* genotoxicity assay

| <i>E. coli</i> strain | Viable <i>E. coli</i> /incubation mix ($\times 10^7$) | | |
|---|---|-----------------|-------------------|
| | Hycanthone concentration (μM) | | |
| | 0 | 22 | 220 |
| 343/765 (LPS ⁺ , <i>uvr</i> ⁺ , <i>rec</i> ⁺) | 70.8 \pm 6.3 | 58.3 \pm 10.4 | 64.7 \pm 3.3 |
| 343/753 (LPS ⁺ , <i>uvrB</i> , <i>recA</i>) | 13.4 \pm 0.33 | 2.46 \pm 0.12 | 0.54 \pm 0.16 |
| 343/801 (LPS ^{def} , <i>uvr</i> ⁺ , <i>rec</i> ⁺) | 33.0 \pm 3.1 | 32.2 \pm 12.8 | 25.0 \pm 4.9 |
| 343/772 (LPS ^{def} , <i>uvrB</i> , <i>recA</i>) | 3.32 \pm 1.34 | 0.34 \pm 0.12 | 0.014 \pm 0.003 |

| | Viable bacteria initially added to incubation ($\times 10^7$) |
|---------|---|
| 343/765 | 67.3 \pm 1.2 |
| 343/753 | 10.8 \pm 3.5 |
| 343/801 | 92.3 \pm 11.0 |
| 343/772 | 9.31 \pm 1.68 |

Overnight cultures of each *E. coli* strain were resuspended in PBS-S to an optical density of 5. Aliquots (0.2ml) of the resuspended cultures were added to incubations containing S9, appropriate cofactors and hycanthone/saline (in a total volume of 1ml) and incubated for 1 hr at 37°C. After the incubation period, 0.1ml was removed and diluted 100 fold in ice cold saline. Further appropriate dilutions were carried out prior to plating on to NRS-S agar (Methods section 7b & 10b). Assays were carried out in duplicate with appropriate dilutions plated in triplicate. Results are given as means \pm SD. Refer also to Fig. 36.

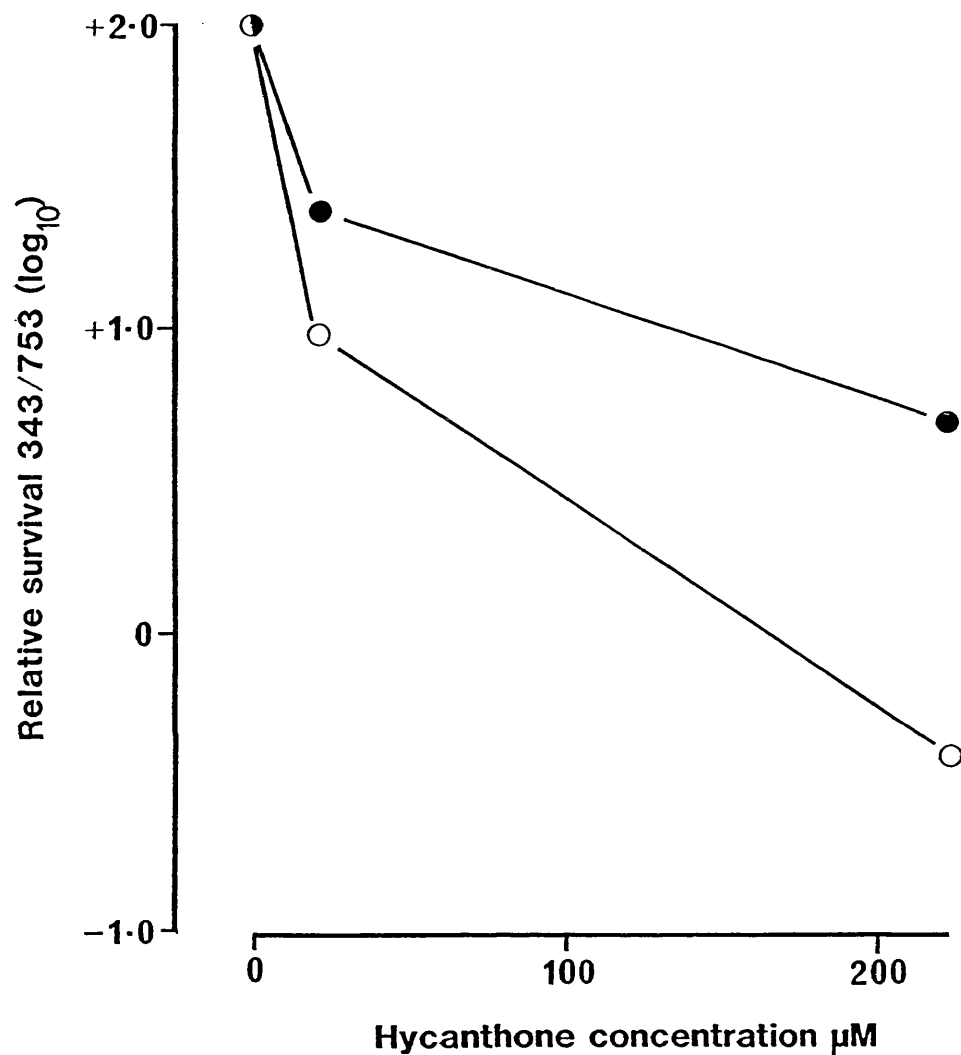


Figure 36 Comparison of LPS^{def} and LPS⁺ *E. coli* indicator bacteria in their ability to detect the genotoxin, hycanthone in an *in vitro* assay. Overnight cultures of the *E. coli* strains 343/765 (Lac⁻,uvr⁺,rec⁺), 343/753 (Lac⁺,uvrB,recA), 343/801 (LPS^{def},Lac⁻,uvr⁺,rec⁺) and 343/772 (LPS^{def},Lac⁺,uvrB,recA) were resuspended in PBS-S to an optical density of 5. Aliquots (0.2ml) of each culture were added to incubations containing S9, cofactors and hycanthone (in a total volume of 1ml). For further details, refer to Table 45 and Methods sections 7b & 10b. Results (see Table 45) are calculated as survival of the repair deficient strains with respect to the appropriate repair proficient strain and given as means of triplicate plates of 2 replicate incubations. LPS⁺ (●); LPS^{def} (○).

is expressed as the percentage relative survival of the repair deficient to the repair proficient strain no genotoxic activity was apparent in these two sets of control samples.

In incubation tubes containing hycanthone a marked decline in the relative survival of both repair deficient strains (*E. coli* 343/753 and 343/772) was observed - the extent of the reduction being dependent on both the concentration of hycanthone and the LPS status of the cell layer (Fig. 36). As the amount of hycanthone present in the incubation increased, the numbers of viable repair deficient bacteria decreased with the greatest loss of viability occurring in the LPS^{def} cells (Fig. 36). At a hycanthone concentration of 22µM, the relative survival of the LPS^{def} strain *E. coli* 343/772 fell by 90% compared to the controls (containing saline in place of hycanthone). This reduction in the relative survival of the LPS^{def} strain decreased by a further 95% at the higher mutagen dose of 220µM hycanthone (Fig. 36). In comparison, the relative survival of *E. coli* 343/753 (LPS⁺) decreased by 78% and then by a further 80% as the concentration of hycanthone increased from 0µM (ie. saline) to 22µM and 220µM hycanthone respectively (Fig. 36). Cell death was apparent in both the repair proficient strains exposed to hycanthone with reductions of 9% and 21% observed for *E. coli* 343/765 and 343/801 respectively at the highest mutagen concentration (Table 45).

Since the LPS^{def} strains of *E. coli* were more sensitive to the genotoxic action of hycanthone, further experiments were attempted to investigate the response of LPS^{def} *E. coli* strains towards hycanthone *in vivo*. However problems arose when using these

strains. Firstly, it was difficult to obtain sufficient overnight culture of the LPS^{def} repair deficient *E. coli* strain 343/772 as required for a host-mediated assay, due to the reduced growth kinetics of this strain. Secondly, the mucoid colony morphology of *E. coli* 343/772 led to a strong tendency to confluence of large sized colonies, making it difficult to make exact measurements of differential killing effects. As a result of these problems I considered it impractical to continue using these strains on a routine basis. Consequently, other methods of improving the sensitivity of the DNA-repair host-mediated assay were investigated.

2. Bacteria in logarithmic growth

Actively growing bacteria are considered to be more sensitive to the genotoxic action of chemicals. An investigation was carried out to determine whether the paired indicator *E. coli* strains 343/765 and 343/753 were more sensitive to the genotoxic activity of nitrosomethylurea when in logarithmic growth phase or when in stationary growth in the *in vitro* genotoxicity assay (Methods 10b). The carcinogen, nitrosomethylurea was used since similar comparisons (of bacteria in logarithmic and stationary growth phase) have been carried out using this compound at the laboratory at University College for other *E. coli* indicator strains.

The growth curve of *E. coli* 343/765 and 343/753 is shown in Figure 37. From this figure it can be seen that both strains are in logarithmic growth 40 minutes after adding the bacterial inoculum to the fresh PEPS-bouillon (Methods 7b). Initially the rates of exponential growth for *E. coli* 343/765 and 343/753 were similar with values of optical density doubling every 60 minutes. However, the

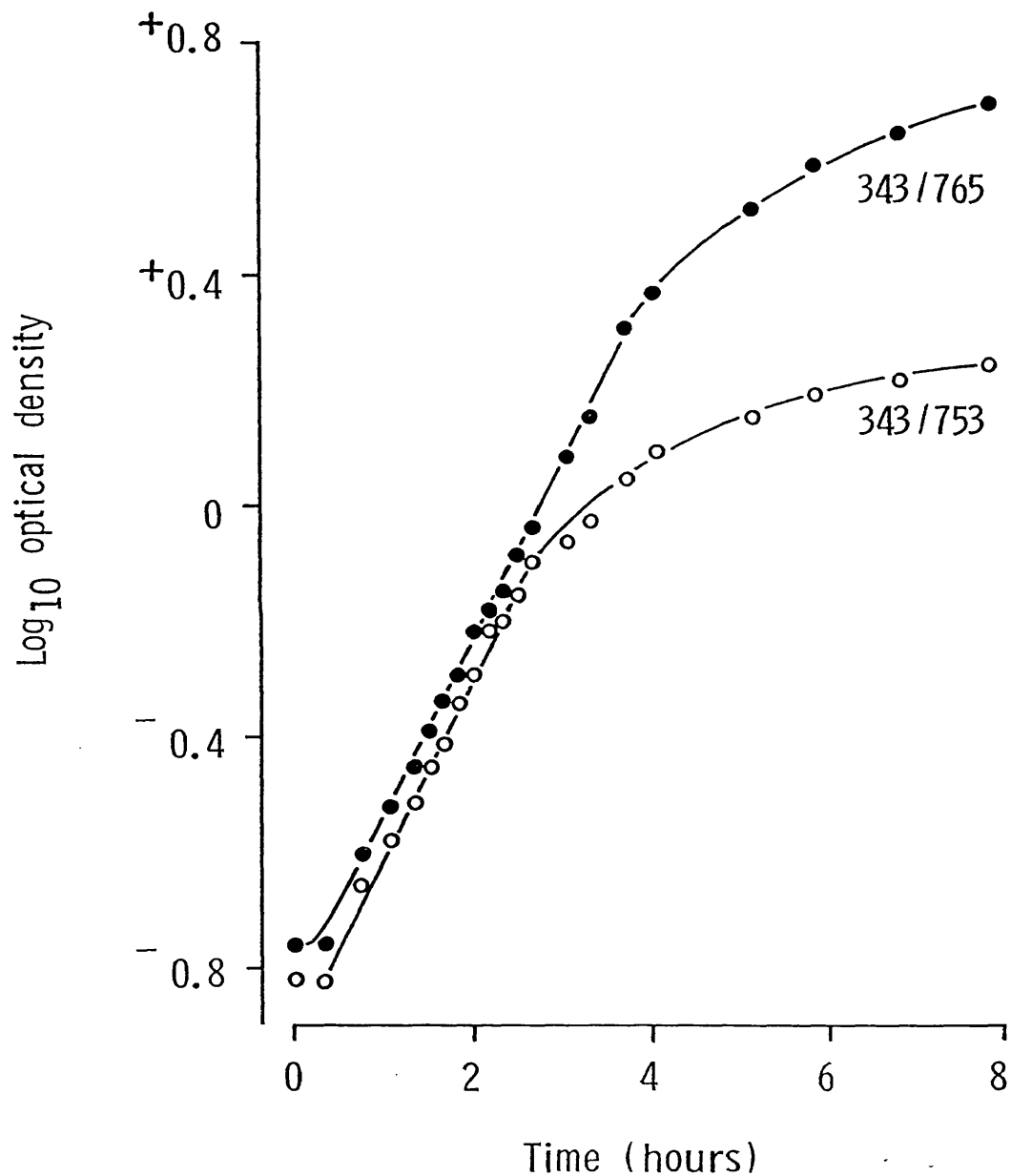


Figure 37 Growth curves of *E. coli* 343/765 and 343/753. An aliquot of overnight culture of each strain was inoculated into fresh growth medium (PEPS-bouillon) to give an optical density of 0.1. The cultures were shaken at 37°C and the optical density measured at regular intervals. For further details see Methods section 7b. *E. coli* strains: 343/765 (Lac^- , uvr^+ , rec^+ ; ●); 343/753 (Lac^+ , $uvrB$, $recA$; ○).

length of the logarithmic growth phase differed for the two bacteria with the repair proficient strain remaining in logarithmic growth for approximately 3.5 hours compared to 2 hours for the repair deficient strain (Fig. 37).

In the *in vitro* genotoxicity assay (Methods section 10b), aliquots of bacteria were removed from the actively growing cultures when optical density readings of 0.90 and 0.78 for *E. coli* 343/765 and 343/753 respectively were obtained. The bacteria (both those in logarithmic growth and aliquots of a stationary overnight culture) were then resuspended to an optical density of 5. As observed in the previous section, the numbers of viable bacteria present in suspensions of similar optical density were lower (by 78%) for the repair deficient strains as compared to the paired repair proficient strain irrespective of growth phase (Table 46). Furthermore, stationary cultures contained approximately 40% fewer viable bacteria than an equivalent volume of actively growing cells (Table 46).

When exposed to nitrosomethylurea an increase was observed in the amount of repairable DNA damage (as measured by the reduction in the relative survival of the repair deficient strain) concomitant with the increase in carcinogen concentration. The extent of the increase in DNA damage was similar for both actively growing and stationary cells. For example, for cells in either growth phase there was an 88% decline in the relative survival of the repair deficient strain as the mutagen concentration increased from 0.2 μ M to 1.2 μ M. Furthermore this increase in repairable DNA damage occurred at a logarithmic rate except for actively growing cells, at

Table 46 Effect of growth phase and exposure to nitrosomethylurea on the survival of *E. coli* 343/765 and its repair deficient derivative 343/753 in the *in vitro* genotoxicity assay

| NMU (μ M) | Viable <i>E. coli</i> /incubation ($\times 10^7$) | | | |
|-------------------|---|----------------|-----------------|-------------------|
| | 343/765 | | 343/753 | |
| | stationary | log | stationary | log |
| 0 | 31.8 \pm 6.9 | 23.7 \pm 3.5 | 8.90 \pm 1.01 | 3.92 \pm 0.58 |
| 0.2 | 20.6 \pm 3.6 | 21.6 \pm 3.2 | 6.92 \pm 1.17 | 2.15 \pm 0.47 |
| 0.6 | 27.0 \pm 8.1 | 17.2 \pm 2.1 | 4.02 \pm 0.81 | 0.69 \pm 0.17 |
| 1.2 | 24.4 \pm 8.8 | 14.3 \pm 2.4 | 1.01 \pm 0.32 | 0.18 \pm 0.04 |
| 2.0 | 12.3 \pm 2.8 | 12.2 \pm 2.3 | 0.30 \pm 0.08 | 0.042 \pm 0.008 |
| | Viable bacteria added initially to incubation ($\times 10^7$) | | | |
| | 36.0 \pm 3.5 | 23.1 \pm 2.5 | 8.1 \pm 0.71 | 4.75 \pm 0.71 |

Each *E. coli* strain was grown in PEPS-bouillon as detailed in Methods 7b. When at the required stage of growth the bacteria were sedimented and resuspended in PBS-S to an optical density of 5. Aliquots (0.2ml) of the resuspensions were added to incubations containing nitrosomethylurea/saline (in a total 0.6ml volume) and incubated for 30 mins at 37°C. After the incubation period, aliquots (0.1ml) were diluted 100 fold in ice cold saline. Further appropriate dilutions were carried out prior to plating on to NRS-S agar (Methods section 10b). Assays were carried out in duplicate with appropriate dilutions plated in triplicate. Results are given as means \pm SD. Refer also to Figs. 37 & 38.

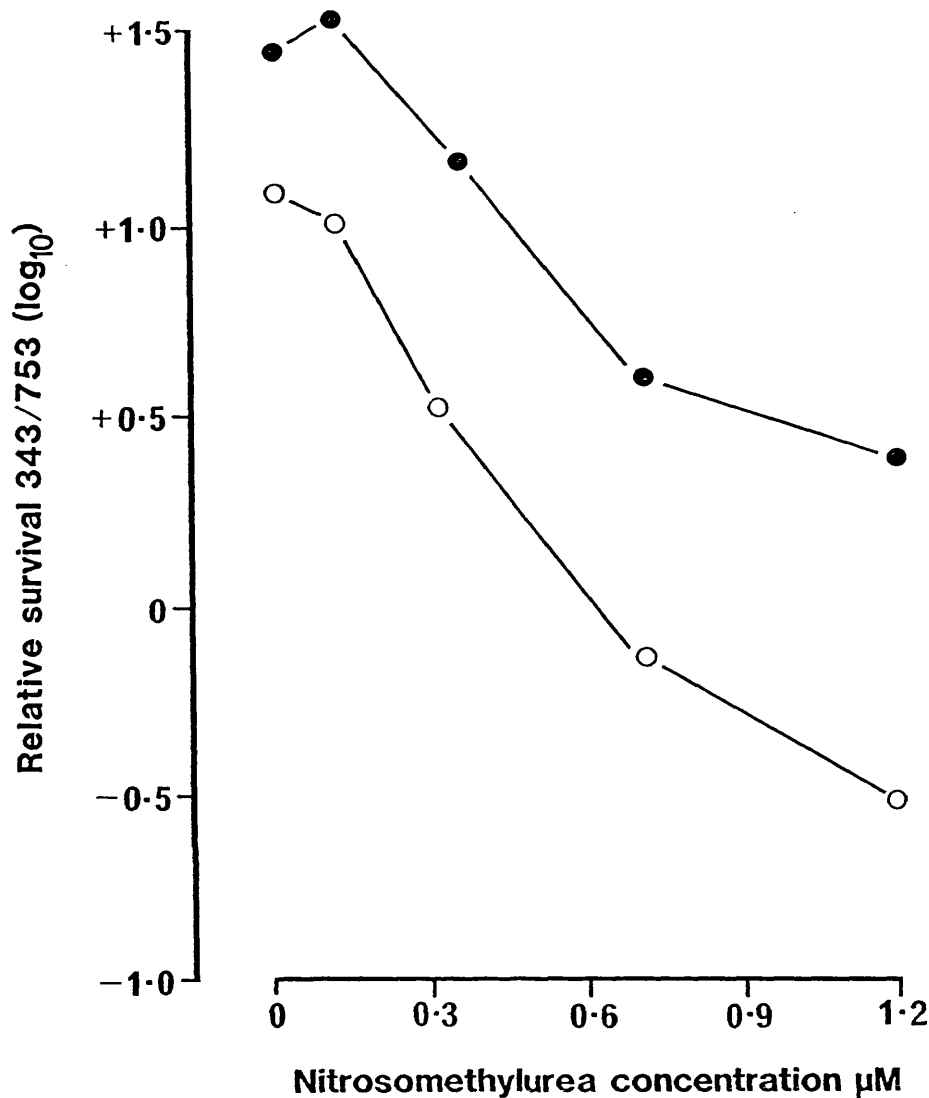


Figure 38 The effect of growth phase on the ability of *E. coli* 343/765 and 343/753 to detect the genotoxin, nitrosomethylurea. An aliquot of overnight culture of each strain was inoculated into fresh growth medium and shaken at 37°C until the cultures were in mid-logarithmic growth (see Fig. 37). These log growth cultures and overnight stationary cultures were then resuspended in PBS-S and aliquots (0.2ml) incubated with different concentrations of nitrosomethylurea (in a total volume of 0.6ml). After the incubation, aliquots (0.1ml) were diluted in ice-cold saline prior to plating onto NRS-S agar. For further details refer to Table 46 and Methods sections 7b & 10b. Results (see Table 46) were calculated as the percentage survival of the repair deficient strain (343/753) with respect to the repair proficient strain (343/765) from triplicate plates of 2 replicate incubations and given as means. Growth status : logarithmic (O); stationary (●).

the highest mutagen concentration (2.0 μ M nitrosomethylurea; Fig. 38) and for those bacteria in stationary growth, the reduced survival of *E. coli* 343/753 was only apparent at concentrations above 0.2 μ M nitrosomethylurea (Fig. 38).

In addition, as the concentration of nitrosomethylurea in the incubation mix increased from no mutagen to 2.0 μ M nitrosomethylurea there was a decline in the survival of the repair proficient strain 343/765 by 61% and 48% for bacteria in logarithmic and stationary growth respectively (Table 46).

3. Treatment of *E. coli* 343/765 and 343/753 with EDTA

Treating bacteria with EDTA as described in Methods 7c increases the permeability of the cell wall by removing some of the lipopolysaccharide. An experiment was carried out to investigate whether this pre-treatment increases the sensitivity of the paired indicator strains *E. coli* 343/765 and 343/753 to the genotoxic activity of the dietary carcinogens, IQ and aflatoxin B₁.

As reported earlier in this chapter (see sections 1 & 2), the number of viable *E. coli* 343/753 per ml overnight culture was lower (by 90%) than that of the repair proficient *E. coli* 343/765. In the *in vitro* genotoxicity assay (Methods section 10b), each strain, whether pretreated with EDTA or untreated was incubated separately with mutagen. The EDTA treatment alone produced a reduction in the number of viable bacteria, by 23% and 67% for *E. coli* 343/765 and 343/753 respectively, compared to the untreated bacteria (Table 47).

When exposed to IQ (5, 50, 500nM) there was no significant loss

in viability of the repair proficient strain (*E. coli* 343/765), irrespective of treatment with EDTA or not (Table 47). For the repair deficient strain (343/753) a decline in survival, of around 66%, was apparent for the untreated cells at the highest concentration (500nM) of IQ. However, the viability of *E. coli* 343/753 pre-treated with EDTA was markedly reduced at all concentrations of IQ tested. For example when 5 or 500nM of IQ was included in the incubation mix the numbers of 343/753 decreased by 80% and 98% respectively with respect to those incubations containing no mutagen (Table 47, Fig. 39).

When aflatoxin B₁ was included in the incubation mix there was a loss of viable repair proficient bacteria (343/765) at the highest aflatoxin concentration (1.6μM), of 12% and 63% for untreated and EDTA-treated cells respectively (Table 47). As a result of this decline in viability of *E. coli* 343/765, the data are expressed as the percentage survival of the repair deficient *E. coli* 343/753 relative to the repair proficient strain *E. coli* 343/765 (Fig. 39). As for IQ, there was a decline in the relative survival of the repair deficient strain as the concentration of aflatoxin in the incubation mix increased. In the control samples (no EDTA treatment) the relative survival of *E. coli* 343/753 decreased to 64% in incubations containing 0.08μM aflatoxin B₁ and to 8% for 1.6μM aflatoxin with respect to incubations containing DMSO in place of mutagen (Table 47; Fig. 39). In comparison the relative survival of *E. coli* 343/753 pre-treated with EDTA at the same aflatoxin B₁ concentrations (0.08 and 1.6μM) was 10% and 2% respectively (Table 47; Fig. 39).

Table 47 The survival of *E. coli* 343/765 and 343/753 after pretreatment with EDTA in the *in vitro* genotoxicity assay

| Mutagen | Concentration | Viable <i>E. coli</i> /incubation (x10 ⁷) | | | |
|--------------------------|---------------|---|------------|--------------|-------------|
| | | Controls | | EDTA-treated | |
| | | 343/765 | 343/753 | 343/765 | 343/753 |
| IQ | 5nM | 52.0 ± 3.6 | 11.9 ± 0.8 | 18.8 ± 10.3 | 1.4 ± 0.3 |
| | 50nM | 49.6 ± 11.0 | 13.4 ± 1.6 | 15.1 ± 5.0 | 2.3 ± 1.4 |
| | 500nM | 60.0 ± 10.1 | 4.6 ± 1.4 | 18.1 ± 2.8 | 0.13 ± 0.01 |
| Aflatoxin B ₁ | 0.08µM | 64.3 ± 4.0 | 8.6 ± 0.8 | 16.4 ± 1.0 | 0.42 ± 0.87 |
| | 0.32µM | 58.7 ± 7.2 | 5.8 ± 1.7 | 21.3 ± 5.6 | 0.14 ± 0.22 |
| | 1.60µM | 56.7 ± 6.7 | 1.1 ± 0.3 | 9.7 ± 1.5 | 0.06 ± 0.02 |
| DMSO | 10µl | 64.7 ± 7.6 | 13.6 ± 7.4 | 26.5 ± 7.1 | 6.8 ± 0.9 |

Viable bacteria added to incubation (x10⁷):
 860 ± 87 254 ± 18 660 ± 57 84 ± 18

Aliquots of overnight cultures of *E. coli* 343/765 & 343/753, resuspended in Tris/HCl pH 8, were exposed for 3 mins to EDTA at 37°C. To stop EDTA activity MgCl₂ was added to the suspensions as described in Methods section 7c. The bacteria (EDTA treated and untreated [controls]) were then resuspended in PBS as required for the *in vitro* genotoxicity assay (Methods section 10b) and aliquots (0.2ml) added to incubations containing S9, cofactors and mutagen (or DMSO) in a total volume of 1ml and incubated for 30 mins at 37°C. After the incubation, 0.1ml was removed and diluted appropriately in saline prior to plating, in triplicate, on NRS-S agar. Each assay was carried out in duplicate. Results are given as means ± SD.

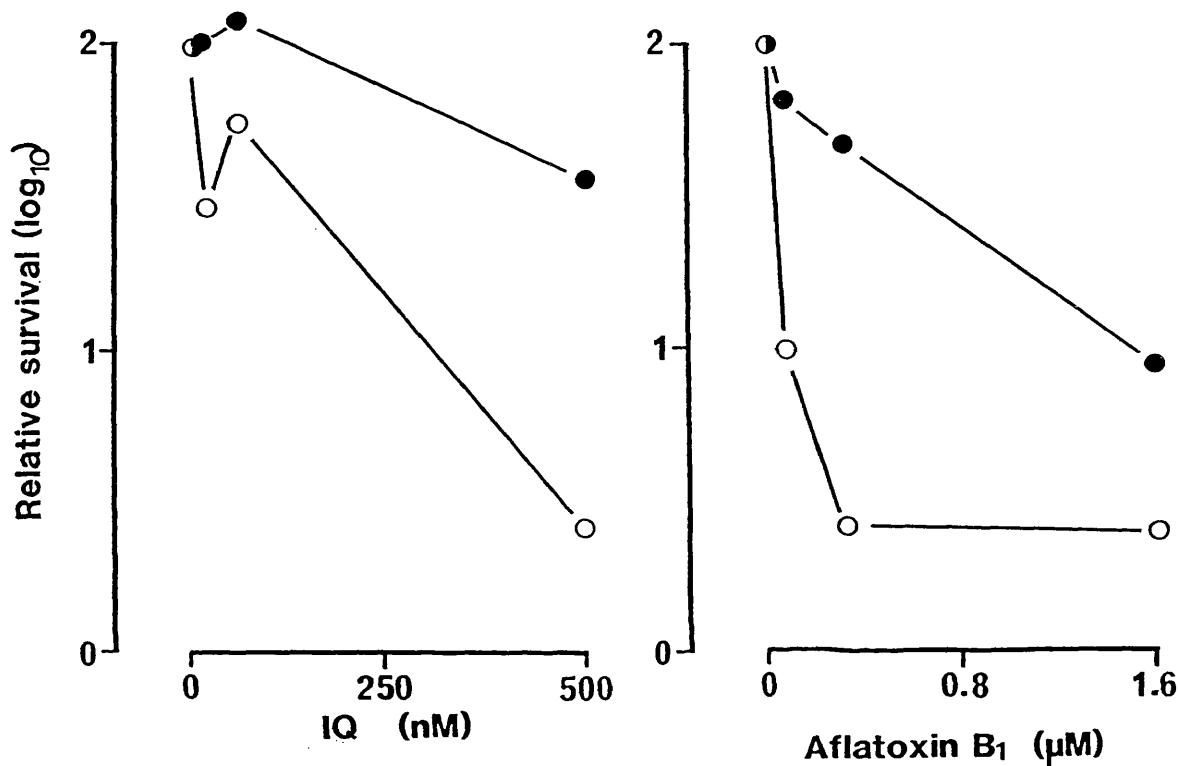


Figure 39 The effect of pretreating *E. coli* bacteria with EDTA on their ability to detect the genotoxic action of two food mutagens. Overnight cultures of *E. coli* 343/765 and 343/753 were exposed for 3 min at 37°C to EDTA (final concentration 0.5mM). MgCl₂ (final concentration 2mM) was added to the cultures to stop EDTA activity. The bacteria were resuspended in saline and used in the *in vitro* genotoxicity assay. Incubations contained (in a total volume of 0.6ml) S9, cofactors and mutagen and were incubated for 30 mins at 37°C, prior to plating appropriate dilutions on to NRS-S agar. Assays were carried out in duplicate with triplicate plates of appropriate dilutions. For further details refer to Table 47 and Methods sections 7c & 10b. Results (see Table 47) are given as means and expressed as the percentage survival of 343/753 relative to 343/765. EDTA treated (○); untreated (●).

4. Location of *E. coli* bacteria in livers of mice used in intrasanguineous host-mediated assays

To investigate the hepatic location of indicator bacteria in the intrasanguineous host-mediated assay two female BALB/c mice, 7 - 8 weeks of age were injected via the tail vein with 0.2ml bacterial suspension in saline (approximately 1×10^{10} cells). The bacteria chosen for this investigation were *E. coli* D494 as this indicator strain was being used predominantly in the laboratory at University College at the time of the study. The mice were left for 30 minutes before removing and preparing sections of liver for electron microscopy (Methods section 13). A short exposure time was chosen since my studies were concerned with nitrosodimethylamine genotoxicity (see Chapter 5). The reactive metabolite of nitrosodimethylamine is short-lived. Consequently damage to the bacterial DNA is likely to occur soon after injection of the compound (together with the bacteria) into the bloodstream. Since the aim of the experiment was to determine the location of bacteria at the point at which they are exposed to DNA-damaging agents it was considered that 30 minutes was a suitable exposure time.

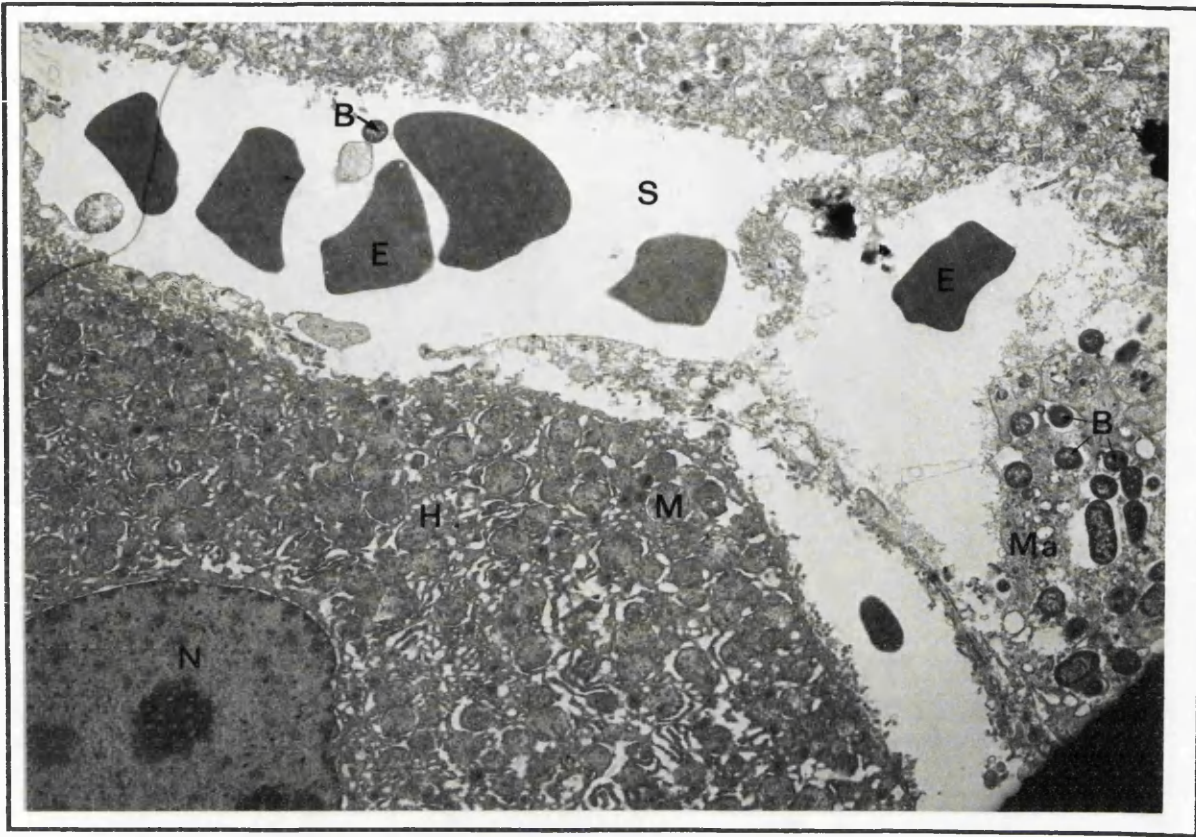
Sections of liver were prepared from all lobes. Ten sections, chosen at random, were examined under the electron microscope with the whole section closely scanned for the presence of bacteria. Two electron micrographs are given in Fig 40 which represent results obtained in this experiment. It was observed that bacteria were located mainly in mesenchymal cells (macrophages, Kupffer cells) lining the liver blood vessels. These cells were often packed with *E. coli* and were found to contain up to 26 bacteria. Since pseudopodia and phagocytosis were not seen and few bacteria

Figure 40 Electron micrographs of liver sections from mice injected intravenously with E. coli D494. Two female BALB/c mice were injected iv with approximately 2×10^{10} E. coli D494 suspended in saline as in the host-mediated assay (Methods section 11b). After 30 mins, the mice were sacrificed, the livers removed and sections prepared for electron microscopy as described in Methods section 13.

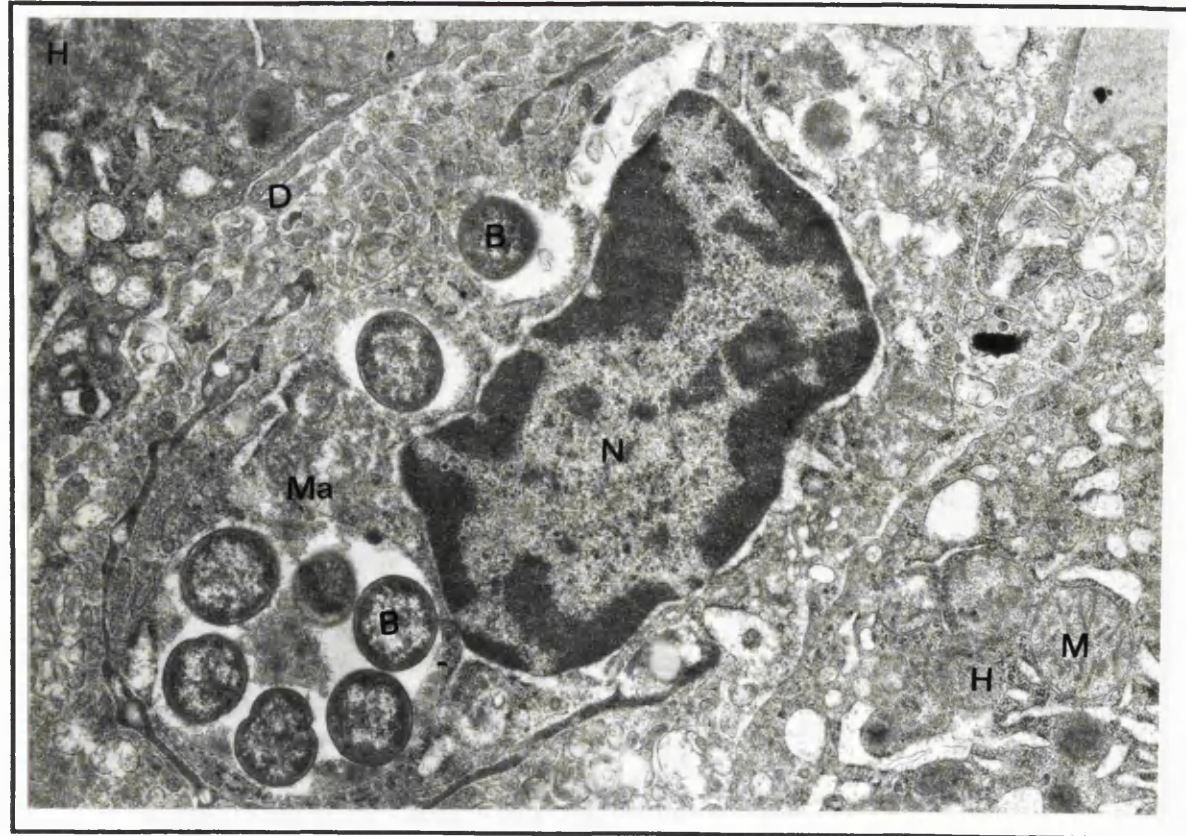
Photograph i. : Liver sinusoid with macrophage containing E. coli D494 and adjacent hepatocytes. Magnif. x 4,500
ii. : E. coli trapped in macrophage. Magnif. x 15,750

Abbreviations : B, bacterium; D, space of Disse; E, erythrocyte; H, hepatocyte; M, mitochondria; Ma, macrophage; N, nucleus; S, sinusoid.

(i)



(ii)



were sited free within the sinusoids, it would indicate that the absorption of bacteria into these cells had occurred very quickly. At no time were bacteria observed in hepatocytes.

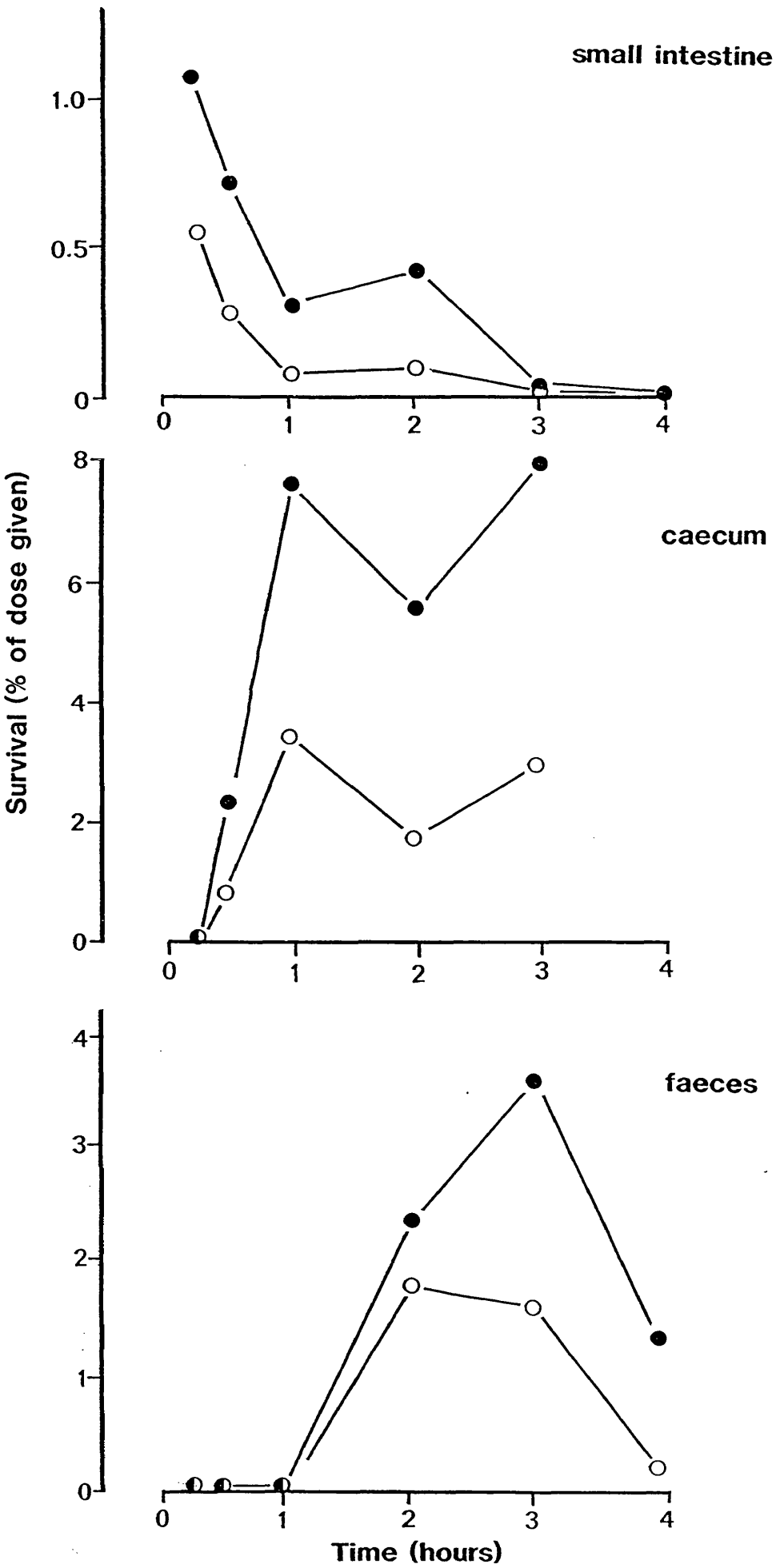
5. Recovery of orally dosed *E. coli* 343/765 and 343/753 from the mouse gastro-intestinal tract

The bacterial strains *E. coli* 343/765 and 343/753 can be given to rodents orally and recovered from the gastro-intestinal tract (since these indicator strains are streptomycin-dependent and can therefore, be distinguished from the streptomycin-sensitive coliforms in the gut). A preliminary investigation to determine the survival of the two strains during their passage through the mouse gut was carried out.

Overnight cultures of *E. coli* 343/765 and *E. coli* 343/753 were mixed in a ratio of 1:20 (v/v), sedimented and resuspended in saline to an optical density of 80 (approximately 8×10^8 cells of each strain/ml). Aliquots (0.2ml) were then dosed *per os* to female BALB/c mice 7 - 8 weeks of age as described in Method section 11b. At various times after dosing mice were sacrificed and the small intestine and caecal contents ^{excised} (in addition to any faecal pellets that had been excreted) and treated appropriately.

Within 15 minutes of dosing the mice up to 4×10^7 indicator bacteria (5.2% of the original dose) were recovered from the small intestine (Fig. 41). Subsequently, the numbers of viable bacteria declined, until 4 hours after dosing, 0.07% of the administered dose were recovered. Coincident with this decline in the small intestine, there was an increase in the numbers of *E. coli* 343/765

Figure 41 The survival and transit of *E. coli* 343/765 and 345/753 through the mouse gastro-intestinal tract. Mice were dosed per os with 0.1ml suspension of *E. coli* 343/765 and 343/753 (approximately $2-3 \times 10^9$ cells of each strain). At regular intervals mice were sacrificed, the caecal sac (and contents) and small intestine (with contents) removed and faeces collected. Each sample was homogenized in PBS-S and appropriate dilutions plated, in triplicate, on to NRS-S agar as described in Methods section 11b. For the caecum and small intestine, results are given as means of 4 samples. For the faecal samples results are given as means of two samples. *E. coli* 343/765 (●); *E. coli* 343/753 (○).



and 343/753 recovered from the caecal sac, rising from 6×10^4 bacteria (0.008% of the given dose) 15 minutes after dosing, to 8×10^7 cells (9% of the dose) at 1 hour (Fig. 41).

The two strains were detected in the faeces 15 minutes after dosing, although the numbers only comprised 0.002% of the dose, approximately 2×10^4 bacteria for the entire faeces excreted (0.2g). At one hour, the numbers of bacteria recovered from the faeces had increased to more than 2×10^6 bacteria and thereafter the number of bacteria rose rapidly, peaking between 2 - 3 hours after administration, where a total of 8×10^6 bacteria were recovered from 0.36g of faeces.

The transit time of the repair deficient strain *E. coli* 343/753 was similar to that of the repair proficient strain. However, the survival of 343/753 was markedly reduced, around 60 - 90%, of that of 343/765 at 15 minutes, and only 20 - 30% at the 4 hour timepoint. It would appear from these results that removing the caeca from mice at least 1 hour after administration of the bacteria, would give optimal recovery of the bacteria.

CHAPTER 8

Discussion

The large epidemiological differences in the occurrence of various forms of cancer indicate a role for environmental factors in carcinogenesis. Of these environmental factors diet and nutrition are thought to contribute a major proportion. However, the task of evaluating the aetiology of dietary components in the development of cancer is considerable since foods are highly complex chemical mixtures. Epidemiological and experimental studies indicate that the macronutrients, fat and protein, may be involved (review by Natl. Acad. Sci. 1982). In general, diets high in fat and protein increase the probability of developing certain cancers, notably of the breast, large bowel and prostate. This increased risk in developing cancer may be associated with observed, diet-related, changes in mixed function oxidase activities. Such changes in metabolic capacity suggests that the ability to metabolize (activate/detoxify) xenobiotics, including carcinogenic presursors, is modified by diet. Using *in vivo* and *in vitro* bacterial mutation assays as a measure of genotoxic potential, I have found that the mutagenic activity of three dietary carcinogens (that require metabolism by the mixed function oxidase system to become active) is dependent upon the type and/or amount of fat and protein in the diet. A summary of these results is outlined in Table 48.

1. Dietary fat

In the series of experiments reported in Chapter 3, high (25% w/w) saturated and monounsaturated fat diets (represented by beef

Table 48 Summary of the effects of dietary fat and protein on the activity of food mutagens in bacterial mutation assays.

| Mutagen | Relative change in mutagenicity ¹ | | | |
|--------------|--|-----------------|----------------|-----------------|
| | High fat | | High protein | |
| | <i>in vivo</i> | <i>in vitro</i> | <i>in vivo</i> | <i>in vitro</i> |
| Aflatoxin B1 | ↓ | NC | ↓ | NC |
| MeIQ | ↑ | ↑ | NC | NC |
| Trp-P-2 | ↑ | ↑ | NC | NC |

Mice were fed different fat or protein diets and either used in the host-mediated assay (*in vivo* data) or were used for the preparation of hepatic S9 fractions and incubated with mutagens in the *Salmonella* mutagenicity assay (*in vitro* data). Refer to Chapters 3 & 4 for further details.

¹ with respect to data from mice fed low fat or low protein control diets

NC - no change.

dripping and olive oil respectively) were compared to a low (1%) fat diet. This level (25%) of dietary fat provided approximately 45% of the total energy supplied, which is similar to that consumed by many western populations. The latest report of the National Food Survey Committee states that in Britain in 1987 the proportion of dietary energy derived from fat was 42.2% (Anon. 1989).

In the initial investigations looking at the effect of dietary fat on the *in vitro* mutagenicity of aflatoxin B₁, MeIQ and Trp-P-2 a dramatic rise in the mutagenicity of the two heterocyclic amines was observed when hepatic S9 fractions from mice fed either high fat diet were included in the incubation mix in place of fractions from mice fed the low fat diet (Figs. 8 - 10). The extent of the increase depended on the source of fat, since hepatic preparations from mice fed olive oil had a greater capacity to activate these heterocyclic amines to bacterial mutagens than those from mice fed beef dripping (Figs. 9 & 10). Similar diet-related increases were reported for the *in vitro* mutagenicity of the heterocyclic amines, MeIQ, IQ and MeIQx (Alldrick *et al* 1987a), the aromatic amide 2-acetylaminofluorene (Castro *et al* 1978) and benzo(a)pyrene (Cassano *et al* 1987). In contrast to these results, Dolara *et al* (1986) demonstrated that a 20% olive oil diet protected mice against the ability of IQ to induce nuclear damage in colon epithelial cells, as compared to a 5% lipid diet. However, measurement of nuclear damage detects both mutagenic and non-mutagenic events. As it is not possible to identify, with certainty, at which point olive oil was having its effect this data may not conflict with the present results.

The diet-related increase in the mutagenicity of heterocyclic compounds may be due to a number of factors. From previous studies of Alldrick *et al* (1987a) it would appear that such diet-related increases in MeIQ and Trp-P-2 mutagenicity are not attributable to organochlorine residues (chemicals known to induce mixed function oxidases; Alvares & Kappas 1977) present in the fat. Alldrick and colleagues have carried out a number of studies on the effects of dietary fat on genotoxicity in the BIBRA laboratories (where part of this thesis project was carried out) and are of particular relevance since they used diets based on the recipe followed in this investigation (see Table 2). Alldrick *et al* (1987a) found that in order to achieve equivalent levels of mutagenicity, the low fat diet required 15 times more Aroclor 1254 to be added to it, than Aroclor equivalents measured in the high fat diet. In addition, Alldrick *et al* (1987a) demonstrated that degradation products within the fat, such as peroxides, did not vary sufficiently to account for the changes observed in heterocyclic amine activity. Nor is it likely that either vitamin E or cholesterol content are important in producing the marked changes in mutagenicity associated with dietary fat. In the first instance, the diets were prepared so that they contained the same amount of vitamin E (by weight) and secondly, Alldrick *et al* (1987b) found that in rats fed diets containing increasing amounts of beef dripping and a constant amount of cholesterol, that it was the triglyceride and not the cholesterol content that was the controlling factor in causing increased conversion of the heterocyclic amines to bacterial mutagens. Alldrick *et al* (1987b) have proposed that fat-related changes in genotoxicity may be related to fatty acid composition since the decrease in the activation of mutagens by S9 fractions from rats fed

a variety of high fat diets (olive oil, beef dripping, sunflower oil and coconut oil) was similar to their monounsaturated fatty acid content. This may indeed be the case with the results reported here for mice, since the extent of the increase in mutagenicity of MeIQ and Trp-P-2, after feeding the olive oil and beef dripping diets (Fig. 9 & 10), corresponds to the monounsaturated fatty acid content of the two diets, 49.6 monounsaturated fatty acid/100g total fatty acid and 84.4g/100g total fatty acid for beef dripping and olive oil respectively.

The greater body and liver weights of mice fed the high fat diets (Table 6) may suggest that the observed effects are a consequence of increased caloric intake. The observation, however, that the effects of the two fats used in this study were different and, in previous investigations, that the degree of enhancement of mutagen metabolism associated with high fat diets is dependent on the chemical nature of the fat fed, strongly suggests that these diet-related changes in genotoxicity are due to fat *per se*.

The present study agrees with the view that dietary fat can stimulate the production of enzymes capable of converting carcinogens to active mutagens. Associated with this are the many reports that dietary fat modifies mixed function oxidase activity (Martin *et al* 1980, Alldrick *et al* 1987a, Newberne *et al* 1979, Fielding & Huges 1976, Wade *et al* 1978), with the nature of these changes dependent on both the amount and type of fat. However, this ability of dietary fat to modify hepatic metabolism of chemical carcinogens is not universal.

Despite the influence of dietary fat on the activation of heterocyclic amines, there was no apparent effect of dietary fat on the *in vitro* activation of aflatoxin B₁ (Fig. 8). It is possible that the differing effects of dietary fat on the activation of aflatoxin B₁ and the heterocyclic amines is a consequence of differential induction of mixed function oxidase enzymes. Whereas the activation of MeIQ and Trp-P-2 is thought to involve cytochrome P448-mediated N-hydroxylation (Ishii *et al* 1980b, Okamoto *et al* 1981, Yamazoe *et al* 1983), aflatoxin B₁ requires epoxidation to form the ultimate reactive species, aflatoxin B₁-2,3-oxide (Swenson *et al* 1977, Essigmann *et al* 1982). Several cytochrome P450 isoenzymes have been identified as having a role in aflatoxin B₁ activation including certain species of cytochrome P448 (Ishii *et al* 1986, Shimada *et al* 1987, Yoshizawa *et al* 1982). Studies of diet-induced changes in specific cytochrome P450 isoenzymes, however, were not part of the present investigation and so it is not possible to confirm whether differential induction of hepatic isoenzymes is the cause of different responses in mutagenicity in fat-fed mice.

In contrast to the *in vitro* studies described above, when mice were dosed with [¹⁴C]MeIQ, [¹⁴C]Trp-P-2 or [³H]aflatoxin in the intrasanguineous host-mediated assay (see Chapter 3, section 2) no marked diet-related increase in the mutagenicity of MeIQ and Trp-P-2 was apparent (Table 7). Furthermore, a reduction in the mutagenicity of aflatoxin B₁ in the liver was observed in those mice fed the high fat diets (Table 7).

One possible explanation for the discrepancy between the *in vitro* and *in vivo* data is that dietary fat alters the

pharmacokinetics of the three compounds used in the present study and in so doing, may modify the amount of carcinogen present in the liver. Indeed, the amount of radioactivity in the livers of the high fat fed mice was lower than that in the animals fed the low fat diet although similar amounts of label were present in the blood (Table 9). Despite this, it is realized that determining the amount of radioactivity in the blood or liver at the time of sacrifice represents events at a single time point and does not indicate the total amount of mutagen (and metabolites) in the liver during the duration of the experiment. Furthermore, this single measurement does not distinguish between the mutagen and its metabolites, or whether the radiolabel is free or bound. However, data from the limited study on the uptake of Trp-P-2 from olive oil-fed mice, suggests that dietary fat does not influence the initial rates of uptake from the small intestine into the blood or liver (Table 10, Fig. 11). The possibility still remains that mutagen turnover in the liver is greater in mice fed the high-fat diet compared with the controls. In support of this, Shinozuka *et al* (1986) and Wade *et al* (1978) have found that dietary fat modifies phospholipid components of cell membranes. Cell membranes are important for the interaction between cytochrome P450 reductase, cytochrome P450 and the substrate and also for the uptake of compounds into hepatocytes - the main site of Phase I and II metabolism.

To return to the uptake study depicted in Fig. 11 & Table 10, it was observed that [¹⁴C]Trp-P-2 was rapidly absorbed from the small intestine, with more than 50% of the given dose absorbed within 3 minutes of injecting the radiolabel into the ligated gut

section, although subsequently, the rate of absorption from the gut declined. Uptake studies for MeIQ and other heterocyclic amines (Howes *et al* 1989, Bergman 1985, Alldrick & Rowland 1988) also show rapid absorption from the small intestine suggesting that the small intestine is the primary site of absorption of these compounds from the gastro-intestinal tract. Since absorption of [¹⁴C]Trp-P-2 (and IQ and MeIQx; Alldrick & Rowland 1988) was also detected from the large intestine (60 - 70% in 30 minutes., Fig. 12) it would indicate that the majority of an oral dose of heterocyclic amine would be absorbed into the blood, with little excreted directly in the faeces. Previous studies (Hayatsu *et al* 1987, Bergman 1985, Alldrick & Rowland 1988) have reported that orally dosed IQ and MeIQx are excreted either in the bile or urine, with a substantial proportion appearing in the urine 6 hours after administration (Alldrick & Rowland 1988), reflecting the rapid absorption of these mutagens from the small intestine. Investigations into the excretion of tryptophan pyrolysates are still required.

In the present study, the amount of radioactivity in the blood was still increasing at a linear rate 15 minutes after administration of radiolabelled Trp-P-2 into the small intestine. In contrast, blood levels of radioactivity for [¹⁴C]MeIQ and [¹⁴C]IQ peaked 5 and 10 minutes, respectively, after administration into the ligated gut section (Howes *et al* 1989, Alldrick & Rowland 1988). This is a possible reflection of the differences in chemical structure (and partition coefficient) of Trp-P-2 from the IQ-type heterocyclic amines. The amounts of [¹⁴C] in the blood, from the present study, were low (less than 1% of the administered dose; Table

10), even though more than 50% of the radiolabel had been absorbed from the small intestine (Table 10). Similarly, when the large intestine was ligated, only 1 - 2% of the radioactivity was measured in the blood (Fig. 12, Table 10). This would indicate that [¹⁴C]Trp-P-2 was rapidly transferred from the blood to other tissues in the body including the liver. Indeed, the amount of radioactivity present in the liver was far greater than that measured in the blood. Similarly for MeIQ and IQ blood radioactivity was relatively low (Howes *et al* 1989, Bergman 1985, Alldrick & Rowland 1988).

Alldrick and Rowland (1988) observed that the uptake of IQ and MeIQx into various organs was different, suggesting that there may be organ specific differences in the uptake or metabolism of these compounds. Differences in the metabolism of various heterocyclic amines have been reported (Bergman 1985, Alldrick *et al* 1986). It is possible that although dietary fat does not alter the initial uptake from the gastro-intestinal tract, it may alter the uptake into different tissues. To determine whether dietary fat does alter the disposition of mutagen, a more detailed distribution study is required. The radiolabelled mutagen would need to be given orally to mice fed different fat diets with the amount of radioactivity measured in a number of tissues at various times after dosing and the excretion rate of label into the urine determined.

It was interesting to find that when the large intestine was ligated (Fig. 12) a substantial amount of radioactivity was measured in the small intestine (approximately 10% of the administered dose 1 hour after dosing). This suggests that [¹⁴C]Trp-P-2 (or its

metabolites) may undergo enterohepatic circulation, in which Trp-P-2 (or its metabolites) that has first passed to the liver, is excreted back into the gut via the bile. The radioactivity measured in the small intestine is presumably in the form of metabolites that are probably not easily absorbed from the intestine. Other groups have noted the presence of radioactivity in the bile after an intravenous injection or an oral dose of [¹⁴C]MeIQ (Sjodin & Jagerstad 1984, Bergman 1985). Similarly Howes *et al* (1989) and Alldrick & Rowland (1988) observed that the amount of radioactivity in blood and internal organs decreased with time but in the small intestine the quantity of radiolabel increased, providing further indirect evidence of enterohepatic circulation.

In contrast to the present study, in which a diet-dependent reduction in the mutagenic activity of aflatoxin B₁ was observed *in vivo*, Krishnamurthy & Neelaram (1986) revealed that *in vivo* aflatoxin B₁-induced higher frequencies of bone marrow chromosomal aberrations in mice fed a high fat diet than in mice fed a low fat diet. In addition Newberne *et al* (1979) demonstrated increased incidence of aflatoxin B₁-induced hepatic tumours in rats fed a high fat diet compared to a control low fat diet. Although these two studies indicate an enhanced conversion of aflatoxin B₁ to a mutagen, in neither study was a mutagenic end-point measured. The present results are also in agreement with a study by Neal & Godoy (1976) who found that rodents pretreated with agents that induce Phase I enzymes show a reduction in the carcinogenic effects of aflatoxin B₁, despite the increased ability of these tissues to convert it to a mutagenic species. Dietary fat is a known promoter of carcinogenesis and since both of these parameters are

measurements of post DNA binding (mutagenic) events, it is possible that fat enhances aflatoxin carcinogenicity by acting as a promoter of post-mutagenic events.

Data has also been presented, in this thesis, on the effect of dietary fat on the *in vivo* mutagenic and genotoxic activity of nitrosodimethylamine (Tables 11 & 12). As with MeIQ and Trp-P-2 only a slight increase in activity was observed in mice fed high-fat diets. Since, in the present study, nitrosodimethylamine was given intravenously, the lack of response in the host-mediated assay could not be due to differences in the uptake from the gastro-intestinal tract. However changes in tissue disposition of the nitrosamine may be a possible explanation. Wade *et al* (1985) has shown that the microsomal metabolism of nitrosodimethylamine is increased with dietary fat, suggesting that the activation of the nitrosamine, as for MeIQ and Trp-P-2, may be enhanced by dietary fat.

The results from the present study, together with previous work, indicate that a reduction in fat intake (both saturated and monounsaturated fats) of humans who normally consume a western diet may be advisable, in order to reduce the conversion of potential genotoxins (eg. heterocyclic amines) to reactive species. A primary source of fat in the Western diet is meat which by its nature also contains (after cooking) heterocyclic amines and a large amount of protein. Since dietary protein has also been linked to carcinogenesis, a further investigation was carried out to determine whether changes in dietary protein concentration may, like dietary fat, modify MeIQ, Trp-P-2 and aflatoxin B₁ mutagenicity (see Chapter 4 for results). A summary of the results for dietary fat and

protein are given in Table 48.

2. Dietary protein

When the protein content of a mouse diet was reduced from 20% to 5% (w/w) (see Table 3 for recipe) the most notable effect observed was the marked increase in the *in vivo* mutagenicity of the carcinogen aflatoxin B₁ (Fig. 13). This may suggest that animals with a restricted protein intake are more susceptible to the genotoxic effects of aflatoxin B₁ compared to those consuming higher levels of protein. This could have important consequences for man since the quantity of protein consumed varies widely throughout the world. It is generally considered that dietary protein levels as low as 6 - 7% meet the basic requirements to maintain normal bodily functions. However, for Western countries, such as the UK, protein intake is usually double this value while for developing countries protein deficiency has become, for children in particular, a major nutritional problem (Meydani 1987). It is possible therefore, that children from developing countries may be particularly susceptible to the initiation of carcinogenesis by this fungal toxin, especially as these countries tend to have the greatest incidence of contamination of food by aflatoxin. Several epidemiological studies have linked the distribution of *A. flavus* with an increased incidence of human hepatoma (review by Moreau 1979). For example, in the study of Purchase and Goncalves (1971), it was found that there was one case of liver cancer per 100,000 inhabitants in countries such as Holland, Norway or Canada, but there were 103.8 cases among the Bantus of the Transvaal and Swaziland of Southern Africa. Furthermore, experimental studies detailed in Chapter 6 (Table 36) of this thesis have found that the *in vivo* mutagenicity of aflatoxin

B₁ is significantly higher in young animals than in animals of an older age group which, if true in man, may further enhance the sensitivity of children to the genotoxic effects of aflatoxin B₁.

In contrast to the results on the effects of dietary protein on aflatoxin B₁ mutagenicity, long-term carcinogenicity studies have found a greater incidence of aflatoxin B₁ induced hepatomas in rats fed a high (20-30%) protein diet compared to those fed a low (5%) protein diet (Madhavan & Gopalan 1968, Wells *et al* 1976). Contrary to this carcinogenicity data, high protein diets cause a reduction in aflatoxin B₁ induced hepatotoxicity (Madhavan & Gopalan 1968). This apparent contradiction is similar to the studies on fat and the results suggest that dietary protein enhances aflatoxin B₁ carcinogenicity by acting as a promoter of post-mutagenic events.

Increased tumour incidence associated with a high protein diet was also found in rodents treated with dimethylhydrazine (Topping & Visek 1976), 2-acetylaminofluorene (Morris *et al* 1948), nitrosodimethylamine (Zeiger 1975, Cyzgan *et al* 1974) and nitrosomethylurea (Hawrylewicz *et al* 1986). In contrast to the present study Kari *et al* (1983) found the mutagenicity of dimethylhydrazine and azoxymethane in the host-mediated assay to increase with an increase in dietary protein intake, although the mutagenicity and carcinogenicity of dimethylbenzanthracene was reduced (Clinton *et al* 1979, 1986, Singletary *et al* 1984). This would indicate, as for dietary fat, that the modification of carcinogen metabolism by dietary protein is inconsistent. This is exemplified in the present study as the mutagenicity *in vivo* (Fig. 14 & 15) of the two heterocyclic amines MeIQ and Trp-P-2 were, in

general, unaltered by quantitative changes in dietary protein.

However, differences in protein consumption in man are attributed primarily to differences in meat consumption and since cooked meats are a major source of heterocyclic amines, an individual with a high protein intake may have a greater exposure to these genotoxins, even if the metabolism of these compounds is unaltered.

It should be noted that differences in the reported effects of dietary protein may be due to differences in the species and strain of rodents used. In addition and of greater significance, are the diets used and these should be carefully analysed. In dietary studies it is usually best for purified diets to be used. However these diets, which are supposed to be deficient in a single nutrient, can provide less than adequate supplies of other components, which may be compounded further by interactions between constituents (Rowland *et al* 1985, Wise *et al* 1982). For example, many of the cited studies use casein as the protein source, but casein also contains phosphorus (Wise 1990) as well as several other trace elements. In addition to the obvious failure to control experimental conditions when phosphorus is ignored, imbalance in the mineral content can cause nephrocalcinosis (Wise 1990). Diets containing various amounts of casein must, therefore, be appropriately supplemented with these substances. However, it is often difficult to analyse experimental diets used in published reports as insufficient information is provided in the manuscript. Another error in experimental protocol is exemplified by the study of Zeiger (1975) in which animals were kept in solid-bottomed cages containing wood shavings for bedding. This provides an

additional source of fibre and allows coprophagy to occur.

A number of possible mechanisms to explain the protein related change in aflatoxin B₁ mutagenicity *in vivo* have been explored in this investigation including, a) changes in the activation capacity of the liver towards aflatoxin B₁, b) modification of detoxification processes, notably hepatic glutathione transferase activity and glutathione concentration, c) differences in the amount of carcinogen available for metabolism by the liver. These further investigations (see Chapter 4, sections 2 - 5) have shown that the influence of dietary protein on the activity of aflatoxin B₁ appears to be unrelated to its activation since hepatic microsomes from mice fed a low protein diet exhibited only a slight, increased ability to activate aflatoxin B₁ (and MeIQ and Trp-P-2) to a bacterial mutagen (Figs. 16 -18). This was, perhaps, unexpected for a number of reasons. First, microsomes derived from mice fed a low protein diet contained 33% more cytochrome P450 (and protein; per gram liver) than microsomes from mice fed the 20% protein diet (Table 17 & 19). Since the liver weights (and body weights) were similar for mice fed the two diets this would be true also for the intact animal (Table 14). A reduction in cytochrome P450 in animals fed a high (15 or 30%) protein diet has also been reported by Clinton *et al* (1979) and Czygan *et al* (1974) although other workers have reported no significant change in hepatic cytochrome P450 content for rats fed different protein diets (Hietanen 1980, Sachan 1975). Second, a number of studies report that a reduction in either the quantity or the quality of dietary protein depresses mixed function oxidase activity (up to 75 - 80% in certain cases) using a range of substrates including ethylmorphine, aniline, phenobarbitone,

strychnine, aminopyrine, theophylline, zoxazolarine, benzo(a)pyrene and heptachlor (Campbell & Hayes 1974, 1976, Hayes *et al* 1978) which would suggest that hepatic activation of mutagens may be altered. However, the reliability of some of this data is unknown since the diets used are not fully detailed (see Discussion p.253). In this investigation with intricately balanced diets, there were no diet-dependent differences in the activities of the cytochrome P450 isoenzymes, benzphetamine N-demethylase and ethoxycoumarin-O-deethylase, although the O-deethylation of ethoxyresorufin (which is used to monitor cytochrome P448 activity; Burke & Majer 1974) was lower in microsomes from mice fed the low protein diet than in microsomes from mice fed the high protein diet (Table 19). Heterocyclic amines, such as MeIQ and Trp-P-2, are thought to be activated specifically by cytochrome P448-dependent N-hydroxylation (Kato 1986). In addition, certain cytochrome P448 isoenzymes may be involved in the activation of aflatoxin B₁ to a bacterial mutagen (Ishii *et al* 1986) which would suggest, contrary to the present study, that the hepatic activation of MeIQ, Trp-P-2 and aflatoxin B₁ may be influenced by dietary protein. However, it has been found that ethoxyresorufin-O-deethylase preferentially reflects the activity of a low spin form of cytochrome P448 (Abu-Shakra *et al* 1986) whereas the activation of both the cooked food mutagens and aflatoxin B₁ is thought to involve high spin species (Kato 1986, Ishii *et al* 1986). Changes, therefore, to ethoxyresorufin-O-deethylase may not necessarily reflect changes in enzymes involved in the conversion of heterocyclic amines and aflatoxin B₁ to reactive species.

Studies by Neal and his group, (personal communication, G.

Neal) investigating the conjugation of aflatoxin B₁ to glutathione in rats fed high or low protein diets, indicate that alterations in this detoxification pathway may be responsible for the diet-related change in the *in vivo* genotoxicity of aflatoxin B₁ in the present study using mice. Neal found that the conjugation of aflatoxin B₁ to glutathione *in vitro* was reduced when using cytosolic hepatic fractions from rats fed a low (5%) protein diet (of the same recipe that was used in the present study; see Table 3) compared to fractions from rats fed a high (20%) protein diet (see Table 3). Similarly, Reed & Beatty (1980) found that animals fed diets deficient in protein had reduced levels of hepatic glutathione and that conjugation via this pathway was impaired. In contrast other workers have reported increased levels of glutathione in rodents fed low protein diets (Allen-Hoffman & Campbell 1977) although subsequent analysis of the diets used in these latter studies indicated that the enhanced glutathione levels were due to supplementation of the casein diet with methionine (Mainigi & Campbell 1981). The present study in female BALB/c mice, however, found no diet dependency in the amount of aflatoxin B₁-glutathione conjugates formed *in vitro* (Fig. 19). A possible explanation for the different results obtained in the present study and that of Neal is the use of different animal species. Animal species differ markedly in their susceptibility to both the acute and chronic toxicity of aflatoxin B₁ (Patterson 1973, Newberne & Butler 1969). Whereas the mouse is relatively resistant to the effects of aflatoxin B₁ (with an LD50 of 15mg/kg; Newberne & Butler 1969), the rat is sensitive with an estimated LD50 value of 4mg/kg. This difference is thought to be due primarily to the efficient detoxification of the epoxide to a glutathione conjugate (O'Brien *et*

al 1983). It is interesting to note that humans may be rapid metabolisers of aflatoxin B₁ (O'Brien *et al* 1983, Moss & Neal 1985) although, in contrast to rodents, it appears that aflatoxin Q₁ is produced rather than the epoxide, with little or no conjugation to glutathione. Although this lack of detoxification may suggest a relatively high susceptibility compared to other species, it may be modified by the observation that epoxidation is not a major pathway.

The present study found that after incubating activated aflatoxin B₁ with hepatic cytosol from mice fed different protein diets that of the total metabolites, only 3% consisted of the dihydrodiol (which estimates the amount of epoxide present; see Fig. 7). The major metabolite was aflatoxin B₁-glutathione conjugate (90%), which supports the view that this detoxification pathway is important in mice. Dietary protein, however, had no apparent effect on the production of the glutathione metabolite. Furthermore there was no marked difference in the amount of reduced glutathione present in liver homogenates from mice fed low and high protein diets, although more oxidized glutathione was present in mice fed the high protein diet (Table 20).

Since mouse cytosol is an efficient deactivator of aflatoxin B₁ a further experiment was carried out to investigate the effect of hepatic cytosol on the microsomal activation of aflatoxin B₁ (and MeIQ and Trp-P-2) to a bacterial mutagen (Fig. 20). Cytosol had no significant influence on the mutagenicity of aflatoxin B₁ but enhanced the mutagenicity of MeIQ and Trp-P-2, as found for IQ (Abu-Shakra *et al* 1986) and 2-acetylaminofluorene (Stout *et al* 1976, Felton *et al* 1976). Consistent with this result, Nemoto *et al*

(1979) observed that the covalent binding of Trp-P-2 to DNA in the presence of microsomes was markedly enhanced by the addition of cytosol, with the amount of enhancement dependent on the amount of cytosol added. The lack of mutagen detoxification observed in these experiments may be related to the fact that frozen cytosolic tissue was used. To determine glutathione levels for instance, in hepatic fractions fresh tissue is required. Alternatively Abu-Shakra *et al* (1986) proposed that cytosol contains enzymes which are able to convert the microsome-generated metabolites to more potent mutagens. Support for this hypothesis comes from the studies of Forster *et al* (1981a, 1981b) in which 2-acetylaminofluorene was activated by hepatic cytosolic fractions from Aroclor-treated rats in the absence of microsomes.

A number of reports exist which in fact question the importance of glutathione in aflatoxin B₁ genotoxicity. For example, Campbell (1982) concluded from his experiments using diethyl maleate (which sequesters glutathione), that hepatic glutathione levels had little effect on either the formation of aflatoxin B₁-macromolecular adducts or mercapturic acid and proposed that glutathione was not significantly involved in the effect of dietary protein on adduct formation. Furthermore, Coles *et al* (1985) using *S. typhimurium* TA100, found that glutathione and glutathione-transferases had no apparent effect on the mutagenicity of microsomally activated aflatoxin B₁ even though glutathione conjugation had been associated with reduced macromolecular binding *in vivo* and *in vitro* (Neal *et al* 1981). Although glutathione conjugation is considered to be the main route of detoxification (Degan & Neumann 1978, 1981) it is not the only one. It is possible that dietary protein may alter the

hydration to the dihydrodiol metabolite, a pathway which then involves conjugation, at one of the hydroxyl groups, with glucuronide or sulphate although it is unlikely that this would account for the marked reduction in the mutagenicity of aflatoxin B₁ in mice fed high protein diets.

When radiolabelled mutagens were dosed orally to mice fed either a high or low protein diet (Tables 13 and 16) it was observed that for all three mutagens, the amount of radioactivity in the livers of those mice fed high protein was reduced by at least 20%. This result is similar to that obtained in the dietary fat study, in which the amount of radioactivity was reduced in those mice fed high-fat diets (Table 9). However this does not fully explain the observed reduction of aflatoxin B₁ genotoxicity *in vivo* in mice fed high protein diets. If changes in the amount of carcinogen (and metabolites) present in the liver were responsible for the change in aflatoxin B₁ activity one would have expected reductions in the *in vivo* mutagenicity of MeIQ and Trp-P-2 since dietary protein had little influence on the microsomal activation of any of the three compounds.

A possible explanation for the observed diet-related reduction in aflatoxin B₁ mutagenicity *in vivo* is that dietary protein alters the distribution of reactive metabolite within the liver. As seen in the electronmicrographs (Fig. 40), bacteria in the host-mediated assay are not located in the hepatocytes, but primarily in the Kupffer cells lining the sinusoidal blood vessels (Discussion p.298). Therefore, in the host-mediated assay, the reactive metabolites

must pass out of the hepatocytes in order to induce mutations in the nearby bacteria. In contrast, in the *Salmonella* mutagenicity assay, the cellular structure is destroyed and the reactive metabolites are in close vicinity to the bacteria. This would suggest that dietary protein alters the cell membrane, with diets high in protein enhancing, in some manner, the passage of the reactive metabolites out of the hepatocyte. If this is the explanation, the effect must be specific for certain chemical structures, as the reactive metabolites of the heterocyclic amines, MeIQ and Trp-P-2 were, in general, equally capable of inducing histidine prototrophy in mice, irrespective of diet, in the host-mediated assay. A possible method of testing this hypothesis would be to use intact hepatocytes in place of microsomes in the *Salmonella* mutagenicity assay.

Both dietary protein and dietary fat appear to alter the metabolism of food mutagens, although the mechanisms and the chemicals affected differ for the two macronutrients. A further study of interest would be an investigation in to the effects of dietary meat concentration (say beef) on the mutagenicity of the three food mutagens considered here, as meat contains substantial amounts of both protein and fat.

3. Age-related changes in hepatic metabolism

In addition to dietary fat and protein modifying the activity of food mutagens, animal age was also found to alter the genotoxicity of aflatoxin B₁, MeIQ and Trp-P-2.

Marked age-related differences were detected in the *in vivo*

activity of the three potent dietary mutagens, MeIQ, Trp-P-2 and aflatoxin B₁ (Table 2). The results suggest that young animals may be more susceptible to the genotoxic effects of these compounds than older animals. It is noteworthy that long-term rodent bioassays of other food-borne carcinogens which require metabolic activation, notably N-nitroso derivatives of diethylamine and dimethylamine (Vesselinovitch *et al* 1984, Peto *et al* 1984) have also revealed age-dependent changes in tumour incidence, with rats exposed from 3 weeks of age exhibiting a 20-fold higher incidence of liver tumours than those in which exposure began at 20 weeks (Peto *et al* 1984).

The *in vitro* mutagenicity studies, using optimal concentrations of hepatic fractions from mice of different ages (Fig. 26) strongly suggest that the developmental changes in *in vivo* mutagenicity of MeIQ and Trp-P-2 are attributable to age-dependent changes in the metabolism of the mutagens. Similar decreases in *in vitro* mutagenicity with increasing age have been reported for 3-methylcholanthrene, benzo(a)pyrene and N-nitrosopyrrolidine (Raineri *et al* 1986). However, contrary to the *in vivo* data, the *in vitro* mutagenicity of aflatoxin B₁ was not affected by age. This is in accord with the results of Jayaraj *et al* (1985) who reported that aflatoxin activation in male F344 rats remained unaltered up until 12 months of age, although Robertson & Birnbaum (1982) recorded a decline in activation for female Long Evans rats by the age of 2.5 months. These conflicting results may be attributable to sex, strain or species differences.

The decrease in mutagenicity with increasing age may be due to a decrease in the activity of the enzymes which initially activate

the chemical to its electrophilic reactive species, which for MeIQ, Trp-P-2 and aflatoxin B₁, as discussed above, are the cytochrome P450-dependent mixed function oxidases (Kato 1986, Ishii *et al* 1986). However, the change in the hepatic cytochrome P450 content with age (Table 38 & 40) did not closely follow the changes in Trp-P-2 and MeIQ activating capacity. Since the microsomal proteins (which include cytochrome P450) comprise only 20% of the total S9 protein, it is possible that developmental changes in the soluble protein may distort measurements of cytochrome P450 when expressed in terms of S9 protein. Furthermore, total hepatic cytochrome P450 content may not accurately reflect the levels of specific haemoprotein isoenzymes involved in the activation of these mutagens, the expression of which is developmentally regulated (Klinger 1982, Mannering 1985).

As discussed above on page 255, heterocyclic amines, such as MeIQ and Trp-P-2, are activated by cytochrome P448-dependent N-hydroxylation (Kato 1986) which may also play a role in the activation of aflatoxin B₁ (Ishii *et al* 1986). Although the O-deethylation of ethoxyresorufin was found not to change significantly with age (Table 40) this does not preclude developmental changes to cytochrome P448 isoenzymes to be responsible for the observed changes in the mutagenicity of MeIQ and Trp-P-2.

Alterations in the detoxification (Phase II metabolism) of these compounds may also play a role in age-related changes in genotoxicity. Although no significant change in glutathione content was observed in the present study between mice of 4 and 24

weeks of age, greater activity for glutathione-transferases that use CDNB as a substrate was detected in the older animals (Table 41). CDNB acts as a suitable substrate for a wide range of glutathione-transferases, whereas DCNB and ENPP, in which no age-dependent change was found, are more enzyme-specific (Ketterer *et al* 1983). Similarly, Chengelis (1988) observed an increase in glutathione-transferase activity using CDNB in Sprague Dawley rats with no consistent age-related changes in total hepatic glutathione apparent. In addition, increases in glutathione transferase activity have been observed for female Swiss Webster mice up until 10 months of age (Stohs *et al* 1982). Age-related changes in glutathione-transferases have been reported by other investigators. Jayaraj *et al* (1985) examined changes in activity in Fischer 344 rats, towards CDNB and DCNB but found no significant differences between rats of 4, 12 and 26 weeks of age. Spearman and Liebman (1984) reported a very similar pattern except for an increase in activity toward CDNB in female (Fischer 344) rats at 24 months of age. Birnbaum and Baird (1979) examined the possibility of age-related changes in glutathione transferase toward styrene oxide and concluded that there were no age-related changes. These findings are not in agreement with the present work, which may indicate that there are strain, species or perhaps dietary differences in the animals used to measure age-related changes in glutathione S-transferase activity. Little is known about the enzymes involved in the conjugation of MeIQ and Trp-P-2 to glutathione although Saito *et al* (1984) has reported that N-OH-Trp-P-2 (the proposed ultimate carcinogen of Trp-P-2) can form three glutathione conjugates. Conjugation to glutathione is a major detoxification pathway of aflatoxin B₁ and since hepatic S9

primarily measures the activation of compounds, changes in the Phase II metabolic pathway may explain age-related differences in aflatoxin B₁ mutagenicity observed *in vivo* but not *in vitro*.

It was considered unlikely that the differential effects of age on *in vivo* mutagenicity (as observed in the present study) were a consequence of differences in the rate of intestinal absorption of foreign compounds from the gut of mice in the two age groups. The uptake of Trp-P-2 and MeIQ from the small intestine of adult mice is extremely rapid (see discussion page 246) although an uptake and distribution study of the three compounds using mice of different ages would be required to validate this hypothesis.

Overall our results indicate that the *in vivo* mutagenicity of certain dietary genotoxins is markedly influenced by the age of the animal and that in the case of the cooked-food mutagens, MeIQ and Trp-P-2, these changes may be due primarily to developmental changes in the enzymes involved in the activation of these compounds, while for aflatoxin B₁ the conjugation of the reactive species to glutathione would appear responsible.

4. Age-related changes in gut flora metabolism.

In contrast to hepatic metabolism, there appears to be no general age-related trend common to the three bacterial enzymes (nitrate reductase, nitroreductase and β -glucuronidase) studied in the caecal contents of mice fed the control (fibre-free) diet (Chapter 6 section 2). For a summary of the results refer to Table 49.

Table 49 Summary of age and diet related changes to gut flora metabolism

| Enzyme | Relative change in caecal enzyme activity | |
|-------------------|---|----------------------------------|
| | Age ¹ | Dietary pectin ² (5%) |
| Nitrate reductase | NC | ↑ |
| Nitroreductase | ↑ | NC |
| β-glucuronidase | ↑ | NC |
| β-glucosidase | - | ↓ |

Enzyme activities in caecal contents of mice of different ages and fed a fibre-free diet or that diet supplemented with 5% pectin were determined as described in Methods section 9a. Refer to Chapter 5 section 1 and Chapter 6 section 2 for further details.

¹ with respect to caecal enzyme activities of mice 4 weeks of age fed a fibre-free diet.

² with respect to caecal enzyme activities of mice fed a fibre-free diet.

NC - no change in activity

Nitrate reductase activity was similar throughout the 24 week experimental period, whereas the rate of reduction of p-nitrobenzoic acid (Fig. 33), when calculated for the whole animal, was found to increase up until the mice were 12 weeks of age. β -glucuronidase activity (Fig. 35), however, altered little during that time, although, at 24 weeks, an increase in activity was observed with a further increase in activity apparent in the parent mice. This is in agreement with Goldin *et al* (1978) who found rat faecal β -glucuronidase activity to increase from 5 months of age up until the end of the experimental period when the animals were 20 months old. β -Glucuronidase activity is possessed by a wide variety of intestinal organisms including the aerobic enterobacteria and the anaerobic bifidobacteria and bacteroides species (Hawksworth *et al* 1971). Consequently, it is not possible to correlate this age-related change in metabolic activity with any particular microbial inhabitant of the gastro-intestinal tract. Many foreign compounds are excreted into the gut as glucuronide conjugates (glucuronides represent the largest class of xenobiotic conjugates excreted in the bile; Larsen 1988) including the drugs morphine, chloramphenicol and warfarin and the carcinogens benzo(a)pyrene, N-hydroxy N-2-(fluorenyl)-acetamide and dimethylhydrazine (Weisburger 1971, Rowland *et al* 1985). Increased glucuronidase activity, as measured in older animals, may therefore result in increased hydrolysis of conjugates allowing the aglycone to be reabsorbed. The source of β -glucuronidase is likely to be the microbial flora since the hydrolysis of many xenobiotic glucuronides is substantially reduced (by more than 90%) in germ-free or antibiotic treated rats (Williams *et al* 1970, Grantham *et al* 1970). The hydrolysis of glucuronide conjugates can lead to enterohepatic

circulation of drugs and other foreign compounds and may have a number of consequences, including increased exposure to the compound with the possible potentiation of any pharmacological effects or an increased exposure to a reactive metabolite. The delay in the appearance of deconjugating enzymes may spare the liver from repeated exposure to reabsorbed material during the period in which the conjugating mechanisms of the liver are being developed. In support of this the present study showed that certain host glutathione-transferase activities increased with age (Table 41). An exception, however, would appear to be the hydrolysis of the diethylstilboestrol glucuronide conjugate in rats, as the rate of hydrolysis was equivalent to adult values, in animals as young as 3 - 4 weeks of age (Fischer *et al* 1973).

Enterohepatic circulation is thought to have been observed in the dietary fat study reported in Chapter 3. When the carcinogenic heterocyclic amine [¹⁴C]Trp-P-2 was injected into the ligated large intestine it was found that radioactivity was rapidly absorbed and furthermore, radioactivity was also found in the small intestine within 30 minutes of dosing the radiolabelled mutagen, presumably via excretion of the [¹⁴C]Trp-P-2 (and/or its metabolites) into the bile. Although no measurement was made of the number of metabolites or their structure, it is likely that the absorption of Trp-P-2 from the large intestine and possible recirculation of it or its metabolites would result in increased exposure of the host animal to the compound and possibly to its reactive metabolites.

Associated with the hydrolysis of β -glucuronide conjugates and the potential consequences of this is a link between faecal

β -glucuronidase activity and colon tumour induction (Kinoshita & Gelboin 1978, Renwick & Draser 1976, Hill *et al* 1971). Further evidence for linking β -glucuronidase activity and cancer is provided by the epidemiology study of Reddy & Wynder (1973), which shows populations at high risk for colon cancer to have an intestinal microflora with an increased ability to hydrolyze glucuronide conjugates. This would support the hypothesis that increased β -glucuronidase activity in caecal contents of adult mice may have important toxicological consequences.

Age-related changes in bacterial nitroreductase activity may also affect the animal host. Although no obvious age-related change in the specific activity of p-nitrobenzoic acid ^{reductase} was apparent in the first 8 - 12 weeks of life, an increase in total nitroreductase activity of mice fed the fibre-free diet was recorded. However several nitroreductase enzymes exist (Scheline 1973) so changes in other nitroreductases may occur with age, particularly as nitroreductase activity of the caecal flora is highly dependent on the substrate used (Rowland *et al* 1983c). Nitroreductases are known to be involved in the *in vivo* reduction of nitrocompounds including the rat hepatocarcinogen dinitrotoluene. Reduction of dinitrotoluene by the gut flora results in the production of the activated metabolite (Rickert *et al* 1981) which can be reabsorbed, as described above for the β -glucuronide aglycones, thus increasing the persistence of, and therefore, the exposure of this genotoxin to mammalian tissues. The results from the present study indicate that as an animal ages, it may be more susceptible to the effects of dinitrotoluene and other nitrocompounds through the increased ability of the gut flora to

reduce such nitrocompounds.

Caecal nitroreductase and β -glucuronidase activities (per gram caecal contents) in preweanling mice, were similar to that of their mothers (Table 42). The activities of these enzymes in terms of bacterial numbers were also similar which is not necessarily unexpected since after birth the sterile gut becomes colonised by organisms derived from the immediate environment including the mother's skin, vagina and faeces (Schaedler *et al* 1965). As regards the intact animal, however, the larger caecal sacs of the parent mice (Table 43) results in these mice having greater nitroreductase and β -glucuronidase activities than their young, which in turn would make them more susceptible to the potential toxic consequences of these enzyme activities as discussed above (refer to page 266). In contrast, nitrate reductase was 40 - 50 fold greater (per g or per 10^{10} bacteria; Table 42) in the preweanling mice than in the adults. Adjusting the values to determine the total activity for the animal resulted in the values being almost identical. The existence of certain bacterial species (such as lactobacilli) in the neonates which possess a substantial nitrate reductase activity but little nitroreductase and β -glucuronidase activity may explain this result, although further studies investigating the metabolic activities of bacterial isolates need to be carried out to substantiate this theory.

This comparison of 2 week old preweanling mice and their mothers has emphasized the importance of the choice of units in expressing enzymic data. Changes to gut bacterial enzyme activities can be minimized or accentuated depending on the manner

in which the results are calculated. When specific enzyme activity is expressed per gram weight, it can be highly variable due to inter-animal differences in the water or dry matter content. Dietary components, including fermentable plant polysaccharides such as pectin, give short chain fatty acids following bacterial degradation and these may be associated with a net influx of water into the caecum diluting the hindgut contents (Leegwater *et al* 1974). In the present study dietary pectin consistently produced an increase in caecal size although the bacterial count per gram caecal contents was, in general, similar to those mice fed the fibre-free diet. A second, and often preferred, method of expressing enzymic data is the total activity per caecum, since this is of biological and toxicological relevance to the host animal. Dilution of the hindgut bacteria becomes irrelevant as the activity of the total microflora is considered. The data presented here was also standardized to 10^{10} bacteria (approximating the microbial content of one gram caecal contents) as this allows consideration of changes in the metabolic activity of the organisms comprising the gut microflora, without the confounding effects of changes in dry matter or water content of the sample. A limitation to this method, however, is that it assumes that all bacterial cells possess an equal ability to catalyse a given reaction. Despite this reservation, the method gives an indication of changes to enzyme activities of gut microorganisms by dietary manipulation.

5. Dietary pectin and gut flora metabolism.

In addition to age, dietary components can alter the activity of gut bacterial enzymes (Rowland *et al* 1985). In agreement with this, two studies presented in this thesis (see Chapter 5, section 1

and Chapter 6, section 2), in which female BALB/c mice were fed a fibre-free diet or that diet supplemented with 5% (w/w) citrus pectin, show certain metabolic activities of the caecal flora to be diet-dependent. For a summary of the results refer to Table 49.

Pectins are not degraded by mammalian enzymes, although they are extensively metabolized within the gut and provide a ready source of carbohydrate to the gastro-intestinal microflora (Mallett & Rowland 1988). The pectin used in this study was derived from citrus fruit and had a high methoxyl content (8% by weight with approximately 70% of the carboxyl groups methoxylated) and is widely used by industry. This type of pectin is almost completely utilized by intestinal bacteria (Gilmore 1966) including those of man (Holloway *et al* 1983).

Dietary pectin, when forming 5% (w/w) of the diet, was found to increase significantly the amount of caecal contents (Tables 22 & 43) compared to mice fed a pectin-free diet. This increase in caecal size has previously been reported in both rats and mice (Wise *et al* 1982, DeBethizy *et al* 1983, Mallett *et al* 1983, Rowland *et al* 1983a,c) although in man pectin appears to have little effect on stool output (Doyle *et al* 1983, Ross & Leklem 1981, Spiller *et al* 1980, Mallett *et al* 1988). Various hypotheses have been proposed to explain caecal enlargement but the most favoured is that pectin, or a product of the bacterial fermentation of pectin, alters the osmotic pressure within the gut and that caecal enlargement is an adaptive homeostatic response to the presence of these osmotically active molecules (Walker 1988, Leegwater *et al* 1974). A consequence of caecal enlargement is that total enzyme activity (the

activity per caecum) will vary even if the specific activity (per gram caecal contents) is unaffected by diet. This effect of altered caecal size may be enhanced by the tendency of mice fed 5% pectin to have lower (by about 7%) body weights than mice fed the fibre-free diet (Table 22) so further increasing the caecal to body weight ratio of pectin fed mice. Wise *et al* (1982) and Rowland *et al* (1983a) also reported the body weights of rats fed 5% pectin to be 5 - 10% lower than those fed the fibre-free diet.

Although pectin increases caecal size, pectin has equivocal effects on the concentration of bacteria in the caecal contents (determined by microscopic count). In the present study (Table 22 & 43) 5% dietary pectin had no significant effect on bacterial numbers although Rowland *et al* (1983a) reported a reduction to occur in the concentration of bacteria in ^{the caecum} of pectin-fed MF1 mice. In the rat, bacterial concentration remained the same (Wise *et al* 1982, DeBethizy *et al* 1983, Rowland *et al* 1983a, Freeman 1986) or increased in response to dietary pectin (Rowland *et al* 1983c). Similarly, no consistent results were apparent for the numbers of bacteria in faeces of humans given pectin (Doyle *et al* 1981, Gargo *et al* 1985). Since, in the present study, the metabolic activities of the caecal flora were found to be independent of the total number of microorganisms, it would suggest that changes in bacterial biotransformation were dependent on either enzyme induction or repression or upon redistribution of bacterial species within the gut population.

A number of previous investigations using rats have found that the effects of dietary pectin on gut flora metabolism show an

increase in β -glucuronidase activity with 5 - 10% pectin diets (Bauer *et al* 1979, Freeman 1986, Rowland *et al* 1983a, DeBethizy *et al* 1983, Mallett *et al* 1983). An exception to this is a study by Shiau & Chang (1983) who found β -glucuronidase activity to decrease in pectin-fed (Fischer F344) rats. This discrepancy with other studies may reflect the different method of expressing the enzyme activity (per mg faecal nitrogen as compared to per gram caecal contents or per mg faecal protein). In the present study, however, using female BALB/c mice (and p-nitrophenol- β -glucuronide as substrate) no significant change in β -glucuronidase was observed (Figs. 21 & 35). Similarly Rowland *et al* (1983a) observed no significant change in the activity of this hydrolytic enzyme in mice, indicating a possible species difference in the expression or inducibility of the enzyme. In humans, a recent study by Mallett *et al* (1988) found that β -glucuronidase activity decreased when the normal diet of 6 healthy volunteers was supplemented with 18g pectin/day. In an earlier study in man where approximately similar amounts of citrus pectin were ingested β -glucuronidase activity increased (Ross & Leklem 1983). The reason for the difference in effect between the two investigations is unknown. Diet-related increases in β -glucuronidase activity have been linked with increased carcinogenesis since an increase in this enzyme activity in pectin-fed rodents was associated with a significant increase in dimethylhydrazine induced colo-rectal tumours, suggesting that glucuronide deconjugation is important for the release of an active intermediate of dimethylhydrazine in the hindgut (Bauer *et al* 1979).

The activity of a second hydrolytic enzyme, β -glucosidase, decreased in response to a 5% pectin diet (Fig. 21) as previously

reported in rats, hamsters (Rowland *et al* 1983a) and man (Mallett *et al* 1988) although not in MF1 mice (Rowland *et al* 1983a). Since β -glucosidase is known to convert glycosides to toxic aglycones as in the hydrolysis of cycasin to yield the carcinogen azoxymethanol or the production of cyanide by the action of this enzyme on amygdalin (Rowland & Walker 1983), these changes in activity may have an important role in the development of cancer by these agents.

Dietary pectin had little effect on the nitroreduction of p-nitrobenzoic acid in female BALB/c mice (Figs. 21 & 34). Earlier investigations show no consistent response of this reductive enzyme to pectin (Rowland *et al* 1985) which may reflect differences in the native microflora of the host animal prior to dietary treatment, or may be the result of the highly dependent nature of caecal nitroreductase activity upon the substrate used (Rowland *et al* 1983c). Despite this, dietary pectin may have important consequences on the reduction of nitrocompounds since pectin-induced nitroreductase has been associated with an increase in hepatic macromolecular covalent binding of [3 H]-2,6-dinitrotoluene (DeBethizy *et al* 1983) and an increased susceptibility to methaemoglobinaemia of rats given nitrobenzene (Goldstein *et al* 1984).

The major effect of pectin on gut flora metabolism was on nitrate reductase activity since marked increases in activity were observed in pectin-fed animals (Figs. 21 & 33). This agrees with similar investigations using MFI mice and rats (Wise *et al* 1982, Mallett *et al* 1983, Rowland *et al* 1983a, DeBethizy *et al* 1983). In the present study using mice of different ages (see Chapter 6,

section 2) this pectin-induced increase in nitrate reductase activity was greatest in 4-week-old mice and thereafter decreased as the animals aged, although even at 24 weeks there was an 8 fold difference in nitrate reductase activity (per g caecal contents) compared to mice fed the fibre-free diet (Fig. 33). Since the pectin-induced nitrate reductase activity was greater in younger than in older animals the enhanced ability to generate nitrite from nitrate may make the younger mice more susceptible to the induction of methaemoglobinaemia and the production of nitrosamines.

Nitrite can, on absorption into the blood, undergo co-oxidation with oxyhaemoglobin to yield nitrate and methaemoglobin. Since methaemoglobin is unable to transport oxygen, this reaction can have important clinical consequences if the production of the methaemoglobin exceeds the capacity of the host enzymes to restore the function of the respiratory pigment. Wise *et al* (1982) has demonstrated nitrate-induced methaemoglobinaemia to occur in pectin-fed rats possessing an increased gut microbial nitrate-reductase. Animals fed the pectin-containing diet typically showed a ten-fold increase in blood methaemoglobin six hours after nitrate administration relative to that observed in the control diet group (Wise *et al* 1982). Where man is concerned, infants are particularly at risk of developing methaemoglobinaemia (especially those from areas where drinking water is rich in nitrate), since, among other reasons, they possess a stomach microflora rich in nitrate-reducing bacteria (Mallett 1988, Green & Tannenbaum 1982). The data presented here would also suggest that children with a high pectin intake may have an increased susceptibility to this medical condition, as compared to adults or

children on a low pectin diet.

Nitrosamines are a class of potent carcinogens which have proved to be active in all of the animal species in which they have been tested. Thus increased production of these compounds as a result of increased nitrate reductase may have serious implications. Mallett *et al* (1983) has shown that pectin-induced increases in caecal nitrate reductase activity in rodents are associated with an increase in the production of nitrosamines *in vitro* (Mallett *et al* 1983). Further evidence for elevated nitrosamine formation in caecal preparations from pectin-fed animals was obtained by incubating the preparations with [¹⁴C]proline and nitrate. Fifteen-fold more nitrosoproline was produced under these conditions by caecal contents from rats fed pectin by comparison to those consuming the fibre-free diet (Mallett 1982). This generation of nitrosamines via microbial reduction of nitrate *in vivo* may explain the increased incidence of gastric cancer in man in areas where drinking water contains high concentrations of nitrate (Mallett 1988). Furthermore, there is evidence to suggest an additional association with a high intake of starchy food (Wynder *et al* 1963, Modan *et al* 1974) which may provide a source of energy for bacterial metabolism and growth. However, recent experiments indicate that the role of dietary pectin on the induction of either methaemoblobinaemia or nitrosamine formation in man may be limited because according to Mallett *et al* (1987, 1988) human faecal nitrate reductase activity was unaltered by the consumption of a high pectin diet.

The importance of pectin-induced nitrate reductase activity on

nitrosamine formation in mice was investigated further in Chapter 5. Although the work is discussed in detail in the next section of this discussion (section 6), there was no evidence from the study to suggest that increased nitrate reductase in the gastro-intestinal tract caused increased production of mutagenic nitroso compounds.

The identification of bacterial species that may be involved in the pectin-related induction of nitrate reductase activity has been attempted. Certain strains of *Escherichia coli* have been found to have particularly high nitrate reductase activities (Hawksworth & Hill 1971). These microorganisms are members of the enterobacteria which were detected in high numbers in the caecal contents of mice fed diets similar to those in the present study (Wise *et al* 1982). In addition certain species of anaerobes, such as bacteroides and clostridia can utilize pectin for growth (Rexova-Benkora & Markovic 1976) and ^{such} species in the gut may possess comparable enzyme systems and so proliferate in animals fed a diet supplemented with pectin. However, changes of this type may be too subtle to quantitate with conventional taxonomic techniques. Wise *et al* (1982) observed no overall difference ^{either} in species diversity or ⁱⁿ number of organisms, that could be correlated with ^{the observed} increased nitrate reductase activity in pectin-fed rats. Nitrate may be reduced by a number of facultative and obligate bacteria and ^{because} nitrate occurs together with pectin in plants, it has been proposed that the gut bacteria of ^{herbivores} have evolved to couple pectin oxidation with nitrate reductase and ATP (adenosine 5'-triphosphate) production.

This influence of dietary pectin on microbial metabolism

appears to be related to the methylated structure of pectin.

Whereas polygalacturonic acid (a non-methylated pectin) does not cause an increase in caecal nitrate reductase activity of rats, low methoxyl pectin causes some induction of nitrate reductase activity but is to a lesser extent than that induced by a high methoxyl pectin (Conning *et al* 1983, Mallett *et al* 1983). This apparent substrate specificity may be because bacterial degradation of D-galacturonic polymers occurs only with specific forms of pectin (Rexova-Benkora & Markovic 1976, Schink & Zeikus 1980).

The use of a 5% pectin diet in the present study is likely to be a far greater amount than normally consumed by humans eating a western type diet, since total fibre intake of this human diet is only around 4% (by weight) calculated from dietary intake data published by Mallett *et al* (1988) and equivalent to 15 - 20g of fibre per day (Stephen 1985). The proportion of this fibre intake that is pectin is unknown but data from the present study suggests that pectin needs to form a relatively large proportion of the diet to induce changes in microbial metabolism, since experiments using a 0.5% pectin diet (see Chapter 5) demonstrate that this amount of pectin had no apparent affect on the four enzyme activities measured (Fig. 21). Furthermore there no change observed in the body, liver or caecal size of these animals fed 0.5% pectin relative to the pectin-free diet.

It must also be noted that in the present study a single form of pectin (8% methoxy pectin) was used whereas in nature the extent of methylation varies. Thus in man any changes in microbial gut flora metabolism will be a balance of the various fibres consumed.

Recently Wise *et al* (1986) demonstrated that the effects of dietary fibre components on caecal microflora are dependent upon the initial fibre content of the diet base. Of particular relevance, this group showed that pectin had no apparent affect on nitrate reduction when this plant-cell component was incorporated into a stock diet (Wise *et al* 1986). This may explain the result of Mallett *et al* (1988) who supplemented a "normal" Western diet with 18g apple pectin/day and observed no change in human faecal nitrate reductase activity although other enzyme activities were altered.

The differences in the bacterial colonisation of the gastro-intestinal tract of different species must also be considered when attempting to extrapolate data from one species, such as the mouse, to another (i.e. man). For instance, the mouse stomach contains a large bacterial population, dominated by lactobacilli, bacteroides and bifidobacteria, whereas in healthy people the stomach normally consists of mainly transient flora (Rowland *et al* 1985). The reasons for these differences may be ascribed primarily to differences in gastric acid secretion but also coprophagy and diet are factors. The colonization of stomach and small intestine of rat and mouse means that a substance has a greater chance of microbial transformation in these animals than in the human, where a drug may be absorbed before reaching the heavily populated regions of the gut. However gastro-intestinal disorders, such as hypochlorhydria, which result in bacterial colonization of the stomach and upper small intestine of man are not uncommon and may modify the metabolism of ingested drugs (Rowland 1986).

Furthermore, the importance of caecal microbial enzyme

activities in the enhancement of toxicity (or detoxification) of any chemical will depend on a number of variables including rate and type of host-mediated metabolism, relative amount of caecal contents, distribution of a given bacterial substrate within the body (especially the concentration within the caecal contents) as well as the enzyme activity of caecal microorganisms and absorption of bacterial reaction products from the caecum. The measurement of enzyme activities in caecal contents only takes two of these variables into account, so *in vivo* studies are required to assess the ultimate disposition of individual compounds.

6. Dietary pectin and nitrosamine formation.

The marked increase in nitrate reductase activity in pectin-fed female BALB/c mice, as reported in Chapters 5 & 6 of this thesis, is by its nature associated with an increased production of nitrite. Although nitrite itself is not carcinogenic or mutagenic, as reflected in the present study by the lack of mutagenic response in mice dosed with sodium nitrite in the host-mediated assay (Table 26 & 27), nitrite can combine *in vivo* with certain amino groups and yield carcinogenic or mutagenic N-nitroso compounds (Bartsch & Montesano 1984). In the present study the tertiary amine, aminopyrine, was administered with sodium nitrite producing a bacterial mutagen, presumably nitrosodimethylamine. The reaction of aminopyrine with nitrite is given in Fig. 3. The ability to detect mutagenicity in the host-mediated assay with animals dosed with aminopyrine and nitrite has been previously reported by Neale & Solt (1981), Baumeister (1982) and Barale *et al* (1981). Neale & Solt (1981) also showed that the rate of mutagen formation from aminopyrine nitrosation is very rapid, since mice given ascorbic

acid (a nitrosation inhibitor), at the same time as the amine and nitrite, exhibited no mutagenic activity. Furthermore when ascorbic acid was given 5 minutes after dosing aminopyrine, some mutagenicity was detected whereas dosing 10 minutes after, resulted in mutation frequencies comparable to that in mice given no ascorbic acid, indicating that by this time nitrosation was complete.

The ease of aminopyrine nitrosation is attributed to both its enamine structure and its low basicity (pK_a 5.04; Mirvish 1975). Only the free, unprotonated amine, and not the amine salt, is available for nitrosation, and so the reaction of aminopyrine with nitrite is heavily pH dependent (Mirvish 1975). Consequently, the stomach is the most likely site for this nitrosation reaction to occur due to its low pH. Using artificial gastric juice, Baumeister (1982) demonstrated the formation of mutagenic nitroso compounds from aminopyrine in the presence of nitrite. Low pH favours the formation of the nitrosating agent, nitrous acid (formed from nitrite in acidic aqueous media), but it also favours the protonation of the amine. Because of the counteracting effects of pH on the concentration of unprotonated amine and the concentration of nitrous acid, there is an optimal pH (2.5 - 3.3) for nitrosation of a given amine. For aminopyrine the optimal pH for nitrosodimethylamine formation is 2.0 (Mirvish 1975).

In the host-mediated assay it was observed that the production of active mutagen was not dependent on the dose of aminopyrine and nitrite alone. When different doses of aminopyrine and nitrite were given (Fig. 23 & Table 26), the mutation frequency measured in the liver initially increased with increasing dose but subsequently at

higher concentrations of aminopyrine and nitrite (above 30mg/kg) the mutation rate began to decline. The only exception to this, was when the the pH of a high dose (50mg/kg) of aminopyrine was reduced from pH 7.5 to pH 3.0 indicating that concentrations of aminopyrine above 30mg/kg raised the stomach pH sufficiently to cause a reduction in mutagen formation. Since aminopyrine nitrosation is so dependent on pH, with the optimal pH being very low this is a possible explanation. The stomach pH of BALB/c mice was found to vary between 3.5 and 7.2 (determined approximately using pH indicator paper placed into the stomach contents). This range in stomach pH is probably associated with flow of gastric juice and buffering capacity of the stomach contents (Shephard *et al* 1987). The marked differences in stomach pH may also explain the large intergroup variation observed in mutagenic activity (see Tables 26 - 28). Because of the pH dependence of nitrosation reactions, any variation in stomach pH from animal to animal or from dosing animals with aminopyrine, would modify the rate of nitrosation which in turn would alter the mutagenic response. However, Neale & Solt (1981) who used concentrations of up to 180 mg aminopyrine/kg observed no decline in mutagenicity as the concentration of aminopyrine increased. Different strains of mice were used compared to the present study which may help explain the discrepancy in the results since it is possible that in the latter experiment stomach pH was on average more acidic. It should also be noted that nitrosation can be catalysed by bacteria at more neutral pH values. Since mice have a resident stomach flora it is also possible that different strains of mice differ in their capacity to nitrosate aminopyrine by bacterial catalysis.

In addition to producing a mutagenic response, the ingestion of nitrite and aminopyrine has been shown to induce carcinogenesis, notably haemangioendothelial sarcomas of the liver and metastases in the lung (Lijinsky *et al* 1973, Taylor & Lijinsky 1975), which are similar to the types of tumour frequently induced by nitrosodimethylamine, as reported by Clapp *et al* (1971) in BALB/c mice. This *in vivo* formation of nitrosocompounds may be important to man as humans are exposed to a both a wide range of nitrogen-containing compounds and nitrosating agents. In support of the view that nitrosocompounds may be formed in the stomach, N-nitroso compounds have been detected in the gastric contents of man (Mysliwy *et al* 1974, Mueller *et al* 1983). N-nitrosocompounds have also been detected in blood which are likely to be derived from gastric sources (Fine *et al* 1977, Kowalski *et al* 1980, Gough *et al* 1983, Lakritz *et al* 1982). The generation of N-nitroso compounds *in vivo* may explain the increased incidence of gastric cancer in areas where drinking water contains high concentrations of nitrate. For example, in Narino, an area of Columbia with a high incidence of gastric cancer it was reported that gastric juice nitrate levels were raised compared to other low incidence areas of Columbia (Fontham *et al* 1986). Furthermore pernicious anaemia and partial gastrectomy (diseases which result in gastric achlorhydia) have been associated with an increased risk of gastric neoplasia, with N-nitroso compounds formed by bacterially-catalysed nitrosation thought to be involved in the pathogenesis of these changes (Reed *et al* 1985).

As discussed above (see page 276), an association exists between nitrosocompound formation and carbohydrate/pectin intake. The provision of extra carbohydrate to the gastro-intestinal

microflora may increase bacterial metabolism or bacterial numbers with a concomitant increase in the ability of the bacteria to reduce nitrate to nitrite (see Chapter 5 & 6). The nitrite^{will} then be available for reaction with nitrogen containing compounds. However in the present study the addition of pectin to the diet of mice did not appear to alter the nitrosation of aminopyrine to a bacterial mutagen eventhough microbial nitrate reductase activity was increased (see Chapter 5 section 3). Similarly the *in vivo* formation of nitrosoproline from proline and nitrate was not increased in pectin-fed mice (personal communication I.R. Rowland) although an increase was measured *in vitro* (see discussion page 276; Mallett et al 1982)

It was found that when aminopyrine was administered alone (ie. with no nitrite) no mutagenic response was apparent. This would indicate that there was not enough nitrite available to allow sufficient nitrosation, for detection in the host-mediated assay, to occur (Table 28). Nitrate and nitrite were present in only negligible amounts in the diet, so the only sources of these two compounds available to the mouse microflora were from the drinking water or from endogenous production. This would be in agreement with an experiment of Lin & Lai (1982) who found that there was no nitrite in the stomach of rats given a control diet, but after two weeks on a 0.5% nitrate diet, the stomach contained 0.83ppm nitrite. However when nitrate was given either chronically, or as an acute dose in the present study (Tables 29 & 30), there was no apparent change to the nitrosation of aminopyrine compared to mice given no nitrate supplement, whether or not the mice were fed a pectin- or pectin-free diet. Thus suggesting that the level of nitrite was

still insufficient for a detectable amount of nitrosation to occur. The doses of nitrate used in the present study had previously been found to increase nitrite levels in rodents with the chronic dose inducing methaemoglobinaemia (personal communication I.R. Rowland). In the present study symptoms of methaemoglobinaemia were not observed which may suggest that for BALB/c mice higher doses of nitrate than used here (1.25g/kg for the acute dose and 2.0% or 0.5% nitrate over 5 days for the chronic administration) are required to induce this condition.

Nitrate is rapidly absorbed from the proximal small intestine and enters the body water reaching equilibrium within all body compartments including the hindgut (Hartman 1982, Witter *et al* 1979). Nitrate is thought to participate in nitrosation reactions following reduction to nitrite by the microbial community colonising the oral cavity and other regions of the gastro-intestinal tract such as the caecum (Hartman 1982). Although no increase in aminopyrine nitrosation was found to occur in the study reported here it may be because the reduced nitrate is not located at the site of aminopyrine nitrosation. For instance, nitrate reduction will occur in the large intestine whereas aminopyrine nitrosation is probably located in the stomach.

A further factor that needs to be considered is the sensitivity of the host-mediated assay in this type of experiment. For the markers ampicillin resistance and differential DNA repair, the change in nitrite concentration required to induce a significant change in genotoxicity was equivalent to a 2mg/kg dose which, in these experiments, approximated 0.5µmols of

nitrite. It is unlikely that feeding a pectin diet, or the dosing of mice with nitrate will produce this degree of increase in nitrite concentration in the stomach.

In order to try and enhance the sensitivity of the host-mediated assay to detect nitrosation of compounds to mutagenic derivatives, thiocyanate was administered with the aminopyrine. Thiocyanate is known to increase the rate of nitrosation of aminopyrine due to the production of the nitrosating species ON-NCS (Mirvish 1975). However, in the present study potassium thiocyanate had no apparent effect on the formation of a genotoxin from aminopyrine and nitrite. A possible explanation for this lack of response is that the stomach pH was not sufficiently acidic. The optimum pH for this reaction is pH 2.0 and as pH becomes less acidic the rate of reaction falls because the thiocyanate becomes protonated (Mirvish 1975). However earlier studies, in which mutagenicity was determined as a measure of nitrosation, showed thiocyanate to markedly promote nitrosation (Baumeister 1982, Barale et al 1981).

It has been established that the carcinogen nitrosodimethylamine is produced from the nitrosation of aminopyrine (Mirvish 1975). In the above experiments mutagenicity was determined in the liver but a further experiment showed that the mutagenic activity of nitrosodimethylamine (given intravenously) can be detected in other organs, notably the kidney, spleen and lung with the order of genotoxicity being greatest in the lung, then the liver and least in the kidney and spleen (Table 25). The greater activity of the nitrosamine in the lung compared to the liver may be due to the greater ability of liver to repair alkylated DNA although

previous studies have found the liver to be the primary target for genotoxic activity (Pueyo *et al* 1979, Kerklaan *et al* 1985).

The detection of mutagenicity in tissues other than the liver in nitrosodimethylamine-treated animals is dependent upon the ability of these tissues to degrade the compound to a methylating agent (Montesano & Bartsch 1976, Pegg 1980). This is because the reactive intermediate of nitrosodimethylamine is highly unstable and does not circulate in the blood after its generation. However, the dose of nitrosamine and its mode of administration is also important in determining the metabolism of nitrosodimethylamine. When the liver is exposed to small doses of nitrosodimethylamine, entering the portal blood supply, the liver is normally able to clear the compound in a "first pass" effect resulting in DNA damage to the liver only. However relatively large doses of the nitrosamine or administration by intravenous injection will result in alkylation of DNA in tissues other than the liver (Diaz Grames 1977, Pegg & Perry 1981). Since the present study used a relatively high dose of nitrosodimethylamine (5 mg/kg) given intravenously it is not surprising that genotoxicity was measured in the kidney, spleen and lungs as well as in the liver.

Although the reactive intermediate of nitrosodimethylamine is known to be unstable it must be sufficiently long-lived in order to diffuse out of cells in which it was generated and induce mutations in bacteria in the host-mediated assay. After intravenous administration in to mice, bacteria are located primarily in the Kupffer cells lining the liver sinusoids (refer to discussion on page 298 & Fig. 40). Consequently reactive metabolites have to pass

from the hepatocytes into both the Kupffer cells and the bacteria. In agreement with this Umbenhauer & Pegg (1981) found alkylation of both intracellular and extracellular DNA by nitrosodimethylamine to occur following activation by isolated hepatocytes. There is much evidence to suggest that the major adduct of nitrosodimethylamine that induces mutation in both bacteria and mammalian cells is O⁶-methylguanine (Pegg 1980).

7. The influence of the gut flora on the hepatic activity of mutagens.

In the present study dietary modification had a marked effect upon gut flora metabolism but had little effect on the metabolism or formation of nitrosodimethylamine. However, the presence of a microbial population in the gut is known to influence metabolism both within the intestinal tract and at other sites of the body, particularly the liver (Wostmann 1984). It is possible that the gut microflora may affect the activities of hepatic drug-metabolising enzymes, some of which are involved in the activation of foreign compounds to carcinogenic or mutagenic derivatives. The studies presented in Chapter 5 of this thesis show that, in mice, the presence of a gut flora increases the *in vivo* mutagenic activity of aflatoxin B₁, MeIQ and Trp-P-2 in the liver. Similarly, carcinogenicity studies using germ-free and conventional flora rodents, such as that of Roe & Grant (1970) and Mizutani & Mitsuoka (1979) found tumour incidence was generally lower in the germ-free animals. For example, Mizutani & Mitsuoka (1979, 1980) found that only 30% of germ-free C3H/He mice had developed hepatic tumours after a year, compared to 75% in their conventional counterparts. Furthermore, liver tumourigenesis in

gnotobiotics was promoted markedly when associated with a bacterial combination of *Escherichia coli*, *Streptococcus faecalis* and *Clostridium paraputrificum*. Further addition of bacterial species such as *Bifidobacterium longum*, *Lactobacillus acidophilus* and *Eubacterium rectale* resulted in suppression of tumour formation. These results suggest that intestinal bacteria can promote or inhibit liver carcinogenesis in mice, depending on the species of bacteria present. The reasons for this variation in tumour development have not been elucidated.

A possible mechanism by which the gut flora may affect mutagenesis or carcinogenesis, is through the bacterial formation of carcinogenic initiators or promoters from dietary or endogenous precursors (such as phenols and cresols from aromatic amino acids or bile acid metabolites). These active intermediates are then absorbed from the gut and transported to the liver via the portal blood system. A further possibility is that the presence of a gut flora modifies the ability of the liver to metabolize dietary contaminants leading to the formation of promoters or active carcinogens (Rowland 1988). Rowland *et al* (1987), at the collaborating laboratory at BIBRA, investigated this hypothesis by comparing the ability of hepatic S9 preparations from germ-free or conventional microflora rats to activate aflatoxin B₁, MeIQ and Trp-P-2 using the Ames mutagenicity assay. The presence of a gut flora significantly affected the hepatic activation to bacterial mutagens of all three compounds. In agreement with the present study, the activity of aflatoxin B₁ was almost twice that when S9 from conventional rats was used. However, the activation of the cooked food mutagens was somewhat greater in germ-free S9

preparations. The conflicting findings for the *in vivo* and *in vitro* studies on the two heterocyclic amines may be due to the use of different rodent species with different capacities to detoxify or due to differences in the rates of uptake of the two compounds from the gastro-intestinal tract of germ-free and conventional flora animals. The evidence suggests that active transport across the gut wall is unaffected by the presence or absence of a microbial flora. However, passive absorption tends to be increased in the germ-free state (Heneghan 1988).

In addition to changes in the absorptive capacity of the gut of the germ-free animal, a number of morphological and physiochemical changes occur. For instance, in the germ-free rodent, the wall of the small intestine is thinner, the villi are more slender, the microvilli of the brush border are longer and the rate of turnover of mucosal epithelial cells is slower (Heneghan 1988). However, such morphological differences would suggest that the germ-free small intestine would be more efficient in absorbing nutrients (Heneghan 1988, Coates 1988). Microbial metabolism also alters the physiochemical characteristics of the gut contents producing a lower redox potential and a generally lower pH, which could have important effects on the metabolism and disposition of xenobiotics (Ward *et al* 1986) such as aflatoxin and the cooked food mutagens. This however requires further investigation.

The present study and that of Rowland *et al* (1987) show that the gut flora can affect the metabolism of known carcinogens in the liver, which in turn may influence the induction of tumours. This difference does not appear to be associated with either *E. coli* or

Bacteroides inhabiting the gut since gnotobiotics contaminated with these bacteria had little effect on the activating capacity of the S9 fractions towards MeIQ and Trp-P-2.

8. *In vivo* and *in vitro* bacterial mutation assays.

In this thesis, several investigations have been presented which examine modifiers of mutagenic activity using *in vivo* and *in vitro* bacterial mutation assays. Mutagens often used in these experiments (see Chapters 3, 4 and 6) were food mutagens aflatoxin B₁, MeIQ and Trp-P-2. It was observed in both the *in vivo* host-mediated assay and the *in vitro* mutagenicity assay that the order of potency of the mutagens, as determined by the numbers of revertants induced per μmol , was MeIQ > Trp-P-2 > aflatoxin B₁.

The observed difference in genotoxicity between the two heterocyclic amines, MeIQ and Trp-P-2, concurs with previous studies (Sugimura 1986) and may be explained by differences in the metabolic pathways followed by the two chemicals when activated to reactive intermediates. For instance, the evidence suggests that MeIQ is N-hydroxylated prior to forming acetyl or sulphate esters (Sato *et al* 1980), whereas the active metabolite of Trp-P-2 is an O-acetyl derivative (Hashimoto & Shudo 1985). A further explanation for *in vivo* differences in genotoxicity between compounds, is that the latter may exhibit different pharmacokinetics. This was demonstrated for MeIQ and Trp-P-2 by Howes (1987), who found that an oral dose of MeIQ (5mg[24 μmol]/kg) in the host-mediated assay, gave rise to a peak of mutagenic activity within 1 hour of dosing, whereas the greatest activity of Trp-P-2 (10mg[39 μmol]/kg) was detected 3 hours after dosing. This would suggest that in the

present study, the maximum activity of Trp-P-2 was not measured, since mutagenicity was only determined 1 hour after dosing Trp-P-2 to the animals. The latter suggestion may indeed be true when the administered doses of Trp-P-2 and MeIQ were similar to that of Howes (1987), but one may not necessarily assume this for other doses of mutagen until further time-course studies are performed which use a range of mutagen concentrations.

A number of reasons may explain the apparent lack of mutagenic potency of aflatoxin B₁ compared to MeIQ and Trp-P-2. For example, the low activity may be associated with the known resistance of mice to the toxic and carcinogenic effects of aflatoxin (see discussion p.256).

In contrast, mice are thought to be sensitive to the genotoxic action of cooked food mutagens as they have a greater ability to activate IQ and MeIQ to bacterial mutagens than other rodent species (Alldrick & Rowland 1985). It has been proposed that this species difference in the activation of heterocyclic amines is related to the higher activities of hepatic N-hydroxylases and other cytochrome P450-mediated activities in mice compared to rats (Litterst *et al* 1975, Alldrick & Rowland 1985).

A second explanation for the low potency of aflatoxin in bacterial mutation assays used in the present investigation, is that *S. typhimurium* TA98 was used as the indicator organism. *S. typhimurium* TA98 is sensitive to frameshift mutagens, such as the heterocyclic amines. Although aflatoxin can produce this type of mutation (by single strand breaks. D'Andrea & Haseltine 1978), the preference is to produce base-pair substitutions. In order to measure the latter, an indicator strain

such as *S. typhimurium* TA100 would be required. The reason for not using this strain is that in the host-mediated assay there is evidence to suggest that the bacteria can lose the pKM101 plasmid (personal communication I.R. Rowland). In order to compare results from the host-mediated assays with those from the *in vitro* mutagenicity assays, *S. typhimurium* TA98 was used in both sets of experiments.

A third factor is that *S. typhimurium* TA98 cells have the potential to metabolize heterocyclic amine precursors to mutagenic species. Evidence for this comes from the work of Saito *et al* (1983) who isolated an acetyl CoA-dependent O-acetyl transferase from *S. typhimurium* which was capable of transforming N-OH-Trp-P-2 to a reactive derivative. It is possible that this second activation pathway results in greater activation of the heterocyclic amines and so enhances any differences in mutagenicity ^{between} this class of compounds and aflatoxin B₁.

In addition to aflatoxin, a low mutagenic response was obtained for the highly potent carcinogen nitrosodimethylamine in the *in vitro* bacterial mutation assay (Fig. 22). The inability to detect nitrosamines is a known disadvantage of this assay (Bartsch *et al* 1976, Yahagi *et al* 1977, McCann *et al* 1975). The prime reason for the lack of activity appears to be the inefficient metabolism of N-nitrosamines to active genotoxins by hepatic fractions (Rowland 1988, Lee & Guttenplan 1981). The use of a pre-incubation step, as in the present study, does improve the sensitivity of the assay as it helps to overcome some of the major problems (Yahagi *et al* 1977). For instance, without the pre-incubation the nitrosamine can

diffuse through the bottom agar before the phase of activation is complete. Furthermore the viscosity of agar slows the migration time of the short-lived activated nitrosamine from the microsomes to the bacteria. In addition it has been suggested that these reactive intermediates react with the media before they have even been absorbed by the bacteria (Dahl 1985). Even when using a pre-incubation step with a range of nitrosodimethylamine concentrations, the mutagenic response was not marked as reflected by the maximal 3 fold increase in activity (Fig. 22). Similarly Kerklaan *et al* (1985), using *E. coli* as the indicator strain, detected only a low level of genotoxic activity with nitrosodimethylamine and Pueyo *et al* (1979) with *S. typhimurium* SV3 also found microsomal activation to be relatively inefficient. To return to the present study it was noted that the background reversion of *S. typhimurium* TA100 was only 33 His⁺ revertants per plate, as compared to the usual reversion rate of 140. This would suggest that the culture had lost the pkM101 plasmid and so resembled *S. typhimurium* TA1535 instead. However, this did not invalidate the experiment as the pkM101 plasmid is not a prerequisite for the detection of base pair mutations (McCann *et al* 1975). In contrast to the *in vitro* bacterial mutation assay, the host-mediated assay is sensitive to the genotoxic effects of nitrosamines. In the present study a 2mg/kg dose induced a marked mutagenic response in the liver using ampicillin resistance as a marker. Using the same marker Solt & Neale (1979, 1980) found that nitrosodimethylamine could be detected at concentrations as low as 50µg/kg.

In *in vitro* mutation assays, the amount of hepatic S9 or microsomes incorporated into the incubation mix can be critical in

determining the size of mutagenic response. Profiles of S9-titration curves were found to differ markedly for the three food mutagens. For example in the investigations of Chapter 6, using hepatic S9 fractions prepared from mice of various ages the mutagenicity of each of the compounds initially increased reaching a peak prior to a decline in the amount of mutagenicity (Fig. 26). The S9 concentration at which maximal mutagenicity was observed was dependent on the mutagen used (Fig. 26). This demonstrates the need for this type of experiment since S9-titration curves can be quite complex and may vary considerably between structurally-related chemicals (Gatehouse 1987). It must be realized that at each S9 concentration the mutagenicity observed is a balance between the activating and detoxifying enzyme systems present. An increase in the S9 concentration may cause different mutagenic species to form. At high S9 concentrations there is ^{also} a possibility that metabolites may bind to other proteins in the S9 and ^{that this} will prevent ^{their} further metabolism to an active form.

During my studies the host-mediated bacterial mutation assay was often used in conjunction with the *in vitro* bacterial mutation assays. For certain compounds, such as nitrosodimethylamine this *in vivo* assay can be more sensitive to certain mutagens than comparable *in vitro* assays. Other mutagens that have been detected by the host-mediated assay include cycasin (Gabridge & Legator 1969); natulan (Solt & Neale 1980) and also the *in vivo* formation of mutagens from morpholine and nitrite (Edwards *et al* 1979) and aminopyrine and nitrite (Solt & Neale 1980).

A few reports exist which compare the host-mediated assay and

in vitro bacterial mutation assays including the Gene-Tox Program (Legator *et al* 1982) in which it was found that the two tests usually detected the same compounds as mutagens. Exceptions to this were the carcinogens 7-bromomethyl-12-methylbenz(a)-anthracene and 3-methyl-4-dimethylaminobenzene which were mutagenic *in vitro* but not in the host-mediated assay.

One advantage of the host-mediated assay is that a range of endpoints are available (forward and backward mutation markers, mitotic gene conversion and recombination, recessive lethal mutations and chromosomal aberrations) using a number of indicator organisms (bacteria, yeast, neurospora and mammalian cells). Furthermore test compounds can be given orally, intraperitoneally, intramuscularly, intravenously and, with antibiotic resistant bacteria, intragastrically. The test compound can also be given before, after or at the same time as the indicator organism and depending on the indicator used, the activity of the compound can be determined in a range of organs which may be of particular importance when using carcinogens whose target organ is not the liver. In the present study radiolabelled mutagens were administered which can provide additional data on the distribution and disposition of the mutagen in the body.

The length of time that bacteria are left in the animal before the cells are recovered is dependent upon the indicator strain used. For *S. typhimurium* TA98, a one hour exposure is the longest feasible period, without there being an excessive decrease in the number of surviving bacteria (Arni *et al* 1977, Glatt *et al* 1985). For the DNA-repair intrasanguineous host-mediated assay it was found that

retention times less than three hours produced no substantial alteration to the proportion of viable repair proficient and repair deficient *E. coli* (Mohn 1984). This can be seen in the present study in which a 90 minute exposure time was used. Control mice (given saline in place of mutagen) exhibited no loss of the repair deficient strain compared to the repair proficient strain (Tables 12 & 30). Unlike the assay using *S. typhimurium* TA98, the DNA-repair assay requires relatively few bacteria as the survival of one strain is compared to another, rather than the need to determine a mutation frequency. With *E. coli* D494, an exposure time of three hours can be used since it is complement-resistant and thus resists the phagocytic action of the immune system. However with such a long exposure time, it has been suggested that the bacteria may divide and increase in number. The electronmicrograph given in Fig. 40 does show that one bacterium may be in the process of cellular division after an exposure time of thirty minutes although the cellular state of this cell at the time of injection into the animal is unknown. Work of Pueyo *et al* (1979) using an exposure time of up to 4 hours, indicated that division of *S. typhimurium* SV3 occurred in the liver and probably the kidneys as well. This division of the indicator bacteria may not be important but it does rely on the mutated cells not growing at different rates to the unmutated bacteria. The DNA repair *E. coli* strains are less susceptible to growing within the tissues of the intact mouse, since both strains carry streptomycin dependent markers. It is particularly important with the DNA repair host-mediated assay that growth of the cells does not occur, as different growth rates of the two cell types would give overgrowth of the repair-proficient strain and could lead, therefore, to false positives.

The electronmicrographs (Fig. 40) clearly show that *E. coli* D494 are retained in macrophage cells, notably Kupffer cells, within the capillaries of the liver. Kupffer cells are fixed macrophages of the reticuloendothelial system. Similarly, Hauser & Matter (1977), Frezza *et al* (1979) and Knasmuller *et al* (1986) have reported that bacteria injected intravenously are located within macrophage cells within the liver sinusoids. This bacterial engulfment by cells of the immune system would appear to be quite rapid, since in both the present study and in that of Hauser & Matter (1977) the bacteria were found at this site within 30 minutes of injecting the bacteria. Furthermore the Kupffer cells show little signs of phagocytic activity suggesting that bacterial engulfment was complete. In support of this, published data suggests that bacteria are rapidly removed from the blood and furthermore, retained in various tissues (Pueyo *et al* 1979, Knasmuller *et al* 1986). This was shown in the present study in which *E. coli* D494 were recovered from lung, kidney and spleen as well as the liver (Table 25). In this experiment the majority of bacteria were recovered in the liver (approximately 60%) compared to 3 - 4% in the lung and spleen and and 0.3% in the kidney. This agrees with data demonstrating that 2 hours after injecting bacteria into mice approximately 80% of the cells are in the liver, 13% in the spleen and the remainder in the kidney and lungs (Benaceraff *et al* 1959, Mohn 1973, Pueyo *et al* 1979). In addition Mohn (1973) observed that maximal concentration of the bacteria was achieved 5 minutes after injecting (except in the spleen when it was sometime later) and that the recovery of cells decreased as the incubation time increased, probably because of effective phagocytosis.

It must be remembered in the host-mediated assay that the indicator cells are exposed either to the circulating test compound before its uptake by body tissues, or to metabolites released from these tissues. The latter will tend to be relatively stable chemicals destined for excretion although this is not always the case as shown by the compound nitrosodimethylamine (see discussion p.280). Although the activities of xenobiotic metabolizing enzymes in nonparenchymal cells of the liver (such as the Kupffer cells) are very low (Lafranconi *et al* 1986) they are detectable and therefore, may have a small role in mutagen activation (Oesch *et al* 1986).

One disadvantage of the host-mediated assay is that the doses of mutagen required to evoke a significant response are often large (in comparison with likely human exposure levels). For substances that are insoluble in water, a sufficient dose level that can be detected by the indicator organism may never be achieved (McGregor 1980), the dose being limited by toxicity, not necessarily to the indicator cells, but to the host mammal.

A further disadvantage of the host-mediated assay is the great variation that occurs both within treatment groups and from day to day. This is exemplified by the large standard deviation values in experiments presented in this thesis (for examples see Tables 27 - 29) and also by the differences in mutagenic response from day to day. One example of the latter is reported in Chapter 4, when a 10mg/kg dose of aflatoxin was given to mice in two experiments. The resulting mutation rates (for mice in the same treatment groups) were, on average 1100 and 350 His⁺/plate. In certain cases this

variability may be an artifact derived from animal husbandry such as the presence of fresh paint which can alter rodent metabolism for a period of weeks. Furthermore the animals were not starved overnight and depending on the time the animals last ate will result in differences in the gastric environment - a factor that may be particularly important in experiments investigating the nitrosation of aminopyrine. To reduce the variation in the host-mediated assay I would recommend that inbred mice should be used (so as to reduce genetic variation), to have at least 6 - 8 animals per treatment group and to keep animals within a narrow weight range.

In addition, in bacterial mutation assays the choice of indicator strain is important. Markers are often limited in the range of compounds that they can detect. For instance, *E. coli* D494 and the forward mutation marker, ampicillin resistance, preferentially detects base-pair substitutions (Solt & Neale 1979, Bosworth 1987) and is relatively insensitive to frameshift mutagens, whereas for *S. typhimurium* TA98 it is the reverse (see discussion p.292). It was found that during my studies the DNA repair host-mediated assay was insensitive to compounds with large molecular configuration, presumably because the compounds were unable to penetrate the bacterial cell wall. During my investigations several modifications to the DNA repair host-mediated assay were tested to try and improve the sensitivity of the assay. Initially, LPD^{def} derivatives (*E. coli* 343/801 and 343/772) of the original indicator organisms (*E. coli* 343/765 and 343/753 respectively) were tested. Although these derivatives were more sensitive to the genotoxicity of hycanthone *in vitro* (Table 45), several problems were associated with the use of these strains.

Firstly, *E. coli* 343/772 (the LPS^{def} derivative of 343/753) had a mucoid colony morphology which caused confluence of the large size colonies, or of the smaller colonies if several were on one plate. Secondly, the reduced growth kinetics of *E. coli* 343/772 in liquid suspensions (stationary overnight cultures of this strain contained only around 1% of the viable cell titre of the wild-type strain) made it difficult to obtain sufficient amounts of bacteria required for a host-mediated assay. Furthermore, this strain did not survive for long periods in 7% DMSO at -80°C (the routine procedure for keeping permanent bacterial stocks in the laboratories at University College and BIBRA). Due to this sensitivity, this strain was lost in both our own laboratory and in that of G. Mohn who originally isolated it. Mohn and his co-workers also reported that this strain was impractical for use on a routine basis (personal communication). In his communication Mohn also reported that the LPS⁻ character was genetically unstable. Therefore, it was considered impractical to use LPS^{def} strains in further experimental work.

An alternative procedure to increase membrane permeability of bacterial cells is a short exposure of the cells to EDTA. As shown by Leive (1965, 1968) mild treatment of *E. coli* with EDTA at pH 8.0 removes large parts of the lipopolysaccharide (LPS) cell layer without concomitant loss of viability. However, this effect is only transient since treated cells, when allowed to grow, repair their permeability barrier. EDTA-exposure has been successfully used before in *in vitro* mutagenicity tests to enhance the surface permeability of various *E. coli* strains (Leonardo *et al* 1984, Mohn *et al* 1981, 1984b, Mitchell & Gilbert 1984, Coratza & Molina 1978,

Knasmuller *et al* 1989). Indeed in the present study EDTA treated bacteria were more sensitive to the genotoxic effects of aflatoxin B₁ and IQ compared to untreated bacteria (Table 47, Fig. 39).

At present, the precise mechanisms by which EDTA exposure leads to an increased surface permeability in coliform bacteria is not completely understood. However, evidence suggests that EDTA acts by binding divalent cations (probably Mg²⁺) followed by a chemical or conformational change in the cell wall (Leive 1968, Weiser *et al* 1968). It is unlikely that variations in membrane permeability between the two strains occur upon exposure to EDTA, since both derivatives are subjected to the same treatment conditions. The regeneration of the permeability barrier requires energy and may occur in absence of cell multiplication (Leive 1968) thus this treatment is only effective over a short duration. However, the results (Table 47, Fig. 39) show that the permeability change produced in *E. coli* K-12 derivatives is sufficiently long-lived to enable aflatoxin and IQ to enter the bacteria and cause DNA damage under the conditions of the *in vitro* genotoxicity assay. A further advantage of this modification is that in contrast to the LPS^{def} strains the colony morphology of the cells was unaffected.

It was observed in the present study that treatment of bacteria with EDTA (0.5mM) produced a slight genotoxic response. As a result, it is recommended that a lower concentration of EDTA be tested in future experiments. Similarly Knasmuller *et al* (1989) reported that the concentration of EDTA in the preincubation mix should not exceed 0.5mM as a weak genotoxic activity of this chelating agent was observed.

There is evidence to suggest that the growth rate of bacterial indicator cells in mutagenicity assays can modify the sensitivity of these assays in detecting potential mutagens. For instance, Hince & Neale (1977) found that the use of bacteria in logarithmic growth phase increased the response of the bacteria to the mutagenic effects of nitrosomethylurea and may also influence the response to compounds which are detoxified by intracellular glutathione (Goggelmann 1980). A possible reason for the increased sensitivity with log-phase cells is that when cells are growing rapidly there is less time for damage to the DNA to be repaired. However in the present study using log-phase cells in the *in vitro* differential DNA repair assay there was no marked increase in the genotoxic response towards nitrosomethylurea as compared to stationary phase cells (Fig. 38). This may be partly explained by the observation that nitrosomethylurea was more toxic to *E. coli* (irrespective of its capacity to repair DNA) when the bacteria were in active growth than when stationary. In the rapidly metabolizing logarithmic cell, efficient drug uptake can result in many DNA lesions created by error-prone repair of the DNA cross-links during rapid DNA replication and cell division, resulting in greater cell death. In the stationary phase cell, where its state and chemical composition differs ^{from} that of the exponential cell, it becomes more resistant to adverse physical and chemical agents. Less efficient uptake or penetration of the chemical through the cell membrane will produce fewer DNA lesions per cell, which may explain why nitrosomethylurea was less toxic to stationary cells than to logarithmic cells. Similarly other workers in the laboratory at University College observed greater toxicity by chemicals towards *E. coli* D494 when in

logarithmic growth than in stationary growth.

It would appear from these initial investigations that of the three modifications to the differential DNA repair assay tested, that EDTA-permeabilized *E. coli* cells had the greatest potential for use with compounds with a large molecular weight. However further investigations using a range of compounds and animal-mediated assays are required in order to validate this assay.

An advantage of the *E. coli* 343 indicator strains over *E. coli* D494 and *S. typhimurium* is that it can be given orally and successfully recovered from the gastro-intestinal tract (Mohn 1984). In the present study *E. coli* 343/765 (*uvr⁺, rec⁺*) and 343/753 (*uvrB, recA*) were recovered from the small intestine, colon and faeces. From the results (Fig. 41) it would appear that within a few minutes significant numbers of bacteria are expelled from the stomach into the small intestine. The bacteria are then passed into the caecum initially at a rapid rate although a plateau in the numbers of bacteria in the caecum is reached within an hour of dosing. Presumably this plateau is a balance of bacteria entering the caecum from the small intestine and those being passed into the colon. Not surprisingly, in the faeces the recovery of bacteria was low for the first hour but then rose rapidly over the next two hours. Throughout the time course the recovery of the repair deficient strain was lower than that of the repair deficient strain even though similar numbers of each strain were dosed to the mice. Knasmuller *et al* (1988) have proposed that this genotoxic response is due to some component of the animal feed. In this context it should be noted that the occurrence of mutagenic products in human faeces has

been reported (Ehrich *et al* 1979) and different factors such as composition of the diet, bile and bile acids as well as metabolic activation through the intestinal flora have been considered as contributing to the genotoxic activities observed (Knasmuller *et al* 1988).

This intragastric host-mediated assay can be carried out at the same time as an intrasanguineous assay. This could be used to measure genotoxic activity of chemicals that are metabolized by both the gut microflora and mammalian enzymes, such as chemicals that are detoxified by glucuronide conjugation. Alternatively, the bacteria could be recovered from the faeces without sacrifice of the experimental animal. Animals could then be given several treatments.

The mutants seen in bacterial mutation assays are considered to consist of two classes of mutants. On the control plates (in which there has been no exposure to a mutagen) the colonies consist of either "pre-existing" mutants - those present in the cell population at the time of plating and/or "plate" mutants which arise during the period of growth on the plate. In practice it is thought that nearly all the "spontaneous" mutants are in this second category (Green & Muriel 1976). It is thought that these "background" point mutations occur by hydrolytic damage to DNA which includes loss of bases and deamination of exocyclic amino groups. The number of plate mutants is dependent on the final number of auxotrophic cells which grow on the selective agar plate and mutate. In the case of the *Salmonella* strains, this is, in turn dependent on the supplement of histidine in the selective agar and is completely independent of

the number of cells plated. However, when the many control cells are plated (within certain limits) the same number of cells will grow on a selective agar plate containing a given amount of histidine and give rise to approximately the same number of plate mutants (Green & Muriel 1976). A consequence of this is that the mutation frequencies of the control samples are not subtracted from the induced mutation frequency, as is sometimes recommended, because this can result in substances that kill bacteria appearing as a mutagen (Green & Muriel 1976). A classic example of this is reported by Green & Muriel (1976) where this miscalculation showed distilled water to be mutagenic. In *E. coli*, spontaneous mutation is primarily the result of error-prone repair (Sargentini & Smith 1985) and in general base substitution is the most common form of spontaneous mutation in bacteria.

Although the host-mediated assay is not suitable as a routine *in vivo* test to screen chemicals for genotoxic potential this assay could have a role in determining modifiers of mutagenicity/ carcinogenicity *in vivo*. For example in the present investigation the host-mediated assay was used to determine the effects of dietary components and host age on the *in vivo* mutagenic activity of food mutagens. Furthermore the assay was used in conjunction with *in vitro* mutagenicity assays which gave an indication of the mechanisms involved in the modification of mutagenic activity.

9. Conclusions

The work presented in this thesis has demonstrated that the mutagenic activity of food carcinogens can be modified by diet (in particular fat and protein) which would support the view that diet

is a contributor to human cancer. However the degree of modulation was dependent on the carcinogen to which the animals were exposed as well as on the type and/or the amount of fat and protein in the diet. In addition to diet the age of the host animal was also important in determining chemical mutagenicity. Ageing appeared to reduce the genotoxic activity of carcinogens suggesting that the young are more susceptible to the initiating events of carcinogens. Since most cancers take many years to develop this result is not contrary to the fact that cancer incidence increases with age.

Furthermore the results of my work show that modifiers of mutagenic activity (for example dietary fat and protein) could affect a variety of parameters including hepatic activation and detoxification, gut flora metabolism, uptake from the gastrointestinal tract into tissues such as the liver and the disposition of a chemical around the body or within a tissue. Consequently the results obtained from *in vivo* studies were a balance of the factors that affect chemical metabolism . If chemical mutagenicity, determined *in vivo*, was unaltered by diet it would indicate that any diet-induced changes in mutagen activation were counteracted by changes to factors that reduce mutagenic activity. The use of *in vitro* mutagen^ccity assays in conjunction with the *in vivo* assay helped to determine whether hepatic metabolism (in particular activation) was modified. The use of these two assays provide a useful tool, which is relatively quick and inexpensive, for assessing the effects of diet on genotoxic activity in the whole animal and also the mechanisms involved which in turn direct the way for further investigations. Various modifications to the *in vitro* and animal mediated bacterial mutation

assays are available (choice of indicator strains, site for dosing bacteria and mutagen, the length of incubation and the tissue from which the bacteria are recovered) and are important for optimizing the experimental conditions and need to be carefully considered for each genotoxin tested.

REFERENCES

- Abu-Shakra, A., Ioannides, C. & Walker, R. (1986) Metabolic activation of 2-amino-3-methylimidazo(4,5-f)-quinoline (IQ) by hepatic preparations; contribution of the cytosolic fraction and its significance to strain differences, *Mutagenesis* 1, 367-370.
- Adams, J.D., Lauterburg, B.H. & Mitchell, J.R. (1983) Plasma glutathione and glutathione disulfide in the rat: regulation and response to oxidative stress, *J. Pharm. Exp. Ther.* 227, 749-754.
- Alldrick, A.J., Lake, B.G., Flynn, J. & Rowland, I.R. (1986) Metabolic conversion of IQ and MeIQ to bacterial mutagens, *Mutation Res.* 163, 109-114.
- Alldrick, A.J. & Rowland, I.R. (1985) Activation of the food mutagens IQ and MeIQ by hepatic S9 fractions derived from various species, *Mutation Res.* 144, 59-62.
- Alldrick, A.J. & Rowland, I.R. (1988) Distribution of radiolabelled [2-¹⁴C]IQ and MeIQx in the mouse, *Toxic. Lett.* 44, 183-190.
- Alldrick, A.J., Rowland, I.R., Lake, B.G. & Flynn, J. (1987a) High levels of dietary fat: alteration of hepatic promutagen activation in the rat, *J.N.C.I.* 79, 269-272.
- Alldrick, A.J., Rowland, I.R. & Wise, A. (1987b) The hepatic conversion of some heterocyclic aromatic amines to bacterial mutagens is modified by dietary fat and cholesterol, *Mutagenesis* 2, 221-224.
- Allen-Hoffmann, B.L. and Campbell, T.C. (1977) The relationship between hepatic glutathione levels and the formation of aflatoxin B₁-DNA adducts as influenced by dietary protein intake, *Fed. Proc.* 36, 1116.

- Altman, D.G., Gore, S.M., Gardner, M.J. & Pocock, S.J. (1983) Statistical guidelines for contribution to medical journals, *Br. Med. J.* 286, 1489-1493.
- Alvares, A.P. & Kappas, A. (1977) Heterogeneity of cytochrome P-450's induced by polychlorinated biphenyls, *J. Biol. Chem.* 252, 6373-6378.
- Alvey, N., Galway, N. & Lane, P. (1982) *An introduction to genstat*, Academic Press, London.
- Ames, B.N., Durston, W.E., Yamasaki, E. and Lee, F.D. (1973) Carcinogens are mutagens: A simple test system combining liver homogenates for activation and bacteria for detection, *Proc. Natl. Acad. Sci.* 70, 2281-2289.
- Ames, B.N., McCann, J. & Yamasaki, E. (1975) Methods for detecting carcinogens and mutagens with the Salmonella/mammalian microsome mutagenicity test, *Mutation Res.* 31, 347-364.
- Anderson, K.E., Conney, A.H. & Kappas, A. (1982) Nutritional influences on chemical biotransformations in humans, *Nutr. Rev.* 40, 161-171.
- Anisimov, V.N. (1985) Relevance of age to some aspects of carcinogenesis, In A. Likhachev, V. Anisimov & R. Montesano (eds), *Age-related factors in Carcinogenesis*, IARC, Lyon, No. 58, pp. 115-126.
- Anonymous (1989) Household food consumption and expenditure: 1987, *Annual report of the National food survey committee*, MAFF. HMSO, London.
- Appleton, B.S. & Campbell, T.C. (1982) Inhibition of aflatoxin-initiated preneoplastic liver lesions by low dietary protein, *Nutr. Cancer* 3, 200-206.
- Appleton, B.S. & Campbell, T.C. (1983) Effect of high and low

dietary protein on dosing and post-dosing periods of aflatoxin B₁ induced hepatic preneoplastic lesion development in the rat, *Cancer Res.* 43, 2150-2154.

Armstrong, B. & Doll, R. (1975) Environmental factors and cancer incidence and mortality in different countries, with special reference to dietary practices, *Int. J. Cancer* 15, 617-631.

Arni, P., Mantel, T., DeParade, E. & Muller, D. (1977) Intravascular host-mediated assay with *Salmonella typhimurium*, *Mutation Res.* 45, 291-307.

Association of Official Analytical Chemists (1970) *Official methods of analysis* 11th ed. Association of Official Analytical Chemists. Washington DC p.14.

Balish, E., Shih, C.N., Croft, W.A., Pamukcui, A.M., Lower, G., Bryan, G.T. & Yale, C.E. (1977) Effect of age, sex and intestinal flora on the induction of colon tumours in rats, *J.N.C.I.* 58, 1103-1106.

Bandiera, S., Ryan, D.E., Levin, W. & Thomas, P.E. (1986) Age- and sex-related expression of cytochromes P450f and P450g in rat liver, *Arch. Biochem. Biophys.* 248, 658-676.

Bansal, B.R., Rhoads Jr., J.E. & Bansal, S.C. (1978) Effects of diet on colon carcinogenesis and the immune system in rats treated with 1,2-dimethylhydrazine, *Cancer Res.* 38, 3293-3303.

Barale, R., Zucconi, D. & Loprieno, N. (1981) A mutagenicity methodology for assessing the formation of N-dimethylnitrosamine *in vivo*, *Mutation Res.* 85, 57-70.

Barbolt, T.A. & Abraham, R. (1978) The effect of bran on dimethylhydrazine-induced colon carcinogenesis in the rat, *Proc. Soc. Exp. Biol. Med.* 157, 656-659.

Barnes, D.S., Clapp, N.K., Scotts, A., Oberst, D.L. & Berry,

S.G. (1983) Effects of wheat, rice, corn and soybean bran on 1,2-dimethylhydrazine-induced large bowel tumorigenesis in F344 rats, *Nutr. Cancer* 5, 1-9.

Barnes, W.S., Lovelette, C.A., Tong, C., Williams, G.M. & Weisburger, J.H. (1985) Genotoxicity of the food mutagen 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) and analogs, *Carcinogenesis* 6, 441-444.

Bartsch, H., Camus, A. & Malaveille, C. (1976) Comparative mutagenicity of N-nitrosamines in a semi-solid and in a liquid incubation system in the presence of rat or human tissue fractions, *Mutation Res.* 37, 149-162.

Bauer, H.G., Asp, N., Oste, R., Dahlquist, A. & Freudlund, P. (1979) Effect of dietary fibre on the induction of colorectal tumors and fecal β -glucuronidase activity in the rat, *Cancer Res.* 39, 3752-3756.

Bartsch H. & Montesano R. (1984) Commentary: relevance of nitrosamines to human cancer, *Carcinogenesis* 5, 1381-1393.

Basu, T.K., Dickerson, J.W.T. & Parke, D.V.W. (1974) Effect of development on the activity of microsomal drug-metabolizing enzymes in rat liver, *Biochem. J.* 124, 19-24.

Baumeister, M. (1982) Experimental models for the biological detection of N-nitroso compounds formed from amines and nitrite, *Toxic. Lett.* 12, 281-288.

Benacerraf, B., Sebestyen, M.M. & Schlossman, S. (1959) A quantitative study of the kinetics of blood clearance of P^{32} -labelled *Escherichia coli* and *stapylococci* by the reticulo-endothelial system, *J. Exp. Med.* 110, 27-48.

Bergman, K. (1985) Autoradiographic distribution of ^{14}C labelled 3H-imidazo[4,5-f]quinoline-2-amines in mice, *Cancer Res.*

45, 1351-1356.

Bidlack, W.R., Brown, R.C. & Mohan, C. (1986) Nutritional parameters that alter hepatic drug metabolism, conjugation and toxicity, *Fed. Proc.* 45, 142-148.

Bingham, S., Williams, D.R.R., Cole, T.J. & James, W.P.T. (1979) Dietary fibre and regional large-bowel cancer mortality in Britain, *Br. J. Cancer* 40, 4546-463.

Birnbaum, L. & Baird, M. (1979) Senescent changes in rodent hepatic epoxide metabolism, *Chem. Biol. Interac.* 26, 245-256.

Bosworth, D., Crofton-Sleigh, C., & Venitt, S. (1987) A forward mutation assay using ampicillin resistance in *Escherichia coli* designed for investigating the mutagenicity of biological samples, *Mutagenesis* 2, 455-467.

Bratton, C.A. & Marshall, F.K. (1939) A new coupling component of sulfonilamide determination, *J. Biol. Chem.* 128, 537-555.

Bresnick, E. (1980) Colon carcinogenesis: an overview, *Cancer* 45, 1047-1051.

Brown, R.C., Kelleher, J. & Losowsky, M.S. (1979) The effect of pectin on the structure and function of the rat small intestine, *Br. J. Nutr.* 42, 357-365.

Buell, P. (1973) Changing incidence of breast cancer in Japanese-American women, *J.N.C.I.* 51, 1479-1483.

Bull, A.W., Soullier, B.K., Wilson, P.S., Hayden, M.T. & Nigro, N.D. (1979) Promotion of azoxymethane-induced intestinal cancer by high fat diet in rats, *Cancer Res.* 39, 4956-4959.

Bullen, C.L., Tearle, P.V. & Stewart, M.G. (1977) The effect of "humanised" milks and supplemented breast feeding on the flora of infants, *J. Med. Microbiol.* 10, 403-413.

Burke, M.D. & Majer, R.T. (1974) Ethoxyresorufin: direct

fluorimetric assay of a microsomal O-dealkylation which is preferentially inducible by 3-methylcholanthrene, *Drug Metab. Disp.* 2, 583-586.

Butler, W.H. & Barnes, J.M. (1966) Carcinoma of the glandular stomach in rats given diets containing aflatoxin, *Nature* 209, 90.

Campbell, T.C. (1982) Nutritional modulation of carcinogenesis, In M.S. Arnott, J. van Eys & Y.-M. Wang (eds) *Molecular interrelations of nutrition and cancer*, Raven Press, New York, pp. 359-367.

Campbell, T.C. & Hayes, J.R. (1974) Role of nutrition in the drug-metabolizing enzyme system, *Pharm. Rev.* 26, 171-197.

Campbell, T.C. & Hayes, J.R. (1976) The effect of quantity and quality of dietary protein on drug metabolism, *Fed. Proc.* 35, 2470-2474.

Capizzi, R.C., Papirmeister, B., Mullins, J.M., Chang, E. (1974) The detection of chemical mutagens using the L5178Y/Asn⁻ murine leukemia *in vitro* and in a host-mediated assay, *Cancer Res.* 34, 3073-3082.

Carroll, K.K. (1975) Experimental evidence of dietary factors and hormone-dependent cancers, *Cancer Res.* 35, 3374-3383.

Carroll, K.K. (1980) Lipid and carcinogenesis, *J. Environ. Path. Toxic.* 3, 253-271.

Carroll, K.K. & Khor, H.T. (1971) Effect of level and type of dietary fat on incidence of mammary tumors induced in female Sprague-Dawley rats by 7,12-dimethylbenz(a)anthracene, *Lipids* 6, 415-420.

Cassano, P., Bannamour, D., Grolier, P., Pelissier, M.A., Amelizao, Z. Albrecht, R. & Narborne, J.F. (1987) The effect of dietary imbalances on the activation of benzo(a)pyrene by the

metabolizing enzymes from rat liver, *Mutation Res.* 191, 67-72.

Castro, C.E., Falkener, I.C. & Yang, S.P. (1978) Dietary lipid dependent activation of the carcinogen N-2-fluorenylacetamide in rats monitored by *Salmonella typhimurium*, *Cancer Res.* 38, 2836-2841.

Chadwick, R.W., Copeland, M.F. & Chadwick, C.J. (1978) Enhanced pesticide metabolism, a previously unreported effect of dietary fibre in mammals, *Fd. Cosmet. Toxic.* 16, 217-225.

Chan, P.C., Ferguson, K.A. & Dao, T.L. (1983) Effect of dietary fats on mammary carcinogenesis, *Cancer Res.* 43, 1079-1083.

Chasseaud, L.F. (1976) Conjugation with glutathione and mercapturic acid excretion, In I.M. Amas & W.B. Jakoby (eds), *Glutathione: metabolism and function*, Raven Press, New York pp. 77.

Chengelis, C.P. (1988) Age- and sex-related changes in epoxide hydroxylase, UDP-glucuronosyl transferase, glutathione S-transferase and PAPS sulphotransferase in Sprague-Dawley rats, *Xenobiotica* 18, 1225-1237.

Clinton, S.K., Truex, C.R. & Visek, W.J. (1979) Dietary protein, aryl hydrocarbon hydroxyase and chemical carcinogenesis in rats, *J. Nutr.* 109, 55-62.

Clinton, S.K., Mulloy, A.C. & Visek, W.J. (1984) Effects of dietary lipid saturation on prolactin secretion, carcinogen metabolism and mammary carcinogenesis in rats, *J. Nutr.* 114, 1630-1639.

Clinton, S.K., Alster, J.M., Imrey, P.B., Nandkumar, S., Truex, C.R. & Visek, W.J. (1986) Effects of dietary protein, fat and energy intake during an initiation phase study of 7,12 dimethylbenz(a)anthracene-induced breast cancer in rats, *J. Nutr.* 2290-2302.

Coates, M.E., Drasar, B.S., Mallett, A.K. & Rowland, I.R.

(1988) Methodological considerations for the study of bacterial metabolism, In I.R. Rowland, (ed), *Role of the gut-flora in toxicity and cancer*, Academic Press, London, pp. 1-21.

Coles, B.F., Smith, J.R.L. & Garner, R.C. (1977) Mutagenicity of 3a,8a-dihydrofuro[2,3-b] benzofuran, a model of aflatoxin B₁ for *Salmonella typhimurium* TA100, *Biochem. Biophys. Res. Commun.* 76, 888-892.

Coles, B., Meyer, D.J., Ketterer, B., Stanton, C.A. & Garner, R.C. (1985) Studies on the detoxication of microsomally-activated aflatoxin B₁ by glutathione and glutathione transferases *in vitro*, *Carcinogenesis* 6, 693-697.

Commoner, B., Vithayathil, A.J., Dolara, P., Nair, S., Madyastha, P. & Cuca, G.C. (1978) Formation of mutagens in beef and beef extract during cooking, *Science* 201, 913-916.

Coratza, G. & Molina, A.M. (1978) Enhanced sensitivity to the dynamic effect of 3,4-benzopyrene in ethylenediaminetetraacetate-treated *Escherichia coli*, *J. Bacteriol.* 133, 411-412.

Croy, R.G., Essigmann, J.M., Reinhold, V.N. & Wogan, G.N. (1978) Identification of the principal aflatoxin B₁-DNA adduct formed *in vivo* in rat liver, *Proc. Natl. Acad. Sci.* 75, 1745-1749.

Czygan, P., Greim, H., Garro, A., Schaffner, F. & Popper, H. (1974) The effect of dietary protein deficiency on the ability of isolated hepatic microsomes to alter the mutagenicity of a primary and a secondary carcinogen, *Cancer Res.* 34, 119-123.

Dahl, A.R. (1985) Mutagenicity of some dialkylnitrosamines, cyclic nitrosamines and N,N-diethanolnitrosamine in *Salmonella typhimurium* with rat and rabbit nasal, lung and liver S9 homogenates, *Mutation Res.* 158, 141-147.

Dales, L.G., Friedman, G.D., Ury, H.K., Grossmann, S. &

Williams, S.R. (1978) A case control study of relationships of diet and other traits to colorectal cancer in American blacks, *Am. J. Epidemiol.* 109, 132-144.

Dallman, P.R. (1971) Malnutrition: incorporation of thymidine-³H into nuclear and mitochondrial RNA, *J. Cell. Biol.* 51, 549-553.

D'Andrea, A.D. & Haseltine, W.A. (1978) Modification of DNA by aflatoxin B₁ creates alkali-labile lesions in DNA at positions of guanine and adenine, *Proc. Natl. Acad. Sci.* 75, 4120-4124.

Dao, T.L. & Chan, P.C. (1983) Effect of duration of high fat intake on enhancement of mammary carcinogenesis in rats, *J.N.C.I.* 71, 201-205.

DeBethizy, J.D., Sherrill, J.M., Rickert, D.E. & Hamm Jr., T.E. (1983) Effects of pectin-containing diets on the hepatic macromolecular covalent binding of 2,6-dinitro-[³H]toluene in Fischer-344 rats, *Toxic. Appl. Pharm.* 69, 369-374.

Degan, G.H. & Neumann, H.G. (1978) The major metabolites of aflatoxin B₁ in the rat is a glutathione conjugate, *Chem. Biol. Interac.* 22, 239-255.

Degan, G.H. & Neumann, H.G. (1981) Differences in aflatoxin B₁ susceptibility of rat and mouse are correlated with the capability *in vitro* to inactivate aflatoxin B₁ epoxide, *Carcinogenesis* 12, 299-306.

Diaz Gomez, M.I., Swann, P.F. & Magee, P.N. (1977) The absorption and metabolism in rats of small oral doses of dimethylnitrosamine, implication for the possible hazard of dimethylnitrosamine in human food, *Biochem, J.* 164, 497-500.

Ditmer, D.S. (1961) Blood and other body fluids, *Federation of American Society for Experimental Biology*, Washington DC, p. 9.

Doll, R. & Peto, R. (1981) The causes of cancer: qualitative

estimates of avoidable risks of cancer in the United States today, *J.N.C.I.* 66, 1191-1308.

Dolara, P., Caderni, G., Bianchini, F. & Tanganelli, E. (1986) Nuclear damage of colon epithelial cells by the food carcinogen 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) is modulated by dietary lipids, *Mutation Res.* 175, 255-258.

Drasar, B.S. & Irving, D. (1973) Environmental factors and cancer of the colon and breast, *Br. J. Cancer* 27, 167-172.

ECETOC (European Chemical Industry Ecology and Toxicology Centre) (1988) Nitrate and drinking water, *ECETOC Technical Report* 27, 26-37.

Edwards, G., Whong, W.-Z., & Speciner, N. (1979) Intrahepatic mutagenesis assay: a sensitive method for detecting N-nitrosomorpholine and *in vivo* nitrosation of morpholine, *Mutation Res.* 64, 415-423.

Ehrich, M., Ashell, J.E., van Tassell, R.L., Wilkins, T.D. (1979) Mutations in the feces of 3 South African populations at different levels of risk for colon cancer, *Mutation Res.* 64, 231-240.

Enstrom, J.E. (1975) Colorectal cancer and consumption of beef and fat, *Br. J. Cancer* 32, 432-439.

Enwonwu, C.O. & Munro, H.N. (1970) Rate of RNA turnover in rat liver in relation to intake of protein, *Arch. Biochem. Biophys.* 138, 532-539.

Epstein, S.M., Bartus, B. & Farber, E. (1969) Renal epithelial neoplasms induced in male Wistar rats by oral aflatoxin B₁, *Cancer Res.* 29, 1045-1050.

Essigmann, J.M., Croy, R.G., Bennett, R.A. & Wogan, G.N. (1982) Metabolic epoxidation of aflatoxin B₁ and its metabolites: Patterns

of DNA adduct formation and removal in relation to biological effects, In E. Nicolimi, (ed) *Chemical Carcinogenesis*, Plenum Press, pp. 47-66.

Felton, J.S., Nebert, D.W. & Thorgeirsson, S.S. (1976) Genetic differences in 2-acetylaminofluorene mutagenicity *in vitro* associated with mouse hepatic aryl hydrocarbon hydroxylase activity induced by polycyclic aromatic compounds, *Mol. Pharm.* 12, 225-232.

Felton, J.S., Knize, M.G., Shen, N.H., Andresen, B.D., Bjeldanes, L.F. & Hatch, F.T. (1986) Identification of the mutagens in cooked beef, *Environ. Health Perspect.* 67, 17-24.

Feuer, G. & Liscio, A. (1970) Effect of drugs on hepatic drug metabolism in the fetus and newborn, *Int J. Clin. Pharm.* 3, 30-33.

Fielding, A.M. & Huges, R.E. (1976) Changes in liver microsomal cytochrome P450 induced by dietary protein and lipid material, *Biochem. Pharmac.* 25, 1916-1919.

Fine, D.H., Ross, R., Rounbehler, D.P., Silvergleid, A. & Song, L. (1977) Formation *in vivo* of volatile N-nitrosamines in man after ingestion of cooled bacon and spinach, *Nature* 265, 753-755.

Fischer, L.J., Kent, T.H. & Weissinger, J.L. (1973) Absorption of diethylstilbestrol and its glucuronide conjugate from the intestines of five and twenty-five day old rats, *J. Pharm. Exp. Ther.* 185, 163-170.

Fleischer, D., Murray, D., MacFarlane, J. & Brown, R.A. (1978) Protective effect of dietary fibre against chemically induced bowel tumors in rats, *Lancet* ii, 552-553.

Fontham, E., Zavala, D. & Correa, P. (1986) Diet and chronic atrophic gastritis: a case-control study, *J.N.C.I.* 76, 621-627.

Forster, R., Green, M.H.L., Priestley, A. & Gorrod, J.W. (1981a) Apparent activation of 2-acetylaminofluorene and some

aromatic amines by cytosolic preparations, *Mutation Res.* 85, 187-198.

Forster, R., Green, M.H. & Priestley, A. (1981b) Enhancement of S9 activation by S105 cytosolic fraction, *Carcinogenesis* 2, 1081-1085.

Freeman, H.J. (1986) Effects of differing purified cellulose, pectin and hemicellulose fiber diet on fecal enzymes in 1,2-dimethylhydrazine-induced rat colon carcinogenesis, *Cancer Res.* 46, 5529-5532.

Frezza, D., Zeiger, E. & Gupta, B.N. (1979) The intrasanguineous host-mediated assay procedure, distribution and retention of yeast in the mouse, *Mutation Res.* 64, 295-305.

Gabridge, M.G. & Legator, M. (1969) A host-mediated microbial assay for the detection of mutagenic compounds, *Proc. Soc. Exp. Biol. Med.* 130, 831-834.

Garner, R.C., Miller, E.C. & Miller, J.A. (1972) Liver microsomal metabolism of aflatoxin B₁ to a reactive derivative toxic to *Salmonella typhimurium* TA1530, *Cancer Res.* 32, 2058-2066.

Gatehouse, D. (1987) Guidelines for testing of environmental agents. Critical features of bacterial mutation assays, *Mutagenesis* 2, 397-409.

de Gerlache, J., Taper, H.S., Lans, M., Preat, V. & Roberfroid, M. (1987) Dietary modulation of rat liver carcinogenesis, *Carcinogenesis* 8, 337-340.

Glatt, H. Bucker, M., Platt, K.L. & Oesch, F. (1985) Host-mediated mutagenicity experiments with benzo(a)pyrene and two of its metabolites, *Mutaiton Res.* 156, 163-169.

Goggelmann, W. (1981) Culture conditions and influence of the number of bacteria on the number of revertants, in A. Kappas (ed),

Progress in mutation research, 2, Elsevier, North Holland, pp.
173-178.

Goldin, B., Dwyer, J., Gorbach, S.L., Gordon, W. & Swenson, L.
(1978) Influence of diet and age of fecal bacterial enzymes, *Am. J. Clin. Nutr.* 31, 136-140.

Goldstein, R.S., Chism, J.P., Sherrill, J.M. & Hamm, T.E.
(1984) Influence of dietary pectin on intestinal microfloral
metabolism and toxicity of nitrobenzene, *Toxic. Appl. Pharm.* 75,
547-553.

Gough, T.A., Webb, K.S. & Swann, P.F. (1983) An examination of
human blood for the presence of volatile nitrosamines, *Fd. Chem. Toxic.* 21, 151-156.

Graham, S., Dayal, H., Swanson, M., Mittleman, A. & Wilkinson,
G. (1978) Diet in the epidemiology of cancer of the colon and
rectum, *J.N.C.I.* 61, 709-714.

Gram, T.E., Guarino, A.M., Schroeder, D.H. & Gillette, J.R.
(1969) Changes in certain kinetic properties of hepatic microsomal
aniline hydroxylase and ethylmorphine demethylase associated with
postnatal development and maturation in male rats, *Biochem. J.* 113,
681-685.

Grantham, P.H., Horton, R.E., Weisburger, E.K. & Weisburger,
J.H. (1970) Metabolism of the carcinogen N-2-fluorenylacamide in
germ-free and conventional rats, *Biochem. Pharm.* 19, 163-171.

Gray, G.E., Pike, M.C. & Henderson, B.E. (1979) Breast cancer
incidence and mortality rates in different countries in relation to
known risk factors and dietary practices, *Br. J. Cancer* 39, 1-7.

Green, M.H.L. & Muriel, W.J. (1976) Mutagen testing using
Trp⁺ reversion in *Escherichia coli*, *Mutation Res.* 38, 3-32.

Green, L.C., Ralt, D. & Tannenbaum, S.R. (1982) Nitrate,

nitrite and N-nitroso compounds: biochemistry, metabolism, toxicity and carcinogenicity, In, A. Neuberger & T.H. Jukes (eds) *Human Nutrition, Current Issues and Controversies*, MTP Press, pp. 87-140.

Gregus, Z., Varga, F. & Schmelas, A. (1985) Age-development and inducibility of hepatic glutathione S-transferase activities in mice, rats, rabbits and guinea-pigs, *Comp. Biochem. Physiol.* 80C, 85-90.

Grover, P.L. & Sims, P. (1964) Conjugations with glutathione. Distribution of glutathione S-aryltransferase in vertebrate species, *Biochem. J.* 90, 603-606.

Grubbs, C.J., Peckham, J.C. & Kato, K.D. (1983) Mammary carcinogenesis in rats in relation to age at time of N-nitroso-N-methylurea administration, *J.N.C.I.* 70, 209-212.

Guengerich, F.P. & Macdonald, T.L. (1984) Chemical mechanisms of catalysis by cytochrome P-450: a unified view, *Acct. Chem. Res.* 17, 9-16.

Habig, W.H., Pabst, J.J. & Jakoby, W.B. (1974) Glutathione S-transferases, the first enzymatic step in mercapturic acid formation, *J. Biol. Chem.* 249, 7130-7139.

Haenszel, W., Berg, J.W., Segi, M., Kurihara, M. & Locke, F.B. (1973) Large bowel cancer in Hawaiian Japanese, *J.N.C.I.* 51, 1765-1779.

Hard, G.C. (1979) Effect of age at treatment on incidence and type of renal neoplasm induced in the rat by a single dose of dimethylnitrosamine, *Cancer Res.* 39, 4965-4970.

Hartman, P.E. (1982) Nitrates and nitrites: ingestion, pharmacodynamics and toxicology, in F.J. de Serres and A. Hollaender (eds) *Chemical mutagens - principles and methods for their detection* Vol. 7 Plenum Press, New York pp. 211-294.

Hashimoto, Y. & Shudo, K. (1985) Chemical modification of DNA with muta-carcinogens, 1. 3-amino-1-methyl-5H-pyrido[4,3-b]indole and 2-amino-6-methyldipyrido[1,2-a:3',2'-d]imidazole: metabolic activation and structure of the DNA adducts, *Environ. Health Perspect.* 62, 209-214.

Hashimoto, S., Yokokura, T., Kawai, Y & Mutai, M (1976) Dimethylnitrosamine formation in the gastro-intestinal tract of rats, *Fd. Cosmet. Toxic.* 14, 553-556.

Hauser, R. & Matter, B.E. (1977) Localisation of *E.coli* K-12 in livers of mice used for intrasanguineous host-mediated assay, *Mutation Res.* 46, 45-48.

Hawksworth, G.M., Drasar, B.S. & Hill, M.J. (1971) Intestinal bacteria and the hydrolysis of glycosidic bonds, *J. Med. Microbiol.* 4, 451-459.

Hawksworth, G.M. & Hill, M.J. (1971) Bacteria and the N-nitrosation of secondary amines, *Br. J. Cancer* 25, 520-527.

Hawrylewicz, F.J., Huang, H.H. & Liu, J.M. (1986) Dietary protein, enhancement of N-nitrosomethylurea-induced mammary carcinogenesis, and their effect on hormone regulation in rats, *Cancer Res.* 46, 4395-4399.

Hayatsu, H., Kasai, H., Yokoyama, S., Miyazawa, T., Yamaizumi, Z, Sato, S., Nishimura, S., Arimoto, S., Hayatsu, T. & Ohara, Y. (1987) Mutagenic metabolites in the urine and feces of rats fed with 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline a carcinogenic mutagen present in cooked meat, *Cancer Res.* 47, 791-794.

Hayes, J.R., Mgbodile, M.U.K., Merrill, A.H. Jr., Nerurkar, L.S. & Campbell, T.C. (1978) The effect of dietary protein depletion and repletion on rat hepatic mixed function oxidase activities, *J. Nutr.* 108, 1788-1797.

Henderson, P.Th. (1978) Development and maturation of drug metabolizing enzymes, *Eur. J. Drug Metab. Pharm.* 3, 1-14.

Heneghan, J.B. (1988) Alimentary tract physiology, in, I.R. Rowland (ed) *Role of the gut flora in toxicity and cancer*, Academic Press, London pp. 39-77.

Hietanen, E. (1980) Modification of hepatic drug metabolizing enzyme activities and their induction by dietary protein, *Gen. Pharm.* 11, 443-450.

Hill, M.J., Drasar, B.S., Aries, V., Growther, J.S., Hawksworth, G. & Williams, R.E.O. (1971) Bacteria and aetiology of cancer of the large bowel, *Lancet* i, 95-100.

Hill, M.J., Drasar, B.S., Williams, R.E.O., Meade, T.W., Cox, A.G., Simpson, J.E.P. & Morson, B.C. (1975) Faecal bile-acids and clostridia in patients with cancer of the large bowel, *Lancet* i, 535-538.

Hill, M., MacLennan, R. & Newcombe, K. (1979) Diet and large bowel cancer in three socioeconomic groups in Hong Kong, *Lancet* 1, 436.

Hince, T.A. & Neale, S. (1977) Physiological modification of alkylating-agent induced mutagenesis. 1. Effect of growth rate and repair capacity on nitrosomethylurea-induced mutation of *Escherichia coli*, *Mutation Res.* 46, 1-10.

Hirayama, T. (1979) Diet and cancer, *Nutr. Cancer* 1, 67-81.

Holdeman, L.V. & Moore, W.E.C. (1975) *Anaerobe Laboratory Manual, 3rd. Ed.*, V.P.I. Anaerobe Laboratory, Blacksburg, Va.

Holloway, W.D., Tasman-Jones, C. & Maher, K. (1983) Pectin digestion in humans, *Am. J. Clin. Nutr.* 37, 253-255.

Hosaka, S., Matsushima, T., Hirono, I & Sugimura, T. (1981) Carcinogenic activity of 3-amino-1-methyl-5H-pyrido[4,3-b]indole

(Trp-P-2), a pyrolysis product of tryptophan, *Cancer Lett.* 13, 23-28.

Howe, G.M. (1986) International variations in cancer incidence and mortality, In, G.M. Howe (ed) *Global geocancerology, a world geography of human cancers*, Churchill Livingstone, pp. 3-44.

Howes, A.J. (1987) Ph.D thesis. *In vitro* and *in vivo* studies of mutagens found in cooked food. University of Surrey.

Howes, A.J., Rowland, I.R., Lake, B.G. & Alldrick, A.J. (1989) Effect of dietary fibre on the mutagenicity and distribution of 2-amino-3,4-dimethylimidazo[4,5-f]quinoline (MeIQ), *Mutation Res.* 210, 227-235.

Hsie, A.W., Machanoff, R., Couch, D.B., Holland, J.M. (1978) Mutagenicity of dimethylnitrosamine and ethylmethanesulphonate as determined by the host-mediated CHO/HGPRT assay, *Mutation Res.* 51, 77-84.

Hsieh, D.P.H. (1986) Biological reactive intermediates of mycotoxins, *Adv. Exp. Med. Biol.* 197, 597-610.

Hsieh, D.P.H. & Wong, J.J. (1982) Metabolism and toxicity of aflatoxins, *Adv. Exp. Med. Biol.* 136B, 847-863.

Huang, C.T.L., Levine, M.M., Woodward, W.E., Gopala Krishna, G.S., Nahn, D.R., Hornick, R.B. & Nicholas, B.L. (1981) Biohydrogenation of cholesterol as an index of bacterial 7- α -dehydroxylase activity, *Lipid.* 16, 670-677.

Huggins, C., Grand L.C. & Brillantes, F.P. (1961) Mammary cancer induction by a single feeding of polynuclear hydrocarbons and its suppression, *Nature* 189, 204-207.

IARC (1977) Dietary fibre, transit time, fecal bacteria, steroids and colon cancer in two Scandinavian populations, *Lancet* ii, 207-211.

I.C.N. Nutritional Biochemicals (1979) *Animal research diets*,
Cleveland, Ohio p.2.

Ioannides C., Lum, P.Y. & Parke, D.V. (1984) Cytochrome P448
and its activation of toxic chemicals and carcinogens, *Xenobiotica*
14, 119-137.

Ishii, K., Ando, M., Kamataki, T., Kato, R. & Nagao, M. (1980a)
Metabolic activation of mutagenic tryptophan pyrolysis products
(Trp-P-1 and Trp-P-2) by a purified cytochrome P-450-dependent
monooxygenase system, *Cancer Lett.* 9, 271-276.

Ishii, K., Maeda, K., Kamataki, T. & Kato, R. (1986) Mutagenic
activation of aflatoxin B₁ by several forms of purified cytochrome
P-450, *Mutation Res.* 174, 85-88.

Ishii, K., Yamazoe, Y., Kamataki, T. & Kato, R. (1980b)
Metabolic activation of mutagenic tryptophan pyrolysis products by
rat liver microsomes, *Cancer Lett.* 40, 2596-2600.

Jacobs, L.R. & Lupton, J.R. (1986) Relationship between
colonic luminal pH, cell proliferation and colon carcinogenesis in
1,2-dimethylhydrazine treated rats fed high fibre diets, *Cancer*
Res. 46, 1727-1734.

Jagerstad, M., Grivas, S., Olsson, K., Laser-Reutersward, A.,
Negishi, C. & Sato, S. (1986) Formation of food mutagens via
Maillard reactions, in I. Knudsen (ed), *Genetic toxicology of the*
diet, Alan R. Liss Inc., pp. 155-167.

Jain, M., Cook, G.M., Davis, F.G., Grace, M.G., Howe, G.R. &
Miller, A.B. (1980) A case-control study of diet and colo-rectal
cancer, *Int. J. Cancer* 26, 757-768.

James, S.P. & Pheasant, A.E. (1978) Glutathione conjugation
and mercapturic acid formation in the developing rat, *in vivo* and *in*
vitro, *Xenobiotica* 8, 207-217.

Jayaraj, A., Hardwick, J.P., Diller, T.W. & Richardson, A.G.
(1985) Metabolism, covalent binding and mutagenicity of aflatoxin
B₁ by liver extracts from rats of various ages, *J.N.C.I.* 74, 95-103.

Jensen, O.M. (1983) Cancer risk among Danish male Seventh-Day
Adventists and other temperance society members, *J.N.C.I.* 70,
1011-1014.

Jensen, O.M., MacLennan, R. & Wahrendorf, J. (1982) Diet,
bowel function, faecal characteristics and large bowel cancer in
Denmark and Finland, *Nutr. Cancer* 4, 5-19.

Kamataki, T., Maeda, K., Shimada, M., Kitani, K., Nagai, T. &
Kato, R. (1985) Age-related alteration in the activities of drug
metabolizing enzymes and contents of sex-specific forms of
cytochrome P-450 in liver microsomes from male and female rats, *J.*
Pharm. Exp. Ther. 233, 222-228.

Kappas, A., Anderson, K.E., Conney, A.H. & Alvares, A.P. (1976)
Influence of dietary protein and carbohydrate and antipyrone and
theophylline metabolism in man, *Clin. Pharm. Ther.* 20, 643-653.

Kari, F.W., Johnston, J.B., Truex, C.R. & Vissek, W.J. (1983)
Effect of dietary protein concentration on yield of mutagenic
metabolites from 1,2-dimethylhydrazine in mice, *Cancer Res.* 43,
3674-3679.

Kasai, H., Nishimura, S., Nagao, M., Takahashi, Y. & Sugimura,
T. (1979) Fractionation of a mutagenic principle from broiled fish
by high-pressure liquid chromatography, *Cancer Lett.* 7, 343-348.

Kasai, H., Nishimura, S., Wakabayashi, K., Nagao, M. &
Sugimura, T. (1980a) Chemical synthesis of 2-amino-3-methylimidazo
[4,5-f]quinoline (IQ), a potent mutagen isolated from broiled fish,
Proc. Japan Acad. 56B, 382-384.

Kasai, H., Yamaizumi, Z., Shiomi, T., Yokoyama, S., Niyazawa,

T., Wakabayashi, K., Nagao, M., Sugimura, T. & Nishimura, S. (1981) Structure of a potent mutagen isolated from fried beef, *Chem. Lett.* 12, 485-488.

Kasai, H., Yamaizumi, Z., Wakabayashi, K., Nagao, M., Sugimura, T., Yokoyama, S., Miyazama, T. & Nishimura, S. (1980b) Structure and chemical synthesis of MeIQ, a potent mutagen isolated from broiled fish, *Chem. Lett.* 11, 1391-1394.

Kato, R. (1986) Metabolic activation of mutagenic heterocyclic aromatic amines from protein pyrolysates, *CRC Crit. Rev. Toxicol.* 16, 307-348.

Katsouyanni, K., Trichopoulos, D., Boyle, P., Xiroushaki, E., Trichopoulou, A., Lisseas, B., Vasilaros, S. & MacMahon, B. (1986) Diet and breast cancer: a case-control study in Greece, *Int. J. Cancer* 38, 815-820.

Kaur, S. & Gill, S.S. (1985) Age-related changes in the activities of epoxide hydrolases in different tissues of mice, *Drug Metab. Disp.* 13, 711-715.

Kerklaan, P.R.M., Bouter, S., van Elburg, P.A. & Mohn, G.R. (1985) Evaluation of the DNA-repair host-mediated assay I. Induction of repairable DNA-damage in *E. coli* cells recovered from liver, spleen, lungs, kidneys and the blood stream of mice treated with methylating carcinogens, *Mutation Res.* 148, 1-12.

Ketterer, B. (1988) Protective role of glutathione and glutathione transferases in mutagenesis and carcinogenesis, *Mutation Res.* 202, 343-361.

Ketterer, B., Coles, B. & Meyer, D.J. (1983) The role of glutathione in detoxification, *Environ. Health Perspect.* 49, 59-69.

Kinlen, M.J. (1982) Meat and fat consumption and cancer mortality: a study of strict religious orders in Britain, *Lancet* i,

946-949.

Kinoshita, N. & Gelboin, H.V. (1978) β -glucuronidase catalysed hydrolysis of benzo(a)pyrene-3-glucuronide and binding to DNA, *Science* 199, 307-309.

Klinger, W. (1982) Biotransformation of drugs and other xenobiotics during postnatal development, *Pharm. Ther.* 16, 377-429.

Knasmuller, S., Szakmary, A. & Wottawa, A. (1989) Investigations on the use of EDTA-permeabilized *E. coli* cells in liquid suspension and animal-mediated genotoxicity assays, *Mutation Res.* 216, 189-196.

Knasmuller, S., Wottawa, A. & Fiedler, W. (1986) Distribution and retention of bacterial cells in various organs of rodents, *Proc. XVI EEMS Conf.*, Brussels, pp. 165-168.

Knasmuller, S., Zeilmaker, M., van der Gen, A., de Wit, P. & Mohn, G. (1988) Differential DNA repair effects in *E. coli* inoculated in intestinal organs of mice. Possible involvement of feed components and/or fecal mutagens, *Mutation Res.* 207, 89-97.

Koizumi, A., Weindruch, R. & Walford, R.L. (1987) Influences of dietary restriction and age on liver enzyme activities and lipid peroxidation in mice, *J. Nutr.* 117, 361-367.

Kosuge, T., Tsuji, K., Wakabayashi, K., Okamoto, T., Shudo, K., Iitaka, Y., Itai, A., Sugimura, T., Kawachi, T., Nagao, M., Yahagi, T. & Seino, Y. (1978) Isolation and structure studies of mutagenic principles in amino acid pyrolysates, *Chem. Pharm. Bull.* 26, 611-619.

Kowalski, B., Miller, C.T. & Sen, N.P. (1980) Studies on the *in vivo* formation of nitrosamines in rats and humans after ingestion of various meals. In, E.A. Walker, L. Grieciute, M. Castegnaro, & Borzsonyi, (eds.), *N-nitroso compounds: analysis, formation and*

occurrence, IARC, Lyon No. 31, pp. 609-617

Krishnamurthy, P.B. & Neelaram, G.S. (1986) Effect of dietary fat on aflatoxin B₁-induced chromosomal aberrations in mice, *Toxicol. Lett.* 31, 229-234.

Kritchevsky, D. (1986a) Diet, nutrition and cancer. The role of fibre, *Cancer* 58, 1830-1836.

Kritchevsky, D. (1986b) Fat, calories and fiber, In, C. Ip, D.F. Birt, A.E. Rogers & C. Mettlin (eds), *Dietary fat and cancer*, Alan R. Liss Inc. pp. 495-516.

Lafranconi, W.M., Glatt, H. & Oesch, F. (1986) Xenobiotic metabolizing enzymes of rat liver nonparenchymal cells, *Toxic. Appl. Pharm.*, 84, 500-511.

Lai, D.Y. & Arcos, J.C. (1980) Dialkylnitrosamine bioactivation and carcinogenesis, *Life Sci.* 27, 2149-2165.

Lake, B.G. (1987) Preparation and characterization of microsomal fractions for studies on xenobiotic metabolism, In *Biochemical Toxicology: A practical approach*, IRL Press, pp. 183-213.

Lakritz, L., Gates, R.A., Gugger, A.M. & Wasserman, A.E. (1982) Nitrosamine levels in human blood, urine and gastric aspirate following ingestion of foods containing potential nitrosamine precursors or preformed nitrosamines, *Fd. Chem. Toxic.* 22, 455-459.

Lang, M. (1976) Depression of drug metabolism in liver microsomes after treating rats with unsaturated fatty acids, *Gen. Pharm.* 7, 415-419.

Laqueur, G., McDaniel, E.G., & Matsumoto, H. (1967) Tumour induction in germ free rats with methylazoxymethanol and synthetic MAM acetate, *J.N.C.I.* 39, 355-371.

Larsen, G.L. (1988) Deconjugation of biliary metabolites by

microflora, β -glucuronidases, sulphatases and cysteine conjugate β -lyases and their subsequent enterohepatic circulation, In, I.R. Rowland, (ed) *Role of gut-flora in Toxicity and Cancer*, Academic, Press, London, pp. 79-108.

Lee, A. & Gemmell, E. (1972) Changes in the mouse intestinal microflora during weaning: role of volatile fatty acids, *Infection and Immunity* 5, 1-7.

Lee, S.Y. & Guttenplan, J.B. (1981) A correlation between mutagenic and carcinogenic potencies in a diverse group of N-nitrosamines determination of mutagenic activities of weakly mutagenic N-nitrosamines, *Carcinogenesis* 2, 1339-1344.

Leegwater, D.C., de Groot, A.P. & van Kaimthout-Kuyper, M. (1974) The aetiology of caecal enlargement in the rat, *Fd. Cosmet. Toxicol.* 12, 687-697.

Legator, M.S., Bueding, E., Batzinger, R., Connor, T.H., Eisenstadt, E., Farrow, M.G., Ficsor, G., Hsie, A., Seed, J. & Stafford, R.S. (1982) An evaluation of the host-mediated assay and body fluid analysis, A report of the U.S. Environmental Protection Agency Gene-Tox Program, *Mutation Res.* 98, 319-374.

Leive, L. (1965) A nonspecific increase in permeability in *Escherichia coli* produced by EDTA, *Proc. Natl. Acad. Sci.* 53, 745-750.

Leive, L. (1968) Studies on the permeability change produced in coliform bacteria by ethylenediaminetetraacetate, *J. Biol. Chem.* 243, 2373-2380.

Leonardo, J.M., Dornfeld, S.S. & Peak, M.J. (1984) Evaluation of *Escherichia coli* K-12 343/113 and derived strains for microbial mutagenicity assays, *Mutation Res.* 130, 87-95.

Lijinsky, W. & Ross, A.E. (1967) Production of carcinogenic

polynuclear hydrocarbons in the cooking of food, *Fd Cosmet. Toxic.* 5, 343-347.

Lijinsky, W. & Shubik, P. (1964) Benzo[a]pyrene and other hydrocarbons in charcoal-broiled meat, *Science* 145, 53-55.

Lijinsky, W., Taylor, H.W., Snyder, C. & Nettesheim (1973) Malignant tumours of liver and lung in rats fed aminopyrine and heptamethyleneimine together with nitrite, *Nature* 244, 176-178.

Lin, J.-K. & Lai, C.-C. (1982) Effect of *Lactobacillus*, antacids and antibiotics on the levels of nitrite in the gastro-intestinal tracts of rats fed sodium nitrate, *Fd. Chem. Toxicol.* 20, 197-204.

Lin, J.-K., Miller, J.A. & Miller, E.C. (1977) 2,3-Dihydro-2-(guan-7-yl)-3-hydroxy-aflatoxin B₁ - a major acid hydrolysis product of aflatoxin B₁-DNA or ribosomal RNA adducts formed in hepatic microsome-mediated reactions and in rat liver *in vivo*, *Cancer Res.* 37, 4430-4438.

Litterst, C.L., Mimnaugh, E.G., Reagan, R.C. & Gram, T.E. (1975) Comparison of *in vitro* drug metabolism by lung, liver and kidney of several common laboratory species, *Drug Metab. Disp.* 3, 259-265.

Longnecker, D.S., Roebuck, D.B., Yager Jr., J.D., Lilja, H.S. & Siegmund, B. (1981) Pancreatic carcinoma in azaserine-treated rats: induction, classification and dietary modulation of incidence, *Cancer* 47, 1562-1572.

Loretz, L.J. & Pariza, M.W. (1984) Effect of glutathione levels, sulphate levels and metabolic inhibitors on covalent binding of IQ and AAF to cell macromolecules in primary monolayer cultures of adult rat hepatocytes, *Carcinogenesis* 5, 895-899.

Lotlikar, P.D., Insetta, S.M., Lyons, P.R. & Jhee, E-C. (1980)

Inhibition of microsome-mediated binding of aflatoxin B₁ to DNA by glutathione S-transferase, *Cancer Lett.* 9, 143-149.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall R.J. (1951) Protein measurement with the folin phenol reagent, *J. Biol. Chem.* 93, 265-275.

Lu, A.I.H., Strobel, H.W. & Coon, M.J. (1969) Hydroxylation of benzphetamine and other drugs by a solubilized form of cytochromes P-450 from liver microsomes: lipid requirement for drug demethylation, *Biochem. Biophys. Res. Commun.* 36, 545-551.

Lyon, J.L. & Sorenson, A.W. (1978) Colon cancer in a low-risk population, *Am. J. Clin. Nutr.* 31, 5227-5230.

MacLennan, R., Jensen, O.M., Mosbech, J. & Vuori, H. (1978) Diet, transit time, stool weight and colon cancer in two Scandinavian populations, *Am. J. Clin. Nutr.* 31, S239-S242.

Madhavan, T.V. & Gopalan, (1968) The effect of dietary protein on carcinogenesis of aflatoxin, *Arch. Pathol.* 85, 133-137.

Magee, P.N. & Barnes, J.M. (1967) Carcinogenic nitroso compounds, *Adv. Cancer Res.* 10, 163-246.

Mainigi, K.D. & Campbell, T.C. (1981) Effects of low dietary protein and dietary aflatoxin on hepatic glutathione levels in F-344 rats, *Toxic. Appl. Pharm.* 59, 196-203.

Mallett, A.K. (1982) Effect of dietary pectin on the metabolic activity of the rat hindgut microflora, *Chemistry and Industry* 18, 984-987.

Mallett, A.K. & Rowland, I.R. (1988) Factors affecting the gut microflora, In, I.R Rowland, (ed) *Role of the gut flora in toxicity and cancer*, Academic Press, London, pp. 347-382.

Mallett, A.K., Rowland, I.R. & Wise, A. (1982) Diet-related production of mutagens by the rat gut flora, *Toxicologist* 2, 146.

Mallett, A.K., Rowland, I.R. & Wise, A. (1983) Interaction between pectin and rat hindgut microflora, *Appl. Environ. Microbiol.* 45, 116-121.

Mallett, A.K., Rowland, I.R. and Wise, A. (1984) Dietary fat and cecal microbial activity in the rat, *Nutr. Cancer* 6, 86-91.

Malling, H.V. (1966) Mutagenicity of two potent carcinogens, dimethylnitrosamine and diethylnitrosamine in *Neurospora crassa*, *Mutation Res.* 3, 537-540.

Mannering, G.J. (1985) Drug metabolism in the newborn, *Fed. Proc.* 44, 2302-2308.

Maron, D.M. & Ames, B.N. (1983) Revised methods for the *Salmonella* mutagenicity test, *Mutation Res.* 113, 173-215.

Martin, C.W., Fjermestad, J. Smith-Barbarro, P. & Reddy, B.S. (1980), Dietary modification of mixed-function oxidases, *Nutr. Rep. Int.* 22, 395-407.

Mata, L.J. & Urrutia, J.J. (1976) Intestinal colonization of breast-fed children in a rural area of low socioeconomic level, *Anal. N.Y. Acad. Sci.* 176, 93-109.

Matsukura, N., Kawachi, T., Morino, K., Oghaki, H & Sugimura, T. (1981) Carcinogenicity in mice of mutagenic compounds from a tryptophan pyrolysate, *Science* 213, 346-347.

McCann, J., Spingarn, N.E., Kobori, J. & Ames, B.N. (1975) Detection of carcinogens as mutagens: bacterial tester strains with R factor plasmids, *Proc. Natl. Acad. Sci.* 72, 979-983.

McGregor, D.B. (1980) The place of the host-mediated assay, *Arch. Toxicol.* 46, 111-121.

McKeown-Eyssen, G.E. & Bright-See, E. (1984) Dietary factors in colon cancer: international relationships, *Nutr. Cancer* 6, 160-170.

Mettlin, C. (1984) Diet and the epidemiology of human breast cancer, *Cancer* 53, 605-611.

Meydani, M. (1987) Dietary effects on detoxification processes, In, J.N. Hathcock (ed), *Nutritional Toxicology II*, Academic Press, London, pp. 1-41.

Miller, J.A. & Miller, E.C. (1979) Perspectives on the metabolism of chemical carcinogens, in: P. Emmelot, P. & E. Kriek, (eds.) *Environmental carcinogenesis: occurrence, risk evaluation and mechanisms*, Elsevier, North Holland, pp. 25-50.

Miller, J.A., Miner, D.L., Rusch, H.P. & Baumann, C.A. (1941) Diet and hepatic tumour formation, *Cancer Res.* 1, 699-708.

Mitchell, I.deG. & Gilbert, P.J. (1984) The effect of pretreatment of *Escherichia coli* CM891 with ethylenediaminetetraacetate on the sensitivity of a variety of standard mutagens, *Mutation Res.* 140, 13-19.

Mizutani, T. & Mitsuoka, T. (1979) Effect of intestinal bacteria on incidence of liver tumors in gnotobiotic C3H/He male mice, *J.N.C.I.* 63, 1365-1370.

Mizutani, T. & Mitsuoka, T. (1980) Effect of intestinal bacteria on incidence of liver tumors in gnotobiotic C3H/He male mice, *Cancer Lett.* 11, 89-95.

Modan, B., Barel, V., Lubin, F., Modan, M., Greenberg, R.A. & Graham, S. (1975) Low-fibre intake as an etiologic factor in cancer of the colon, *J.N.C.I.* 55, 15-18.

Mohn, G. (1973) Revertants of an *Escherichia coli* K-12 strain with high sensitivity to radiations and chemicals, *Mutation Res.* 19, 349-355.

Mohn, G.R. (1981) Bacterial systems for carcinogenicity testing, ICPEMC. working paper 2/7, *Mutation Res.* 87, 191-210.

Mohn, G.R. (1984) The DNA repair host-mediated assay as a rapid and sensitive *in vivo* procedure for the determination of genotoxic factors present in various organs of mice. Some preliminary results with mitomycin C, *Arch. Toxicol.* 55, 268-271.

Mohn, G., Kerklaan, P. & Ellenberger, J. (1984) Methodologies for the direct and animal-mediated determination of various genetic effects in derivatives of strain 343/113 of *Escherichia coli* K-12, In B.J. Kilbey, M. Legator, W. Nichols & C. Ramel (eds.), *Handbook of Mutagenicity Test Procedures*, Elsevier, 2nd ed., pp. 189-214.

Mohn, G.R., Kerklaan, P., de Knijff, P. & Bouter, S. (1981) Influence of phenotypic expression lag and division delay on apparent frequencies of induced mutations in *Escherichia coli* K-12, *Mutation Res.* 91, 419-425.

Mohn, G.R., Maier, P. & Burki, K. (1977) The use of bacteriophages as indicators in host-mediated assays. Longevity of T4 in the blood-stream of mice, *Mutation Res.* 48, 367-370.

Mohn, G.R. & van Zeeland, A.A. (1985) Quantitative comparative mutagenesis in bacteria, mammalian cells and animal-mediated assays. A convenient way of estimating genotoxic activity *in vivo*, *Mutation Res.* 150, 159-175.

Montesano, R. & Bartsch, H. (1976) Mutagenic and carcinogenic N-nitroso compounds: possible environmental hazards, *Mutation Res.* 32, 179-228.

Moreau, C (1979) *Moulds, toxins and food*, M. Moss (ed), John Wiley & Sons p.130.

Morishita, Y. & Miyaki, K. (1979) Effects of age and starvation on the gastrointestinal microflora and the heat resistance of fecal bacteria in rats, *Micro. Immun.* 23, 455-470.

Morotomi, M., Nanno, M., Watanabe, T., Sakurai, T. & Mutai, M.

(1985), Mutagenic activation of biliary metabolites of 1-nitropyrene by intestinal microflora, *Mutation Res.* 149, 171-178.

Morris, H.P., Westfall, B.B., Dubnik, C.S. & Dunn, T.B. (1948) Some observations on carcinogenicity, distribution and metabolism of N-acetyl-2-aminofluorene in the rat, *Cancer Res.* 8, 390.

Moss, E.J. & Neal, G.E. (1985) The metabolism of aflatoxin B₁ by human liver, *Biochem. Pharm.* 34, 3193-3197.

Mucklow, J.C., Rawlins, M.D., Brodie, M.J., Boobis, A.R. & Dollery, C.T. (1980) Drug oxidation in Asian vegetarians, *Lancet* 2, 151.

Mysliwy, T.S., Wick, E.L., Archer, M.C., Shank, R.C. & Newberne, P.M. (1974) Formation of N-nitrosopyrrolidine in a dog's stomach, *Br. J. Cancer* 30, 279-283.

Nagao, M., Fujita, Y., Wakabayashi, K & Sugimura, T. (1983) Ultimate forms of mutagenic and carcinogenic heterocyclic amines produced by pyrolysis, *Biochem. Biophys. Res. Commun.* 114, 626-630.

Nagao, M., Honda, M., Seino, Y., Yahagi, T., Kawachi, T. & Sugimura, T. (1977) Mutagenicities of protein pyrolysates, *Cancer Lett.* 2, 335-340.

Nagasawa, H., Yanai, R. & Taniguchi, H. (1976) Importance of mammary gland DNA synthesis on carcinogen-induced mammary tumorigenesis in rats, *Cancer Res.* 36, 2223-2226.

National Academy of Sciences (1982) *Diet, nutrition and cancer*, National Academy of Sciences, National Research Council, Washington DC.

National Research Council (1978) *Nutrient requirements of laboratory animals*, 3rd ed., Natl. Acad. Sci., Washington DC, p 7.

Neal, G.E. & Godoy, H.M. (1976) The effects of pretreatment with phenobarbitone on the activation of aflatoxin B₁ by rat liver,

Chem. Biol. Interac. 14, 279-289.

Neal, G.E., Judah, D.J., Stirpe, F. & Patterson, D.S.P. (1981)

The formation of 2,3-dihydroxy-2,3-dihydroaflatoxin B₁ by the metabolism of aflatoxin B₁ by liver microsomes isolated from certain avian and mammalian species and the possible role of this metabolite in the acute toxicity of aflatoxin B₁, *Toxic. Appl. Pharm.* 58, 431-437.

Neims, A.H., Warner, M., Loughnan, P.M. & Aranda, J.V. (1976)

Developmental aspects of the hepatic cytochrome P450 monooxygenase system, *Ann. Rev. Pharm. Toxic.* 16, 427-445.

Neale, S. & Solt, A.K. (1981) The effect of ascorbic acid on the amine-nitrite and nitrosamine mutagenicity in bacteria injected into mice, *Chem. Biol. Interac.* 35, 199-205.

Nemoto, N., Kusumi, S., Takayama, S., Nagao, M. & Sugimura, T.

(1979) Metabolic activation of 3-amino-5H-pyrido[4,3-b]indole, a highly mutagenic principle in tryptophan pyrolysate, by rat liver enzymes, *Chem. Biol. Interac.* 27, 191-198.

Newberne, P.M., Hunt, C.E. & Wogan, G.N. (1967) Neoplasms in

the rat associated with administration of urethan and aflatoxin, *Exp. Mol. Path.* 6, 285-299.

Newberne, P.M. & Butler, W.H. (1969) Acute and chronic effects

of aflatoxin B₁ on the liver of domestic and laboratory animals: a review, *Cancer Res.* 29, 236-250.

Newberne, P.M. & Rogers, A.E. (1973) Rat colon carcinomas

associated with aflatoxin and marginal vitamin A, *J.N.C.I.* 50, 439-448.

Newberne, P.M., Weigert, J. & Kula, N. (1979) Effects of

dietary fat on hepatic mixed-function oxidases and hepatocellular carcinoma induced by aflatoxin B₁ in rats, *Cancer Res.* 39,

3986-3991.

Nigro, N.D., Bull, A.W., Klopfer, B.A., Pak, M.S. & Campbell, R.L. (1979) Effect of dietary fibre on azoxymethane-induced intestinal carcinogenesis in rats, *J.N.C.I.* 62, 1097-1102.

Norred, W.P. & Marzuki, A. (1984) Effects of dietary saturated or polyunsaturated fat on hepatic glutathione S-transferase activity, *Drug Nutr. Interac.* 3, 11-20.

Nutter, R.L., Gridley, D.S., Kettering, J.D., Goude, A.G. & Slater, J.M. (1983) BALB/c mice fed milk or beef protein: differences in response to 1,2-dimethylhydrazine carcinogenesis, *J.N.C.I.* 71, 867-874.

O'Brien, K., Moss, E., Judah, D. & Neal, G. (1983) Metabolic basis of the species difference to aflatoxin B₁ induced hepatotoxicity, *Biochem. Biophys. Res. Commun.* 114, 813-821.

Oesch, F., Lafranconi, M. & Glatt, H. (1986) Role of parenchymal versus non-parenchymal cells in the control of biologically reactive intermediates, *Adv. Exp. Med. Biol.* 197, 53-61.

Okamoto, T., Shudo, K., Hashimoto, Y., Kosuge, T., Sugimura, T. & Nishimura, S. (1981) Identification of a reactive metabolite of the mutagen 2-amino-3-methylimidazo[4,5-f]quinoline, *Chem. Pharm. Bull.* 29, 590-593.

Omura, T. & Sato, R. (1964) The carbon monoxide-binding pigment of liver microsomes, *J. Biol. Chem.* 239, 2379-2385.

Parke, D.V. & Gray, T.J.B. (1978) A comparative study of the enzymic and morphological changes of livers of rats fed butylated hydroxytoluene, safrole, ponceau Mx or 2-acetamidofluorene, In B.H. Remmer, H.M. Bolt, R. Bannasch & H. Popper (eds), *Primary Liver Tumours*, MTP Press, Lancaster, pp. 335-346.

Parke, D.V., & Ioannides, C. (1982) Role of mixed-function oxidases in the formation of biological reactive intermediates, *Adv. Exp. Med. Biol.* 136A, 23-38.

Patterson, D.S.P. (1973) Metabolism as a factor in determining the toxic action of aflatoxins in different animal species, *Fd. Cosmet. Toxic.* 11, 287-294.

Peers, F.G., Gilman, G.A. & Linsell C.A. (1976) Dietary aflatoxins and human liver cancer. A study in Swaziland, *Int. J. Cancer* 17, 167-176.

Peers, F.G. & Linsell, C.A. (1973) Dietary aflatoxins and human liver cancer - a population study based in Kenya, *Br. J. Cancer* 27, 473-484.

Pegg, A.E. (1980) Metabolism of dimethylnitrosamine, In R. Montesano, H. Bartsch & L. Tamatis (eds), *Molecular and cellular aspects of carcinogen screening tests*, IARC, Lyon pp. 3-22.

Pegg, A.E. & Perry, W. (1981) Alkylation of nucleic acids and metabolism of small doses of dimethylnitrosamine in the rat, *Cancer Res.* 41, 3128-3132.

Peto, R., Gray, R., Brantom, P. & Grasso, P. (1984) Nitrosamine carcinogenesis in 5120 rodents: Chronic administration of sixteen different concentrations of NDEA, NDMA, NPYR and NPIP in the water of 4440 inbred rats, with parallel studies on NDEA alone of the effect of age starting (3, 6 or 20 weeks) and of species (rats, mice or hamsters), In I.K. O'Neill, R.C. Von Borstel, C.T. Miller, J. Long & H. Bartsch (eds) *N-nitroso-compounds: occurrence, biological effects and relevance to human cancer*, IARC, Lyon No. 57, pp. 627-665.

Phillips, R.L., (1975) Role of life-style and dietary habits in risks of cancer among Seventh-Day Adventists, *Cancer Res.* 35,

3513-3522.

Powrie, W.D., Wu, C.H., Rosin, H.P. & Stitch, H.F. (1981) Clastogenic and mutagenic activities of Maillard reaction model systems, *J. Food Sci.* 46, 1433-1438.

Preston, R.S., Hayes, J.R. & Campbell, T.C. (1976) The effect of protein deficiency on the *in vivo* binding of aflatoxin B₁ to rat liver macromolecules, *Life Sci.* 19, 1191-1198.

Pueyo, C., Frezza, D. & Smith, B. (1979) Evaluation of three metabolic activation systems by a forward mutation assay in *Salmonella*, *Mutation Res.* 64, 183-194.

Purchase, I.F.H. & Goncalves T. (1971) Preliminary results from food analyses in the Inhambane area, In I.F.H. Purchase (ed), *Mycotoxins in human health*, pp.263-269.

Raineri, R., Andrews, A. & Polley, J. (1986) Effect of donor age on the levels of rat, hamster and human liver S9 preparations in the *Salmonella* mutagenicity assay, *J. Appl. Toxic.* 6, 101-108.

Reddy, B.S. (1986) Fecal mutagens as a function of diet, In I. Knudsen (ed) *Genetic Toxicology of the Diet*, Alan R. Liss Inc., pp. 213-224.

Reddy, B.S., Cohen, L.A., McCoy, G.D., Hill, P., Weisburger, J.H. & Wynder, E.L. (1980) Nutrition and its relationship to cancer, *Adv. Cancer Res.* 32, 237-345.

Reddy, B.S., Hedges, A.R., Laakso, K. & Wynder, E.L. (1978) Metabolic epidemiology of large bowel cancer: fecal bulk and constituents of high-risk North American and low-risk Finnish population, *Cancer* 42, 2832-2838.

Reddy, B.S. & Maeura, Y. (1984) Tumour production by dietary fat in azoxymethane-induced colon carcinogenesis in female F-344 rats: influence of amount and source of dietary fat, *J.N.C.I.* 72,

745-750.

Reddy, B.S., Maeura, Y. & Wayman, M. (1983) Effect of dietary corn bran and autohydrolysed lignin on 3,2'-dimethyl-4-aminobiphenyl-induced intestinal carcinogenesis in male F344 rats, *J.N.C.I.* 71, 419-423.

Reddy, B.S., Narisawa, T., Maronpot, R., Weisburger, J. & Wynder, E.L. (1975) Animal models for the study of dietary factors and cancer of the large bowel, *Cancer Res.* 35, 3421-3426.

Reddy, B.S., Narisawa, T. & Weisburger, J.H. (1976) Effect of a diet with high levels of protein and fat on colon carcinogenesis in F344 rats treated with 1,2-dimethylhydrazine, *J.N.C.I.* 57, 567-569.

Reddy, B.S., Watanabe, K. & Weisburger, J.H. (1977) Effect of high fat diet on colon carcinogenesis in F344 rats treated with 1,2-dimethylhydrazine, *Cancer Res.* 37, 4156-4159.

Reddy, B.S., Weisburger, J.H., Narisawa, T. & Wynder, E.L. (1974) Colon carcinogenesis in germ-free rats with 1,2-dimethylhydrazine and N-methyl-N-nitro-N-nitrosoguanidine, *Cancer Res.* 34, 2368-2372.

Reddy, B.S. & Wynder, E.L. (1973) Large-bowel carcinogenesis: fecal constituents of populations with diverse incidence rates of colon cancer, *J.N.C.I.* 50, 1437-1442.

Reddy, B.S. & Wynder, E.L. (1977) Metabolic epidemiology of colon cancer. Fecal bile acids and neutral sterols in colon cancer, *Mutation Res.* 72, 511-522.

Reed, D.J. & Beatty, P.W. (1980) Biosynthesis and regulation of glutathione: toxicological implications, In E. Hodgson, J.R. Bend, R.M. Philpot (eds) *Reviews in Biochemical Toxicology* 2, Elsevier, pp. 213-241.

Renwick, A.G. & Drasar, B.S. (1976) Environmental carcinogens and large bowel cancer, *Nature* 263, 234-235.

Reynolds, E.S. (1963) The use of lead citrate at high pH as an electron-opaque stain in electron microscopy, *J. Cell Biol.* 17, 208-212.

Rexova-Benkova, L. & Markovic, O. (1976) Pectic enzymes, *Adv. carbohydr. chem. biochem.* 33, 323-397.

Rickert, D.E., Long, R.M., Krakowka, S. & Dent, J.G. (1981) Metabolism and excretion of 2,4-[¹⁴C] dinitrotoluene in conventional and axenic Fischer-344 rats, *Toxic. Appl. Pharm.* 59, 574-579.

Robertson, I.G.C. & Birnbaum, L.S. (1982) Age-related changes in mutagen activation by rat tissues, *Chem. Biol. Interac.* 38, 243-252.

Roe, F.J.C. & Grant, G.A. (1970) Inhibition by germ-free status of development of liver and lung tumours in mice exposed neonatally to 7,12-dimethylbenzanthracene: implications in relation to tests for carcinogenicity, *Int. J. Cancer* 6, 133-144.

Rose, D.P., Boyar, A.P. & Wynder, E.L. (1986) International comparisons of mortality rates for cancer of the breast, ovary, prostate and colon and *per capita* food consumption, *Cancer* 58, 2363-2371.

Ross, M.H. & Bras, G. (1973) Influence of protein under- and over-nutrition on spontaneous tumour prevalence in the rat, *J. Nutr.* 103, 944-963.

Ross, J.K. & Leklem, J.E. (1981) The effect of dietary citrus pectin on the excretion of human fecal neutral and acid steroids and the activity of 7- α -dehydroxylase and β -glucuronidase, *Am. J. Clin. Nutr.* 34, 2068-2077.

Rowland, I.R., Mallett, A.K., Cole, C.B. & Fuller, R. (1987)

Mutagen activation by hepatic fractions from conventional, germ-free and monoassociated rats, *Arch. Toxicol. (Suppl.)* 11, 261-263.

Rowland, I.R., Mallett, A.K. & Wise, A. (1983a) A comparison of the activity of five microbial enzymes in caecal contents from rats, mice, hamsters, and response to dietary pectin, *Toxic. Appl. Pharm.* 69, 143-148.

Rowland, I.R., Mallett, A.K. & Wise, A. (1985) The effect of diet on the mammalian gut flora and its metabolic activities, In *CRC Crit. Rev. Toxicol.* 16, 31-103.

Rowland, I.R., Mallett, A.K., Wise, A. & Bailey, E. (1983c) Effect of dietary carrageenan and pectin on the reduction of nitro-compounds by the rat caecal microflora, *Xenobiotica* 13, 251-256.

Rowland, I.R., Robinson, R.D., Doherty, R.A. & Landry, T.D. (1983b) Are developmental changes in methylmercury metabolism and excretion mediated by the intestinal microflora?, In T.W. Clarkson, G.F. Nordberg & P.R. Sager (eds) *Reproductive and Developmental Toxicity of Metals*, Plenum Press pp. 745-758.

Rowland, I.R. & Walker, R. (1983) The gastrointestinal tract in food toxicology, In D.M. Conning & A.B.G. Lansdown (eds), *Toxic Hazards in Food*, Croom Helm, London and Canberra, pp. 183-274.

Rozen, P., Hellerstein, S.M. & Horwitz, C. (1981) The low incidence of colorectal cancer in a "high risk" population: its correlation with dietary habits, *Cancer* 48, 2692-2695.

Ryan, T.A., Joiner, B. & Ryan, B.F. (1976) *Minitab Students Handbook*, Duxbury Press, Boston.

Sachan, D.S. (1975) Effects of low and high protein diets on the induction of microsomal drug metabolizing enzymes in rat liver, *J. Nutr.* 105, 1631-1639.

Saito, K., Shinohara, A., Kamataki, T. & Kato, R. (1985)
Metabolic activation of mutagenic N-hydroxyarylamines by
O-acetyltransferase in *Salmonella typhimurium* TA98, *Arch. Biochem.
Biophys.* 239, 286-289.

Saito, K., Yamazoe, Y., Kamataki, T. & Kato, R. (1984)
Glutathione transferase-mediated and non-enzymatic activation and
detoxification of the N-hydroxy derivative of Trp-P-2, a potent
pyrolysate promutagen, *Xenobiotica* 14, 545-548.

Sargentini, N.J. & Smith, K.C. (1985) Spontaneous mutagenesis:
the roles of DNA repair, replication and recombination, *Mutation
Res.* 154, 1-27.

Sato, S., Negishi, C., Umemoto, A. & Sugimura, T. (1986)
Metabolic aspects of pyrolysis mutagens in food, *Environ. Health
Perspect.* 67, 105-109.

Savage, D.C. (1977) Microbial ecology of the gastrointestinal
tract, *Ann. Rev. Microbiol.* 31, 107-133.

Savage, D.C. (1981) The microbial flora in the
gastrointestinal tract, In *Nutrition in Health and Diseases and
International Development: Symposia from the XII International
Congress of Nutrition*, Alan R. Liss, Inc. pp. 893-908.

Schaedler, R.W., Dubos, R. & Costello, R. (1965) The
development of the bacterial flora in the gastrointestinal tract of
mice, *J. Exp. Med.* 122, 59-66.

Scheline, R.R. (1973) Metabolism of foreign compounds by
gastro-intestinal microorganisms, *Pharm. Rev.* 25, 451-523.

Shay, H., Gruenstein, M. & Shimkin, M.B. (1964) Effect of
casein, lactalbumin and ovalbumin on 3-methylcholanthrene-induced
mammary carcinoma in rats, *J.N.C.I.* 33, 243-253.

Shiau, S.-Y. & Chang, G.W. (1983) Effects of dietary fiber on

fecal mucinase and β -glucuronidase activity in rats, *J. Nutr.* 113, 138-144.

Shibamoto, T. (1982) Occurrence of mutagenic products in browning model systems, *Food Technol.* 36, 59-62.

Shinozuka, H., Katyal, S.L. & Perera, M.I.R. (1986) Fat, lipotropes, lypolipidemic agents and liver cancer, In, C. Ip, D.f. Birt, A.E. Rogers & C. Mettlin (eds) *Dietary fat and cancer*, Alan R. Liss Inc. pp. 461-486.

Shimada, T., Nakamura, S.-I., Imaoka, S. & Funae, Y. (1987) Genotoxic and mutagenic activation of aflatoxin B₁ by constitutive forms of cytochrome P-450 in rat liver microsomes, *Toxic. Appl. Pharm.* 91, 13-21.

Short, C.R., Kinden, D.A. & Stith, R. (1976) Fetal and neonatal development of the microsomal monooxygenase system, *Drug Metab. Rev.* 5, 1-42.

Siegel, J.S. (1981) Demographic background for international gerontological studies, *J. Gerontol.* 36, 93-102.

Silverstone, H. & Tannenbaum, A. (1951) Proportion of dietary protein and the formation of spontaneous hepatomas in the mouse, *Cancer Res.* 11, 442-446.

Singletary, K.W., Milner, J.A. & Martin, S.E. (1984) Effect of dietary protein concentration on rat liver S9 bioactivation of 7,12-dimethylbenz(a)anthracene in the *Salmonella*/microsome assay, *Mutation Res.* 126, 19-24.

Sinha, K. & Dao, T.L. (1980) Induction of mammary tumours in ageing rats by 7,12-dimethylbenz(a)anthracene: role of DNA synthesis during carcinogenesis, *J.N.C.I.* 64, 519-521.

Snedecor, G.W. & Cochran, W.G. (1967) *Statistical methods*, 6th edition, Iowa State University Press.

Sjodin, P. & Jagerstad, M. (1984) A balance study of ¹⁴C-labelled 3H-imidazo[4,5-f]quinoline-2-amines (IQ and MeIQ) in rats, *Fd. Chem. Toxic.* 22, 207-210.

Smith, A.H., Pearce, N.E. & Joseph, J.G. (1985) Major colorectal cancer aetiological hypotheses do not explain mortality trends among Maori and non-Maori New Zealanders, *Int. J. Epidemiol.* 14, 79-85.

Solt, A.K. & Neale, S. (1979) Induction of bacterial mutants in rodents treated with N-nitroso compounds, *Mutation Res.* 64, 147.

Solt, A.K. & Neale, S. (1980) Natulan, a bacterial mutagen requiring complex mammalian metabolic activation, *Mutation Res.* 70, 167-171.

Spearman, M.E., Liebman, K.C. (1984) Effects of aging on hepatic and pulmonary glutathione-S-transferase activities in male and female Fischer 344 rats, *Biochem. Pharm.* 33, 1309-1313.

Spiegelhalder, B., Eisenbrand, G. & Preussmann, R. (1976) Influence of dietary nitrate on nitrite content of human saliva: possible relevance to *in vivo* formation of N-nitroso compounds, *Fd. Cosmet. Toxic.* 14, 545-548.

Spiller, G.A., Chernoff, M.C., Hill, R.A., Gates, J.E., Nassar, J.J. & Shipley, E.A. (1980) Effect of purified cellulose, pectin and a low-residue diet on fecal volatile fatty acids, transit time, and fecal weight in humans, *Am. J. Clin. Nutr.* 33, 754-759.

Spingarn, N. & Garvie, C. (1979) Formation of mutagens in sugar-ammonia model systems, *J. Agric. Food Chem.* 27, 1319-1321.

Stemmerman, G.N., Nomura, A.M.Y. & Heilbrun, L.K. (1984) Dietary fat and the risk of colorectal cancer, *Cancer Res.* 44, 4633-4637.

Stenback, F. & Arranto, A. (1985) Initiation and promotion in

young and old animals. Implications for human tumour formation, In A. Likhacher, V. Anisimov & R. Montesano (eds), *Age and related factors in carcinogenesis*, IARC Lyon pp. 151-166.

Stohs, S.J., Al-turk, W.A., Angle, C.R. (1982) Glutathione S-transferase and glutathione reductase activities in hepatic and extrahepatic tissues of female mice as a function of age, *Biochem. Pharm.* 31, 2113-2116.

Stout, D.L., Baptist, J.N., Matney, T.S. & Shaw, C.R. (1976) N-hydroxy-2-aminofluorene: the principle mutagen produced from N-hydroxy-2-acetylaminofluorene by a mammalian enzymic preparation supernatant, *Cancer Lett.* 1, 269-274

Sugimura, T. (1982) Mutagens, carcinogens and tumor promoters in our daily food, *Cancer* 49, 1970-1984.

Sugimura, T. (1985) Carcinogenicity of mutagenic heterocyclic amines formed during the cooking process, *Mutation Res.* 150, 33-41.

Sugimura, T. (1986) Past, present and future of mutagens in cooked foods, *Environ. Health Perspect.* 67, 5-10.

Sugimura, T. (1986) Studies on environmental chemical carcinogenesis in Japan, *Science* 233, 312-318.

Sugimura, T., Kawachi, T., Nagao, M., Yahagi, T., Seino, Y., Okamoto, T., Shudo, K., Kosuge, T., Tsuji, K., Wakabayashi, K., Iitaka, Y. & Itai, A. (1977) Mutagenic principles in tryptophan and phenylalanine pyrolysis products, *Proc. Japan Acad.* 53, 58-61.

Swenson, D.H., Miller, J.A. & Miller, E.C. (1975) The reactivity and carcinogenicity of aflatoxin B₁-2,3-dichloride, a model for the putative 2,3-oxide of aflatoxin B₁, *Cancer Res.* 35, 3811-3823.

Swenson, D.H., Lin, J-K, Miller, E.C. & Miller, J.A. (1977) Aflatoxin B₁-2,3-oxide as a probable intermediate in the covalent

binding of aflatoxins B₁ and B₂ to rat liver DNA and ribosomal RNA *in vivo*, *Cancer Res.* 37, 172-181.

Takayama, S., Nakatsuru, Y., Masuda, M., Ohgaki, H., Sato, S. & Sugimura, T. (1984) Demonstration of carcinogenicity in F344 rats of 2-amino-3-methyl-imidazo[4,5-f]quinoline from broiled sardine, fried beef and beef extract, *Gann* 75, 467-470.

Takayama, S., Nakatsuru, Y., Ohgaki, H., Sato, S. & Sugimura, T. (1985) Carcinogenicity in rats of a mutagenic compound, 3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole, from tryptophan pyrolysate, *Gann* 76, 815-817.

Tannenbaum, A. (1942) The genesis and growth of tumors III. Effects of a high fat diet, *Cancer Res.* 2, 468-475.

Tannenbaum, A. & Silverstone, H. (1949) The genesis and growth of tumors. IV. Effects of varying the proportion of protein (casein) in the diet, *Cancer Res.* 5, 616-625.

Taylor, H.W. & Lijinsky, W. (1975) Tumor induction in rats by feeding aminopyrine or oxytetracycline with nitrite, *Int. J. Cancer* 16, 211-215.

Temcharoen, P., Anukarahanonta, T. & Bhamarapravati, N. (1978) Influence of dietary protein and vitamin B₁₂ on the toxicity and carcinogenicity of aflatoxins in rat liver, *Cancer Res.* 38, 2185-2190.

Tipping, E. & Ketterer, B. (1981) The influence of soluble binding proteins on lipophile transport and metabolism in hepatocytes, *Biochem J.* 195, 441-444.

Tomkins, A.M., Bradley, A.K., Oswald, S. & Drasar, B.S. (1981) Diet and the faecal microflora of infants, children and adults in rural Nigeria and urban UK, *J. Hyg. Camb.* 86, 285-293.

Topping, D.C. & Visek, W.J. (1976) Nitrogen intake and

tumorigenesis in rats injected with 1,2-dimethylhydrazine, *J. Nutr.* 106, 1583-1590.

Turesky, R.J., Skipper, P.L., Tannenbaum, S.R., Coles, B. & Ketterer, B. (1986) Sulfamate formation is a major route for detoxification of 2-amino-3-methylimidazo[4,5-f]quinoline in the rat, *Carcinogenesis* 7, 1483-1485.

Umbenhauer, D.R. & Pegg, A.E. (1981) Alkylation of intracellular and extracellular DNA by dimethylnitrosamine following activation by isolated rat hepatocytes, *Cancer Res.* 41, 3471-3474.

Vahouny, G.V. & Kay, R. (1981) Working group III: Lipid metabolites and intestinal flora, *Cancer Res.* 41, 3755-3756.

Vesell, E.S. (1984) Commentary: Complex effects of diet on drug desposition, *Clin. Pharm. Ther.* 36, 285-296.

Vesselinovitch, S.D., Koda, M., Mihailovich, N. & Rao, K.V.N. (1984) Carcinogenicity of diethylnitrosamine in newborn, infant and adult mice, *J. Cancer Res. Clin. Oncol.* 108, 60-65.

Vesselinovitch, S.D., Rao, K.V.N., Mihailovich, N. & Rice, J.M. (1974) Development of broad spectrum of tumors by ethylnitrosourea in mice; modifying role of age, sex and strain, *Cancer Res.* 34, 2530-2538.

Vince, A., Down, P.F., Murison, J., Twig, F.J. and Wrong, O.M. (1976) Generation of ammonia from non-urea sources in a fecal incubation system, *Clin. Sci. Molec. Med.* 51 313.

Wade, A.E., Norred, W.P. & Evans, J.S. (1978) Lipids in drug detoxification. In J.N. Hathcock & J. Coon (eds) *Nutrition and Drug Interactions*, Academic Press, New York pp. 475-503.

Wade, A.E., White, R.A., Walton, L.C. & Bellows J.T. (1985) Dietary fat - a requirement for induction of mixed function oxidase activities in starved-refed rats, *Biochem. Pharm.* 34, 3747-3754.

Ward, F.W., Coates, M.E. & Walker, R. (1986) Nitrate reduction, gastro-intestinal pH and N-nitrosation in gnotobiotic and conventional rats, *Fd. Chem. Toxic.* 24, 17-22.

Ward, J.M., Yamamoto, R.S. & Weisburger, J.H. (1973) Cellulose, dietary bulk and azoxymethane-induced intestinal cancer, *J.N.C.I.* 51, 713-715.

Watanabe, J., Kawajira, K., Yonekawa, H., Nagao, M. & Tagashira, Y. (1982) Immunological analysis of the roles of two major types of cytochrome P-450 in mutagenesis of compounds isolated from pyrolysates, *Biochem. Biophys. Res. Commun.* 104, 193-199.

Watanabe, J., Reddy, B.S., Weisburger, J.H. & Kritchevsky, D. (1979) Effect of dietary alfalfa, pectin and wheatbran on azoxymethane- or methylnitrosourea-induced colon carcinogenesis in F344 rats, *J.N.C.I.* 63, 141-145.

Weisburger, J.H. (1971) Colon carcinogens: their metabolism and mode of action, *Cancer* 28, 60-70.

Weisburger, J.H. (1986) Nutrition and cancer prevention: gastrointestinal cancer, In K. Inokuchi, G.P. Murphy, H. Sugano, T. Sugimura & U. Veronesi (eds), *Gann monograph on cancer research* 31. Digestive tract tumors: fundamental and clinical aspects, Plenum Press, New York, pp. 265-274.

Weiser, R., Ascher, A.W. & Wimpenny, J. (1968) *In vitro* reversal of antibiotic resistance by ethylenediamine tetracetic acid, *Nature* 219, 1365-1366.

Wells, P., Aftergood, L. & Alfin-Slater, R.B. (1976) Effect of varying levels of dietary protein on tumor development and lipid metabolism in rats exposed to aflatoxin, *J. Am. Oil Chem. Soc.* 53, 559-562.

Wheeler, L.A., Soderberg, F.B. & Goldman, P. (1975) The

reduction of N-hydroxy-4-acetylamino-biphenyl by intestinal microflora of the rat, *Cancer Res.* 35, 2962-2968.

Whong, W.-Z., & Ong, T.-M. (1979) Metabolic activations of dimethylnitrosamine by male and female mice and rats to metabolites mutagenic in *Neurospora carassa*, *Mutation Res.* 61, 393-398.

Williams, J.R., Grantham, P.H., Marsh, H.H., Weisburger, J.H. & Weisburger, E.K. (1970) Participation of liver fractions and of intestinal bacteria in the metabolism of N-hydroxy-N-2-fluorenylacetylamide in the rat, *Biochem. Pharm.* 19, 173-188.

Williams, C.H. & Kamin, H.J. (1962) Microsomal triphosphopyridine nucleotide-cytochrome c reductase in liver, *J. Biol. Chem.* 237, 587-595.

Wilson, R.B., Hutcheson, D.P. & Wideman, L. (1977) Dimethylhydrazine-induced colon tumours in rats fed diets containing beef-fat or corn oil with and without wheatbran, *Am. J. Clin. Nutr.* 30, 176-181.

Wise, A. (1990) Diet formulation and experimental design for laboratory animal studies, in I.R. Rowland (ed) Nutrition, Toxicity and Cancer, Telford Press, New Jersey (in press).

Wise, A., Mallett, A.K. & Rowland, I.R. (1982) Dietary fibre, bacterial metabolism and toxicity of nitrate in the rat, *Xenobiotica* 12, 111-118.

Wise, A., Mallett, A.K. & Rowland, I.R. (1983) Dietary protein and cecal microbial metabolism in the rat, *Nutr. Cancer* 4, 267-272.

Wise, A., Mallett, A.K. & Rowland, I.R. (1986) Effect of mixtures of dietary fibres on the enzyme activity of the rat caecal microflora, *Toxic.* 38, 241-248.

Witter, J.P., Balish, E. & Gatley, S.J. (1979) Distribution of nitrogen-13 from labelled nitrate and nitrite in germfree and

conventional flora rats, *Appl. Environ. Microbiol.* 38, 870-874.

Wostmann, B.S. (1984) Other organs, In M.E. Coates & B.E. Gusstafsson (eds) *The germ-free animal in biomedical research*, Laboratory animals, London, pp. 215-231.

Wynder, E.L. & Gori, G.B. (1977) Contribution of the environment to cancer incidence: an epidemiological exercise, *J.N.C.I.* 58, 825-832.

Yahagi, T., Nagao, M., Seino, Y., Matsushima, T., Sugimura, T. & Okada, M. (1977) Mutagenicities of N-nitrosamines on *Salmonella*, *Mutation Res.* 48, 121-130.

Yamaiguchi, K., Shudo, K., Okamoto, T., Sugimura, T & Kosuge, T. (1980) Presence of 3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole in broiled beef, *Gann* 71, 745-746.

Yamaizumi, Z., Shiomi, T., Kasai, H., Nishimura, S., Takahashi, Y., Nagao, M. & Sugimura, T. (1980) Detection of potent mutagens, Trp-P-1 and Trp-P-2, in broiled fish, *Cancer Lett.* 9, 75-83.

Yamazoe, Y., Shimada, M., Kamataki, T. & Kato, R. (1983) Microsomal activation of 2-amino-3-methylimidazo[4,5-f]quinoline, a pyrolysate of sardine and beef extracts, to a mutagenic intermediate, *Cancer Res.* 43, 5768-5774.

Yang, C.S. & Yoo, J-S.H. (1988) Dietary effects on drug metabolism by the mixed-function oxidase system, *Pharm. Ther.* 38, 53-72.

Yoshizawa, H., Uchimar, R., Kamataki, T., Kato, R. & Ueno, Y. (1982) Metabolism and activation of aflatoxin B₁ by reconstituted cytochrome P-450 system of rat liver, *Cancer Res.* 42, 1120-1124.

Zeiger, E. (1975) Dietary modifications affecting the mutagenicity of N-nitroso compounds in the host-mediated assay, *Cancer Res.* 35, 1813-1818.