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THE EFFECTS OF HIGH FAT AND HIGH CHOLESTEROL
DIETS ON THE DISPOSITION OF BENZO(a)PYRENE
IN THE GUT

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ABSTRACT

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THE EFFECTS OF HIGH FAT AND HIGH CHOLESTEROL DIETS
ON THE DISPOSITION OF BENZO(a)PYRENE
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The possible interactions of high dietary levels of fat (HF) or fat + cholesterol (HC) with the lipid-soluble carcinogen benzo(a)pyrene (BP) have been investigated in the male guinea pig.

Bile was the major route of elimination of [^{14}C]BP (25μCi i.v.) in normal animals, with a mean of 33% $\pm$ a standard deviation of 13% of the dose excreted within 4h. Animals fed HF or HC diets showed a similar biliary elimination (27$\pm$13% and 37$\pm$15% respectively). However, the pattern of biliary metabolites was altered, such that a greater proportion of the recovered dose was present as BP dihydrodiols and their conjugates in HF and HC groups. The most marked increase was found in the 4,5-dihydrodiol glucuronide fraction, which increased from 2.4% of the ^{14}C in bile to 13.5% and 11.6% in HF and HC groups respectively ($p < 0.001$). The 7,8-dihydrodiol increased from 4% to 5% or 6% respectively, but this was not statistically significant. The 9,10-dihydrodiol increased from 3% in normal guinea pigs to 5% and 6% in HF and HC groups respectively ($p < 0.01$).

Incubation of a number of BP metabolites with pure cultures of intestinal bacteria or guinea pig caecal contents resulted in hydrolysis of conjugated metabolites, particularly glucuronides, but primary oxidative metabolites of BP were stable to degradation.

Cytochrome P450 and aryl hydrocarbon hydroxylase (AHH) were present in liver and small intestinal mucosa from normal animals, but were undetectable in colon and rectum. The administration of HF and HC diets, and/or repeated doses of BP (3mg/kg p.o. twice weekly for 3 weeks prior to sacrifice), did not increase these levels in colon and rectum.

The administration of [^{3}H]BP (2-4mCi p.o.) resulted in low levels of ^{3}H binding to DNA of the gut mucosa in normal animals, much of which could be accounted for by tritium exchange. The feeding of HF and HC diets did not increase this binding.

Therefore the administration of HF and HC diets increased the metabolism of BP via the dihydrodiol pathways. Conjugates of these metabolites are hydrolysed by intestinal bacteria, and the aglycones appear stable to further degradation. However, due to the low and apparently non-inducible levels of AHH in guinea pig colon and rectum, there was no observed increase in DNA binding in these tissues.

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ACKNOWLEDGEMENTS

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Chapter 1

INTRODUCTION

1.1 Epidemiology of colon cancer

Cancer of the colon accounts for 5% of all deaths in the United Kingdom, and 20% of the mortality rate from cancer (Holmes, 1982). The disease occurs with far greater frequency in North America, New Zealand, and Western Europe than in countries such as Africa, Asia, and South America (Doll, 1969). During the past 20 years, a number of studies have shown that these geographical differences originate in the environment rather than the genetics of the populations studied.

Evidence for an environmental aetiology of large bowel cancer is partly provided by the study of migrant populations. It is not possible to carry out planned experiments on the aetiology of cancer in man, but migrants have provided an excellent opportunity to examine the relative contributions of environmental and genetic factors in the causation of a number of diseases. The majority of researchers in this field have studied migrants to the United States, particularly from Japan, but other data are also available.

Buell and Dunn (1965) compared cancer mortality rates of Japanese migrants to California with those for native Caucasian Californians, and Japanese living in Japan. The Japanese migrants were divided into Issei (those who were born in Japan), and Nisei, (descendants of Issei, born in California). They found that the mortality rates from colon cancer in both male and female Issei and Nisei were greater than those for Japanese living in Japan, and approached the higher mortality rate of the Californians. Haenszel and Kurihara (1968) also observed that colon cancer incidence rates of Issei and Nisei, particularly males, were almost as high as those for native United States whites. Japanese migrants in Hawaii also display an elevated risk of developing large bowel cancer than those Japanese

remaining in their native country (Stemmermann, 1970), and within Japan itself a case-control study revealed a greater risk of the disease among individuals who have adopted a western life-style and diet (Wynder et al., 1969).

Staszewski and Haenszel (1965) reported increased mortality from large bowel cancer in Polish immigrants living in the United States, and Indians moving to Kenya were shown to have a higher incidence of colon cancer than either Indians remaining in India, or indigenous Africans (Chopra et al., 1975). Within the group of Kenyan Asians, the occurrence of the disease was particularly high in Gujarati males, despite the fact that the non-migrating Gujaratis have a very low risk. Both Kenyan Africans and Indian Gujaratis live in rural communities, whereas the more privileged Asian immigrants in Kenya tend to live in urban areas, and consume a more western diet. All these results point to environmental rather than genetic factors in the aetiology of colon cancer.

Haenszel (1961) studied mortality data for foreign-born whites living in America, their countries of birth being Ireland, USSR, Czechoslovakia, Canada, England and Wales, Germany, Italy, Sweden, Austria, Poland, Norway or Mexico. The standard colon cancer mortality rates in each of these 12 groups differed little from each other, or from that of native U.S. whites, despite the lower mortality rates found in their countries of origin. This implies that environmental factors were altering the risk of the disease to that prevailing in the United States.

Incidence has also been found to vary with national origin (Mass and Modan, 1969). A study of colorectal cancer in Israel revealed the incidence to be significantly higher among European-born Jews than among those born in Asia or Africa, again indicating the importance of

cultural and nutritional habits rather than genetic factors in the aetiology. Possible environmental factors which have been implicated are the typical western diet, and increased pollution in developed countries.

Wynder and Shigematsu (1967) reported a positive correlation between the incidence of colon cancer and a high intake of dietary fat. Subsequent epidemiological studies have revealed similarly high correlations (Drasar and Irving, 1973; Wynder and Reddy, 1975; Jain et al., 1980). Intake of animal protein has also been observed to be greater in high risk than in low risk areas (Armstrong and Doll, 1975; Gregor et al., 1969; Haenszel et al., 1973; Howell, 1975). However, animal protein and fat are not independent variables, since fat constitutes more than 30% of beef, and 35-45% of our dietary fat intake is estimated to come from meat (Wynder and Reddy, 1975).

Other reports have concerned the fibre and/or the refined carbohydrate content of the diet (Burkitt, 1971; Bingham et al., 1979; Cummings et al., 1978; Modan et al., 1975; Burkitt, 1971a; Burkitt, 1978). Fibre is reported to be negatively correlated with the incidence of colon cancer, that is, a high fibre diet appears to protect against the disease. This finding will be discussed later.

Studies of different socioeconomic and religious groups within a country have also pointed to a dietary component in the aetiology of large bowel cancer. In high risk countries such as North America and Western Europe, no socioeconomic risk gradient has been detected (Cohart and Muller, 1955; Graham et al., 1960). However, differences have been observed in low risk countries such as Columbia. The incidence of colon cancer in Cali, Columbia, is one fifth of that in the United States (Correa and Llanos, 1966; Haenszel et al., 1975), but within Cali the upper socioeconomic classes show a 4-fold excess risk

of developing large bowel cancer (Haenszel et al., 1975). The meat

consumption in Cali differs greatly between the socioeconomic classes;

the lack of a socioeconomic gradient for development of colon cancer in

the United States may be due to the minimal differences in dietary

intake of fat, protein and carbohydrate within the more developed

country.

However, within developed countries certain religious groups

follow strict dietary regimes, which enables comparison of dietary

differences in a relatively small geographical area. Paymaster et al.

(1968) reported that differences in incidence of colon cancer among

religious groups in India could be accounted for on the basis of diet,

the incidence being lowest in Hindus from Gujarat, who adhere to a

strict vegetarian diet. Wynder and Shigematsu (1967) have reported that

Seventh-Day Adventists, who eat little meat, have a lower incidence of

colorectal cancer than the national average. In 1974, Phillips reported

that the cancer mortality rate among Adventists aged 35 and over was

59% of the rate for all Californians; a later study of colon cancer

mortality rates among Seventh-Day Adventists and non-Seventh-Day

Adventists again suggested that the low rate in the former group was

related to a protective effect of their lacto-ovo-vegetarian diet

(Phillips, 1975).

Apart from dietary differences between populations exhibiting

varying risks of developing colon cancer, it has been noted in several

studies that urban populations generally have higher risks for the

disease than rural populations (Levin et al., 1960; Haenszel and Dawson, 1965;

Mass and Modan, 1969). Haenszel and Dawson (1965) noted an increase in

mortality from colon cancer with migration from a rural to an urban

domicile. In urban air, the concentration of environmental pollutants

such as polycyclic aromatic hydrocarbons (PAHs) is far greater than in

rural areas. For example, the concentration of a typical PAH, benzo(a)-pyrene, has been estimated to be between 0.1 and 0.5 ng/m^3 in rural air, whereas in an industrial environment it may be as much as 150 times higher (cited in Lee et al., 1981). PAHs are not solely atmospheric pollutants, however. They also contaminate soil, waterways, the oceans, and are present in the food chain. They are generated by both natural and anthropogenic sources, the latter being by far the major contributor. The occurrence of these environmental pollutants will be discussed in detail in a later section.

1.2 Diseases predisposing to large bowel cancer

A number of other diseases, common in Western cultures but comparatively rare in countries such as Asia and Africa where the incidence of large bowel cancer is also uncommon, were discussed by Burkitt (1973). These included non-infective diseases of the large intestine, namely appendicitis, diverticular disease, benign tumours and ulcerative colitis. All of these diseases may be correlated geographically and chronologically, and in some individual patients, with the adoption of a western life-style. Although the geographical distribution and chronological increase in prevalence of these typically western diseases is not proof of a common cause, it does strongly imply that the adoption of western life-style plays a major part in their aetiology.

Of these diseases, many reports have shown chronic ulcerative colitis to predispose to colon cancer, and other workers have indicated the precancerous nature of familial polyposis and adenomatous polyps.

(a) <u>Familial polyposis</u>

Hill (1975), in his review on the aetiology of colon cancer, has cited reports dating back to the late nineteenth century which link polyposis with colorectal cancer. Familial polyposis is an hereditary disease, characterised by the development of numerous (hundreds, or even thousands) of adenomatous polyps from the mucosa of the colon and rectum. The condition is generally diagnosed at an average age of 25 years by the presence of more than 100 polyps (Morson, 1974), with colorectal cancer detected on average approximately 15 years later. Only a very small percentage of the polyps become cancerous – perhaps only one polyp. Lipkin (1974) has described the proliferative changes leading to the development of cancer in colonic epithelial cells, involving the initial inability to repress DNA synthesis, followed by development of abnormal kinetics of cell proliferation with resultant accumulation of cells in the mucosa, and continuation of enhanced DNA synthesis. These proliferative lesions are essentially those occurring in familial polyposis (Lipkin, 1974).

(b) <u>Ulcerative colitis</u>

The first reports of cancer of the large bowel occurring with higher frequency in patients with ulcerative colitis than in the general population date back to the 1920's (cited in Hill, 1975; Morson, 1966). Estimates of the increased risk factor have varied; references cited by MacDougall (1964) have suggested figures of 5 to 30 times the risk prevailing in the general population. MacDougall reported a 30-fold increased risk of developing colorectal cancer among ulcerative colitis patients when compared with healthy controls, but

only in those individuals in whom most of the colon was inflamed with colitis. Those patients in whom the disease was confined to the distal colon or rectum were found to be at no higher risk of developing large bowel cancer than were the controls. Hinton (1966) also concluded that total or extensive involvement of the colon in ulcerative colitis was required before an increased risk of large bowel cancer was observed. Despite varying reports of the value of the increased risk factor, there is agreement in the literature that extensive inflammation of the large bowel in ulcerative colitis leads to changes in the epithelial surface which predispose to colorectal cancer. However, the number of ulcerative colitis patients developing cancer is relatively small compared to those with adenomatous polyps.

(c) Adenomatous polyps

In 1974, Morson discussed the evolution of colorectal cancer from the three main predisposing diseases. He reported isolated polyps to be more common than either familial polyposis or chronic ulcerative colitis in this respect. Furthermore, he stated the opinion that only those polyps that were classified as adenomatous polyps or villous adenomas were precancerous. As there is a discrepancy between the prevalence of these polyps and that of large bowel cancer, the former being more widespread than the latter, not all adenomatous polyps and villous adenomas become cancerous. The risk of developing cancer appears to depend upon the size of the polyp; approximately 50% of those over 2cm in diameter become cancerous, but very few which are less than 1cm do so. The polyp-cancer sequence appears to average about 10 years, ranging from 5 to more than 15 years (Morson, 1974). Fenoglio and Lane (1974) confirmed Morson's opinion, reporting that there was no

evidence to support the theory that cancerous cells arose de novo from normal colonic epithelial cells, and that "early" invasive cancer arose from adenomas. The absence of benign material from carcinomas is not evidence of de novo origin of the tumour; Morson has shown that the presence of benign material is inversely related to the stage of development of the tumour, and therefore total absence of such tissue may be due to its destruction by the malignant component of an advanced carcinoma.

In 1978, Hill et al. proposed an hypothesis to explain the development of large bowel cancer. They postulated that an environmental factor initially causes the development of adenomas in the epithelial cells of the large intestine, and that a further such agent promotes the growth of small adenomas. The final event in the sequence is the action of a carcinogen, which causes a high percentage of large adenomas to become malignant. They also reported that a genetic component has been postulated in the aetiology of adenomas, and that some evidence in support has been published. A recessive gene has been proposed; individuals homozygous for this gene (pp) would thus be adenoma-prone. If this is so, then there must presumably be as many adenoma-prone individuals in migrant populations as there are in persons remaining in their native country. The difference in the incidence of colon cancer noted between migrants in a high risk country and individuals from the same genetic stock remaining in a low risk country can therefore be explained by differences in the environmental factors which allow the gene to be expressed; these agents must be more prevalent in the high risk areas, and high enough to allow production of adenomas in all susceptible people in these countries. Hill et al. (1978) also state that the agent causing adenoma development, and that causing malignant change in large adenomas are different entities.

Evidence for this is cited; within populations showing similarly low incidence of colorectal cancer, the adenoma incidence is very variable. Furthermore, adenomas and carcinomas exhibit different sub-site distribution within the large bowel.

The information which has been amassed so far regarding the geographical distribution of colon cancer, dietary differences between religious and socioeconomic groups at different risk for the disease, and case-control studies, points to the involvement of environmental aetiological factors, particularly a high fat, low fibre diet, and environmental pollutants. This is backed up by studies of migrant populations. Individuals moving from a low risk country such as Japan, to one at high risk such as the United States develop an increased risk for colon cancer, approaching that of the adopted domicile within one generation. This trend contrasts sharply with that observed for stomach cancer, where the rate prevailing in the native country of an individual continues to be displayed after migration to a country with a different risk for the disease. A largely genetic control is therefore implicated for stomach cancer, whereas this would seem to play a very minor role in colon cancer aetiology. However, individuals suffering from the hereditary disease familial polyposis show an increased risk of developing colon cancer, and a genetic component has been postulated to be involved in the aetiology of adenomatous polyps, again a predisposing disease for large bowel carcinoma. In this case, it has also been proposed that at least two environmental agents are required, one for expression of the gene, the other for malignant transformation of the resulting adenoma. The implicated dietary and pollution factors will now be considered.

1.3 Influence of diet

Since there seems little doubt that dietary factors are involved in the production of colon cancer, a number of hypotheses have been proposed to explain the possible mechanisms by which certain food constituents may either predispose towards or, in the case of fibre, protect against the disease.

(a) Dietary fat

To explain the relationship between dietary fat and colon cancer, it has been proposed that:

(a) the quantity of fat consumed determines both the concentration of acid and neutral sterols secreted in the bile, and also the composition of the intestinal microflora which use these sterols as substrates, and

(b) the gut flora metabolise these acid and neutral sterols to carcinogens or co-carcinogens which act locally within the colon (Aries et al., 1969; Hill et al., 1971; Wynder and Reddy, 1977).

Animal studies have corroborated epidemiological data regarding the involvement of dietary fat in the aetiology of colon cancer. Reddy et al. (1976) studied the effect of both the quantity and type of dietary fat on dimethylhydrazine (DMH)-induced colon carcinogenesis in rats which had been exposed to a particular dietary regime for two generations prior to administration of the carcinogen. Diets used contained 5% or 20% lard, or 5% or 20% corn oil with a control group fed Purina chow. The animals fed fat at the 20% level were more susceptible to DMH-induced colon tumours than the other groups, but there was no major difference between the 20% lard and 20% corn oil diets. At the 5% level, however, corn oil produced more DMH-induced

colon tumours than lard. Reddy et al. (1976a) have also shown that a diet containing 40% beef protein and 20% beef fat, or 40% soybean protein and 20% corn oil resulted in a greater number of DMH-induced tumours in rats than diets containing normal quantities of protein and fat (20% and 6% respectively). They further reported that the incidence of colon tumours in Fischer rats treated with methylazoxymethanol (MAM) acetate intraperitoneally, DMH subcutaneously, or N-methyl-N-nitroso-urea (MNU) intrarectally, was greater when the rats were fed a diet containing 20% beef fat rather than 5% beef fat (Reddy et al., 1977). Rogers and Newberne (1973) reported that a high fat diet increased DMH-induced colon carcinogenesis in rats which were lipotrope-deficient, and Reddy and Ohmori (1981) showed that the incidence of 3,2'-dimethyl-4-amino-biphenyl (DMAB)-induced colon tumours was higher in conventional than in germ-free rats, and significantly higher in conventional rats fed high fat when compared to conventional rats fed low fat diet. Consumption of a high fat diet (20% lard or corn oil) increased faecal excretion of neutral sterols and bile acids which is consistent with the co-carcinogen hypothesis initially proposed by Aries et al. (1969). Excretion of deoxycholic, lithocholic and 12-keto-lithocholic acid was elevated in rats fed high fat diet (Reddy et al., 1977a).

(b) Bile acids

A number of workers have examined the potential carcinogenicity of certain bile acids; their overall structure is similar to that of carcinogenic PAH compounds, they may be chemically converted to the potent carcinogen 3-methylcholanthrene (Haddow, 1958), and full aromatisation of the bile acid nucleus would produce a carcinogenic

metabolite based on cyclopentaphenanthrene (Coombs et al., 1973). These reactions are shown in Figures 1.1 and 1.2.

Deoxycholic acid itself was shown by Cook et al. (1940) to be weakly carcinogenic in mice when administered subcutaneously in sesame oil, and also by skin-painting. These results were confirmed by Salaman and Roe (1956). Deoxycholic acid has also been shown to be mutagenic in bacteria (Jensen et al., 1951).

Animal studies have indicated a promoting effect of bile acids in colon carcinogenesis. Reddy et al. (1977b) gave intrarectal doses of sodium cholate or sodium chenodeoxycholate to germ-free and conventional F344 rats which had previously received intrarectal N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). Conventional rats treated with MNNG plus either bile acid had a higher incidence ($p < 0.05$) of colon tumours than did those given MNNG alone; an increase was also noted in germ-free animals, but this was not statistically significant. No tumours were evident in the colons of either germ-free or conventional rats given sodium cholate or chenodeoxycholate alone, and it was therefore concluded that these bile acids had a promoting effect in MNNG-induced colon carcinogenesis in rats. Lithocholic and tauro-deoxycholic acids were also found to act as promoters of MNNG-induced colon carcinogenesis in rats (Narisawa et al., 1974), as was deoxy-cholic acid (Reddy et al., 1976b). Reddy and Watanabe (1979) similarly tested the promoting effect of sodium lithocholate, cholesterol, cholesterol-5$\propto$,6$\propto$-epoxide (cholesterol epoxide) and cholestane-3β,5$\mathsf{\chi}$,6β -triol (triol) in female Fischer rats after administration of intra-rectal MNNG. None of these substances when given without MNNG produced colon tumours in either conventional or germ-free rats, but sodium lithocholate increased MNNG-induced colon tumour incidence in both groups. Cholesterol, cholesterol epoxide and triol were found to be

without promotional activity in this model. Neither were the microbial products of these three sterols effective as promoters, as evidenced by the similarity between tumour incidence in germ-free and conventional rats. This information is summarised in Table 1.1.

The concentration of secondary bile acids (deoxycholic, litho-cholic, and other microbially modified bile acids) in bile increases in patients after removal of the gall bladder (Pomare and Heaton, 1973; Hepner et al., 1974). Since these acids possess promotional activity in experimental colon cancer in rats, Linos et al. (1981) tested the hypothesis that cholecystectomy predisposes to colon cancer. They followed up over 1,500 cholecystectomy patients, and found that a greater number developed colon cancer than expected. The difference was significant in female patients, particularly for right-sided carcinoma. Similar findings were reported by Vernick and Kuller (1981).

(c) Cholesterol

In 1978, Cruse et al. tested the ability of dietary cholesterol to promote DMH-induced colon cancer in the rat. Addition of cholesterol to basic diet significantly reduced both the time to tumour presentation and the survival of the group, and they therefore concluded that dietary cholesterol facilitates the development, growth and spread of DMH-induced colon cancer in rats. They went on to propose the hypothesis that dietary cholesterol is co-carcinogenic for colon cancer in man (Cruse et al., 1979). Support for this hypothesis has been provided by Liu et al. (1979), who analysed mortality data from 20 industrialised countries for 1967-1973, and food disappearance data for 1954-1965; the partial correlation of dietary cholesterol with colon cancer remained highly significant when fat or fibre was controlled,

and suggests the involvement of dietary cholesterol in the aetiology of colon cancer.

(d) Intestinal bacteria

(i) Differences in flora between high and low risk populations

Since intestinal bacteria are also implicated in the aetiology of colon cancer, the interaction of diet and gut flora have been investigated in a number of different ways. The nature of the microbial flora, the concentration of bile acids and cholesterol metabolites, and mutagenicity have been evaluated in human faecal samples from populations at high and low risk of developing carcinoma of the large bowel.

In 1969, Aries et al. compared the faecal flora of an English population (at high risk for colon cancer) with that of a low risk, Ugandan population. They found no qualitative differences in the strains of bacteria present in the two populations, but there were significant quantitative differences. Bacteroides and bifidobacteria, non-sporing anaerobes, predominated in both groups, but there were 30 times more Bacteroides in English than in Ugandan faeces. Conversely, Ugandans had significantly greater quantities of Streptococci, enterococci, lactobacilli and yeasts in their faeces than did the high risk subjects. Clostridia, enterobacteria, veillonellae and filamentous fungi showed no difference between the two populations. English and Ugandan strains of the same bacteria were equally active in degrading bile salts; Bacteroides and Clostridia were the most active species in both deconjugation and further degradation of bile salts. The higher number of Bacteroides in English specimens presumably accounts for the observation that bile salts in English faeces are more degraded than

those in Ugandan faeces.

Drasar et al. (1976) isolated and identified strains of Clostridia from faeces from subjects in Britain, U.S.A., Hong Kong, Uganda and Japan; these workers found Clostridium paraputrificum to be more common in samples from the high risk countries, namely Britain and the U.S.A., but rare in those from the low risk subjects. This observation was related to the bile acid/co-carcinogen hypothesis proposed by Aries et al. (1969). In 1971, Aries et al. reported the ability of a strain of Clostridium paraputrificum to dehydrogenate the C1-C2 and C4-C5 bonds of a steroid nucleus. Goddard and Hill (1972) also found that a strain of C. paraputrificum isolated from human faeces was capable of introducing double bonds into a steroid ring in vitro, using 3-oxocholanic acid (see Figure 1.2). The ability of these nuclear dehydrogenating clostridia (NDC) to carry out such reactions is particularly relevant to the hypothesis.

Hill et al. (1971) determined the pattern of faecal bacteria in subjects from England, Scotland and the U.S.A., and compared the results with those from Uganada, South India and Japan. Subjects from the high risk countries again had more Bacteroides in their faeces, and fewer enterococci and other aerobic bacteria; their faeces also contained a higher concentration of steroids than the low risk subjects, and these steroids were degraded to a greater extent in the high risk groups. The non-sporing anaerobes isolated were classified in more detail in a later paper (Peach et al., 1974). Maier et al. (1974) studied the effect of feeding a high beef diet on the faecal flora of male volunteers. Their preliminary results, for five subjects only, indicated an increase in Bacteroides during the period on the high protein diet. Finegold et al. (1974) examined the faecal flora of two groups of Japanese Americans, one group consuming a traditional

Japanese diet, the other a typically western diet. They found significantly higher counts of Streptococcus faecalis in the faeces of the group consuming the Japanese diet. These results do not agree with those of Hill et al. (1971), who reported that 64% of the enterococci isolated from English faeces were S. faecalis, whereas this species comprised only 22% of the enterococci isolated from faeces of Indians, a low risk group. Conversely, the most common enterococcus (65% of the total) in Indian faeces was S. faecium, but this accounted for only 33% of enterococci in English subjects; since the total quantity of enterococci was 30-fold greater in Indian than in English samples, the actual difference in the total count of S. faecium was therefore 60-fold. Finegold et al. (1974) did find an increase, although not a statistically significant increase, in Bacteroides counts in the faeces of Japanese subjects on the western diet, and higher counts of certain facultative or aerobic bacteria in the subjects on the Japanese diet.

In 1975, Finegold et al. carried out a case-control study in which they characterised the faecal bacteria of colonic polyp patients, a high risk group for development of colon cancer, and those of a matched control group. They found certain statistically significant differences between the cases and the controls, although these did not correspond with the differences obtained in their study of the Japanese Americans on Japanese or western diets. They suggested that some of the differences observed in the case-control study may have occurred by chance. Aries et al. (1971a) studied two groups of British subjects, one consuming a mixed western diet, the other a strict vegetarian diet with no animal products at all. They found anaerobic sarcinae in faeces of the vegetarians, which had not previously been isolated from faeces of English subjects, although they had been detected in faeces of a number of Ugandans and Indians studied previously. This was the only

notable quantitative difference between the two groups. However, bacteria isolated from the faeces of the vegetarian group were found to be less active in degrading bile salts and acids. The total concentration of acid steroids was much lower in faecal samples from vegetarians, and in those subjects on a mixed diet, faecal steroids were found to be degraded to a greater extent. Finegold et al. (1977) compared the faecal flora of vegetarian and non-vegetarian Seventh-Day Adventists, and also compared both groups with a group of non-Adventists consuming a conventional American diet. There were relatively few statistically significant differences between the two groups of Adventists, but the non-Adventists had higher counts of C. septicum and C. tertium. Lactobacilli were far more common, and C. perfringens and fusobacteria much less common in the Adventist groups than in other subjects studied previously (Japanese Americans on either Japanese or western diet, or Caucasians consuming a conventional American diet).

Comparison of two Scandinavian populations by the International Agency for Research on Cancer Intestinal Microecology Group (1977) with reference to dietary habits and faecal composition revealed the presence of a greater number of Bacteroides in the faeces of subjects from Copenhagen when compared with Kuopio from Finland, where the incidence of colon cancer is a quarter of the rate in Denmark. Eubacteria and enterococci were more common in the low risk population. These data are summarised in Table 1.2.

Reddy et al. (1977c) studied the concentrations of faecal bile acids and cholesterol metabolites in patients with ulcerative colitis (at high risk) as compared with family controls, patients with other digestive disorders, and unrelated healthy controls. The dietary habits of all four groups were similar, that is, they all ate a mixed western

diet. The faecal excretion of cholesterol, coprostanol, and cholestane-
3 β ,5α ,6β -triol was elevated in patients with ulcerative colitis when
compared with the three control groups, indicating the possible
involvement of cholesterol metabolites in the aetiology of colon
cancer. There were no detectable differences in faecal bile acid
concentrations between the four groups. Previous studies by this group
(Reddy et al., 1976c; Reddy and Wynder, 1977), in which bile acids and
neutral sterols were examined in faeces of familial polyposis and
adenomatous polyp patients respectively, revealed somewhat different
results. The latter patients excreted higher levels of both bile acids
and cholesterol metabolites than did controls, but those patients with
familial polyposis excreted similar levels of both bile acids and
neutral sterols to controls, although the cholesterol was degraded to a
lesser extent in the polyposis patients. The common factor linking
these three high risk diseases therefore appears to be an alteration in
cholesterol metabolites. Mudd et al. (1980), in examining colon cancer
patients and patients at high risk of colon cancer (those with
adenomatous polyps, ulcerative colitis, rectal cancer and previous
colon carcinoma), did not find any difference in the concentration of
faecal 3-hydroxy bile acids when compared with healthy age- and sex-
matched controls. Similarly, the IARC study did not confirm previous
findings that faecal bile acid concentrations are higher in high risk
populations. Possible reasons for these discrepancies will be discussed
later.

(ii) Mutagenicity of faeces

More recently other workers, rather than examining cholesterol
and bile acid metabolites in faeces, have tested faecal samples

collected from populations at different risk of colon cancer for mutagenicity using Salmonella typhimurium TA100 and TA98 tester strains. In 1979, Ehrich et al. determined the mutagenicity of faeces from three South African populations; 19% of samples from the high risk urban white group were mutagenic in an S. typhimurium TA100/mammalian microsome system, significantly higher than samples from urban blacks and rural blacks (2% and 0% respectively). A similar pattern was found with strain TA98.

Reddy et al. (1980) compared a high risk population in New York consuming a high fat, high meat diet with a group of vegetarian Seventh-Day Adventists living in the same area, and finally a group living in rural Kuopio in Finland. Only faeces from the high risk group were found to be mutagenic. Three populations consuming different diets but all living in the same region (Vancouver) were investigated by Kuhnlein et al. (1981). The three groups were ovo-lacto-vegetarians, strict vegetarians and non-vegetarians; the last group was found to have significantly greater faecal mutagenic activity than either vegetarian group, and the presence of at least two major mutagens was indicated. Mower et al. (1982) studied two groups of Japanese subjects, one living in Hawaii at high risk, the other in Japan, at low risk. Mutagens to strain TA98 were recovered to a significantly greater extent from faeces of Japanese living in Hawaii. Neither population produced many mutagens to strain TA100 however. This group of workers also investigated water-soluble mutagens, using E. coli rec⁻; these mutagens were less common in the rural, low risk population living in Japan.

(e) Dietary fibre

High dietary fibre intake has been associated with a low incidence of colon cancer for a number of years (Burkitt, 1971; Burkitt et al., 1972; Walker, 1978). Fibre is an ill-defined term, generally referring to that part of dietary plant matter which is resistant to digestion by intestinal secretions. This consists of a heterogeneous group of carbohydrates from cereal, fruit or vegetable sources, which includes cellulose, hemicellulose, pectin and the non-carbohydrate, lignin. Dietary fibre fractions of wheat bran consist principally of hemicellulose, with a smaller proportion of lignin and cellulose, whereas the more readily fermentable vegetable and fruit fibres have a different percentage composition of hemicellulose, lignin and cellulose.

The possible protective effect of fibre has been attributed to a number of factors:

1. increased faecal bulk resulting in dilution of colonic contents, and thus any carcinogens or co-carcinogens which may be present,

2. adsorption of such compounds to the fibre and thus reducing the liklihood of their interacting with cells of the colonic mucosa,

3. decreased transit time, reducing the time available for carcinogenic, co-carcinogenic or promoting agents to exert their effect,

4. alteration of the pH of the colonic environment, thus affecting the enzyme activity of the gut flora, or

5. altered bacterial growth either directly, by forming a nutrient for the organisms, or indirectly, by binding nutrients which would otherwise be absorbed in the small intestine and carrying them to the colon where they would be available to the bacteria.

(i) General theory

In 1974, Hill discussed the fibre theory hypothesised by Burkitt, outlining the evidence for and against each point of the theory. Crowther et al. (1973) reported that the degradation of cholesterol and bile acids in faeces of British subjects consuming a western diet was decreased significantly when they were put onto a synthetic soluble diet for 10 days, although the transit time actually increased by approximately 12 days. Therefore these results do not support the argument that decreased transit time results in decreased bacterial degradation of acid and neutral sterols.

Hill suggested that the relationship between the extent of degradation of a compound and transit time could be an S-shaped curve (see Figure 1.3). Below time A, transit is so rapid that bacterial degradation of the gut contents is minimal. At times greater than B, degradation is maximal, and any increase in transit time will not increase the degree of degradation. Between points A and B, the degradation of compounds in the gut increases with transit time. If the addition of fibre to the diet does not reduce the time taken for the gut contents to move through the intestine to a value below point B, no decreased degradation of colonic contents will be observed. Points A and B will be dependent upon the activity of the enzymes of the bacterial strains present in the gut. The extent of metabolism will depend upon substrate concentration, enzyme concentration, pH, and

reducing potential of the colonic environment, rather than the rate of transit through the gut. The chance of a carcinogenic event occurring in the colon will similarly depend upon the concentration of carcinogenic material in the intestinal contents, rather than transit time.

(ii) Studies in man

Reddy et al. (1978) examined dietary differences between high risk Americans living in New York, and a low risk Finnish population from rural Kuopio. Fat and protein intake in these two populations are similar, although the fat source is different, being mainly dairy produce in Finland, but meat in New York. Fibre intake is much higher in Kuopio, however, and the Finnish subjects had an increased faecal bulk, decreased faecal secondary bile acid concentration (although total daily excretion of bile acids was the same in the two populations), and a more rapid transit time when compared with the high risk group.

Antonis and Bersohn (1962), in their study of the effects of dietary alterations on the faecal constituents of white and Bantu prisoners in South Africa, reported that an increase in dietary fibre intake increased faecal bulk, and these stools also contained appreciably greater quantities of fatty acids, bile acids and neutral sterols. Cummings et al. (1978) reported that the consumption of pentose-containing polysaccharides gave an increase in faecal bulk, and more rapid transit time. Bran gave a greater bulk than cabbage, which in turn was more bulking than carrot, apple, and finally guar gum. In 1979, Bingham et al. studied British colon cancer mortality data; regional variations correlated with differences in consumption of the

pentose component of dietary fibre, and vegetables other than potatoes
- these substances appeared to exert a protective effect against colon
cancer.

Modan et al. (1975) reported the results of their case-control
study investigating dietary fibre intake of colon cancer patients and
matched controls. The patients were found to eat significantly less
fibre of various types than the controls. Drasar et al. (1976a) studied
the effects of feeding a high fibre diet to four volunteers; two
subjects consumed a low fibre diet for 3 weeks prior to a 3 week period
on the high fibre diet, and the other subjects received the high fibre
diet first. No change was detected in the concentration of bacteria in
any of the strains studied. Based on these limited observations, they
therefore suggested that alterations in dietary intake of fibre do not
play a major part in the control of intestinal microflora in man.

(iii) Animal studies

The effects of fibre on large bowel carcinogenesis in animal
models have been largely contradictory. Fleiszer et al. (1978) reported
that the incidence of DMH-induced colon tumours in rats decreased as
the proportion of dietary fibre increased. This work has subsequently
been criticised by Heaton and Williamson (1978) and Cruse et al.
(1978a), who correctly pointed out that fibre was not the only dietary
component to alter in these experiments, and furthermore, the animals
were killed at 28 weeks which could have given a false low incidence
value if tumours had not developed sufficiently by this time to be
recognised as such at necropsy. Cruse et al. (1978b) carried out a
similar study, but over a period of 12 months. They reported that
dietary fibre, the only experimental variable, did not significantly

affect either time to tumour presentation or survival of DMH-treated rats. The tumour incidence after 12 months was 100% in all dietary groups. This work, however, has also been criticised. Newcombe (1979), Thorne (1979) and Lowenfels (1979) pointed out that the results did not necessarily disprove Burkitt's hypothesis, and that the quantity of DMH administered may have been so great as to mask any protective effects of bran. Indeed, Crofts (1979) has questioned the validity of administration of DMH by subcutaneous route in experiments designed to determine the effects of dietary fibre on colon carcinogen metabolism when it is not known whether systemically administered DMH can produce colon tumours from the luminal side with negligible effects from the systemic side.

Wilson et al. (1977), examining the differences in incidence of colon tumours in rats fed 20% fat diet with either 20% wheat bran or no bran, after intragastric DMH administration, reported no differences in the occurrence of malignant tumours between rats receiving the high fibre and no fibre diets. Freeman et al. (1978) reported that cellulose protected against DMH-induced colon cancer in rats, and Nigro et al. (1979) that fibre was able to reduce the incidence of azoxymethane (AOM)-induced colonic neoplasms in the same model if the level of fat in the diet was normal. The latter workers also observed a reduction in faecal steroid concentration in groups of rats with the lower frequencies of colon tumours.

Watanabe et al. (1979) administered either AOM subcutaneously or MNU intrarectally, to rats fed control diet, or diets containing 15% pectin, wheat bran or alfalfa. Pectin and wheat bran had no effect on MNU-induced colon cancer, but both reduced the incidence of AOM-induced tumours. Alfalfa, however, had no effect on the latter type of tumour, whereas the number of tumours following MNU-induction were actually

increased in alfalfa-fed animals when compared to controls. Therefore the observed effect seems to depend upon the type of dietary fibre consumed, as well as the carcinogen used to induce the cancer. Alfalfa has been reported to change the ultrastructure of the colonic mucosa in rats, and this effect could be involved in the alteration of colon cancer incidence by this type of fibre (Cassidy et al., 1978; 1981).

Ward et al. (1973) looked at the effect of addition of 20% or 40% cellulose to a low residue semi-synthetic diet on the incidence of AOM-induced intestinal tumours in rats; colonic neoplasms were little affected by the different diets. Bauer et al. (1979) similarly found that dietary fibre, in this case wheat bran or carrot fibre, had no effect on colon tumour incidence in rats, using DMH as the inducer. With a diet containing citrus pectin, there was actually an increase in colonic neoplasms. Possibly the dose of carcinogen (15mg/kg/week for 15 weeks) was too great, and rendered any protective effect of pectin unobservable, as Lowenfels (1979) has pointed out with regard to the work of Cruse et al. (1978b).

(iv) Binding of bile salts to dietary fibre

It seems that the type of dietary fibre is important in studies of fibre effects on gut flora and bile acid metabolism. Eastwood and Hamilton (1968) reported the adsorption of bile salts and acids to a component of vegetable fibre, which they identified as lignin. The adsorption appeared to have some hydrophobic character, as affinity was greater for less polar bile acids. Kern et al. (1978) studied in vitro binding of bile salts to dietary fibre, and reported that dihydroxy bile acids were adsorbed by all the food residues tested to a greater extent than were trihydroxy bile acids. Furthermore, the degree of

binding increased with increasing bile salt concentration, and was greater at low pH.

Story and Kritchevsky (1976) noted that the ability of different types of fibre to bind bile salts and acids was very variable. Generally, lignin bound most bile acids and bile salts to a greater extent than did alfalfa, with bran showing less binding still, and cellulose least of all.

(v) Summary of fibre effects

Effects of fibre therefore apparently depend upon the type of fibre under study. Some show greater bulking ability than do others, and will thus lead to a greater dilution of carcinogenic, co-carcinogenic or promoter compounds present in the gut. Adsorption of bile acids, tumour promoters in animal experiments, to non-nutritive dietary residues is also dependent upon the type of fibre involved. Transit time decreases with increased faecal bulk, but this factor is not thought to be important in colon carcinogenesis.

Fibre itself seems to have negligible effect on the intestinal flora present in the gut, but may alter the physiological conditions, for example, the pH within the colon, thus exerting an indirect effect on the activity of bacterial enzymes.

Certain types of fibre may alter the structure of the colonic mucosa, rendering the surface more or less susceptible to the action of colon carcinogens. In animal experiments, the type of carcinogen, as well as the route of administration, are important considerations in the interpretation of the results.

Conversely, data collected regarding the effects of dietary fat on colon carcinogenesis seem less confusing. Increased fat intake

alters the biliary excretion of bile acids, the quantity of some of the most active species of intestinal anaerobes which are capable of converting these substrates to possible carcinogens, and the physiological conditions within the gut under which these reactions take place. Since high fat diets are generally low in fibre, and vice versa, then the effects of one of these dietary constituents can probably never be said to be solely responsible for predisposing towards, or protecting against, colon cancer. The aetiology of the disease is undoubtedly extremely complex, and, as pointed to in epidemiological studies, seems also to be dependent upon the extent of environmental pollution. This factor will now be considered.

1.4 Environmental factors

Polycyclic aromatic hydrocarbons are formed during the combustion of organic material. Natural events such as forest and prairie fires contribute in only a minor way to the total environmental concentration of these compounds. Volcanic eruptions also release PAHs into the atmosphere. Anthropogenic sources include emissions from transportation systems, burning of refuse, the generation of heat and power, industrial processes, and oil contamination by disposal of effluent or oil spills into waterways (Yang et al., 1978).

For the past 30 years, data has been collected relating to the levels of several of the most common PAHs in urban air and water in a number of world-wide locations. Much information on the occurrence of benzo(a)pyrene (BP), in particular, has been accumulated. This gives only an approximation of total environmental PAH concentrations. Although a number of methods of varying accuracy have been used, the body of data serves as a very useful guide to changing world-wide

environmental pollution by these compounds.

The mechanism or mechanisms by which PAHs are formed during combustion of fossil fuels is not entirely understood. A two-step reaction is believed to be involved, the first being pyrolysis, the second pyrosynthesis. Pyrolysis yields unstable molecules, mostly radicals, from the organic compounds, and pyrosynthesis converts these labile intermediates into larger and relatively stable aromatic hydrocarbons. Generally, all organic compounds containing carbon and hydrogen may be used as PAH precursors; substances which have branched chains, are unsaturated, or have cyclic structures, lead to a greater production of PAH on pyrolysis. Optimum temperatures for PAH formation range from 650° to 740° C; that for benzo(a)pyrene is reported to be 710°C (Badger et al., 1964).

Until the beginning of this century, the production of PAHs was balanced by natural degradation of these compounds, resulting in a low and steady background concentration of these substances. Increase in industrial activity during the twentieth century has disrupted this natural balance, resulting in an accelerated production of PAH which can no longer be counteracted by natural decay, and so has lead to the accumulation of these compounds in the environment. Their stability is variable, being greater in sediment than in air or water; in intense sunlight, the half-life of these substances may be only hours or days.

(a) PAH in air

Airborne PAHs are associated with both man-made and natural aerosols; the latter include sea-spray, forest fires, volcanic and meteoritic dust, and some vegetation. The production of aerosols by combustion and industry is of much greater significance, however, and

it is thought that the major proportion of airborne PAHs are associated with carbonaceous aerosols produced during incomplete combustion of fossil fuels. They are distributed throughout the atmosphere by wind currents, and fallout through wet and dry precipitation. It has been reported that PAHs are distributed differently depending on aerosol size (cited in Lee et al., 1981). The more reactive compounds decompose rapidly in the atmosphere by reaction, for example, with ozone and oxides of nitrogen and sulphur.

(b) PAH in water

The concentration of carcinogenic PAHs in groundwater unpolluted by human activities is 0.001-0.1 $\mu g/dm^3$; that in similarly uncontaminated fresh water is 0.01-0.025 $\mu g/dm^3$. The PAHs in groundwater are assumed to be leached from upper soil layers, whilst those in freshwater have arisen from aquatic organisms in sediments and soil (for a review, see Harrison et al., 1975). Polluted river water has been found to contain 0.05 to more than 1 $\mu g/dm^3$ of carcinogenic PAHs; benzo(a)pyrene concentration ranged from 0.001-0.05 $\mu g/dm^3$ (Hites and Biemann, 1972). PAHs are hydrophobic molecules of high molecular weight, resulting in limited water solubility, and it is consequently assumed that a large percentage of the PAH content of contaminated water is adsorbed onto solids suspended in the medium. Photo-decomposition of water pollutants is dependent upon the depth of water, fluctuations in solar radiation, ambient temperature, and the quantity of oxygen dissolved in the water (Harrison et al., 1975).

(c) PAH in recent sediments

Polycyclic compounds found in bottom sediments arise from their precipitation to the water bed, or gradual incorporation into the sediment of the less soluble PAHs which have become adsorbed to particulate matter. Some PAH production has been attributed to microorganisms and plants, but there is some uncertainty about this (Blumer and Youngblood, 1975). It has been found that distribution of PAH compounds between water and sediment varies with molecular weight (cited in Lee et al., 1981); the more water soluble naphthalenes were found only in the water of a New England river, whilst the larger, more hydrophobic molecules with molecular weight greater than 228, were found only in sediment of the river bed. Those compounds of intermediate molecular weight and solubility, such as anthracenes and phenanthrenes, were present in both the water and the sediment.

(d) PAH in soil and ancient sediments

The complexity of the mixture of PAHs in the Earth's crust is very variable, ranging from simple mixtures such as in mineral ores (Geissman et al., 1967) to those in ancient sedimentary rocks and fossils where there may be thousands of components. Precipitation and fallout from airborne particles is the most likely source of PAH in the soil. Low-temperature pyrolysis of wood, or biosynthesis, could contribute to soil PAH levels in more remote areas. The concentration of benzo(a)pyrene has been found to range from 40 to 1300 µg/kg in U.S. soil (Blumer, 1961), and up to 21 mg/kg in Swiss soil.

(e) PAH in fossil fuel

The PAH composition of emissions from fossil fuel processes has recently been reviewed (Guerin, 1978).

(f) PAH and other contaminants in food

The contamination of food by polycyclic aromatic hydrocarbons may have an extremely important bearing on the aetiology of intestinal cancer in man. Sources of food contamination are many and varied. Environmental contamination by PAHs raises the PAH content of vegetation. Samples of grain from industrial regions contain higher PAH levels than do samples from rural areas. The removal of the husks from wheat and oats may reduce the benzo(a)pyrene content of these crops by as much as 60 and 40% respectively (Baum, 1978). These compounds may enter plant tissue by absorption from soil and water via the roots, or adsorption of atmospheric aerosol by the leaves. The concentration of PAHs in vegetables, fruit and cereals collected near industrial sites was found to be 2- to 5-fold greater than in those collected from rural areas. The benzo(a)pyrene concentration in lettuce from polluted areas was on average 14 μg/kg (Soos, 1979). Levels of 3 typical PAHs in various foodstuffs are shown in Table 1.3.

As well as PAHs which are intrinsic to the food, cooking methods may add to the level of contamination. Frying, grilling, roasting and smoking of meat and fish produce the highest levels of benzo(a)pyrene associated with food. Concentrations average at about 50 μg/kg, ranging from a few to several hundred μg/kg of dry material. An average of 0.1 μg/kg has been reported to be present in pork and beef (Baum, 1978). The smoking or broiling of meat increases these PAH

levels by the pyrolysis of fat as well as by contamination with smoke. Hot-air drying and roasting are potential sources of contamination of grain and coffee. The source of fuel used for grilling also greatly affects the resultant contamination of the food; Fritz (1973) compared PAH levels in roast beef, pork chops and sausages after grilling over charcoal (commercially produced, or from discarded wood), paper, or pine cones. Benzo(a)pyrene was present in meat grilled over both types of charcoal to a similar extent (8-12 µg/kg in sausage, 0.6-0.8 µg/kg in roast beef, with intermediate values for pork loin chops), whereas considerably higher levels (18-140 µg/kg) were detected in meat grilled over paper or pine cones. Fabian (1968) found higher levels of carcinogenic PAHs in sausage after charcoal grilling or grilling over pine cones than after pan frying or infra-red grilling. Other studies on cooking techniques and subsequent contamination of meat or fish by PAHs have confirmed these results (Binneman, 1979; Fritz and Soos, 1980). The quantity of carcinogens absorbed by smoked meat is dependent upon the temperature at which the meat is smoked, the duration of the smoking procedure, and also the subsequent storage time of the smoked product (Dikun, 1962). A lowering of the smoking temperature is recommended, in order to reduce food contamination by PAH (Schober, 1979; Dikun et al., 1969; Hamm, 1977).

PAHs are not the only carcinogenic food contaminants known, however. N-Nitrosamines have been found in certain foods, particularly meat and fish treated with nitrite (Crosby et al., 1972). N-Nitrosamines are formed by the interaction of nitrite and secondary or tertiary amines, and some workers have shown that certain naturally occurring quaternary ammonium compounds can also form these carcinogens with nitrite (Fiddler et al., 1972). Dimethylnitrosamine and nitrosopyrrolidine are the two most common nitrosamines found in cured meat

products, the latter being formed during the frying of bacon (Sen et al., 1973; 1973a). Pyrolysis of certain amino acids can yeild mutagens. Two pyrolytic products of tryptophan, Trp-P-1 and Trp-P-2, which are very potent mutagens, have been detected in broiled sardines (13.3 and 13.1 ng/g, respectively) (Yamaizumi et al., 1980). Sugimura and Sato (1983) have recently reviewed this subject.

Aflatoxins, toxic metabolites produced by the fungus Aspergillus flavus, have been extracted from a number of foodstuffs including corn, rice, barley, wheat, millet, sesame seed, nuts, peas, beans and sweet potatoes. Aflatoxin B_1 has been positively associated with human cancer; in certain regions of Africa and Asia this mycotoxin is commonly a contaminant of human foods, and is thought to be responsible for the higher incidence of primary liver cancer in these areas. The metabolism, carcinogenicity and interaction of aflatoxins with nucleic acids has been reviewed in detail by Garner and Martin (1979).

Therefore there are a number of environmental carcinogens and mutagens to which man is exposed, particularly in industrialised areas. Here, atmospheric and water pollution is greater than in rural regions, and plants which are grown in such surroundings consequently take up more of these pollutants.

Thus, polycyclic aromatic hydrocarbons and aflatoxin, for example, can enter the body simply by the ingestion of certain foodstuffs containing these compounds. Cooking of meat and fish leads to a greater contamination by PAHs, the formation of nitrosamines from nitrite and secondary or tertiary amine compounds, and the production of mutagenic pyrolytic products of protein. The extent of contamination varies with the method of cooking, the type of fuel used, and the temperature at which the food is cooked, but certainly a number of

carcinogenic compounds enter our bodies by the simple process of ingestion of food, and may also be formed in vivo from precursors present in the diet. Soos (1979) estimated the uptake of benzo(a)pyrene from food to be 400-600 µg/man/year, based on her measurements of PAH levels in food, and Hungarian food consumption data.

1.5 Xenobiotic metabolism

The ability of the body to oxidise xenobiotic molecules such as drugs and environmental pollutants has been known since the late nineteenth century (see Schenkman and Gibson, 1981). The metabolism of such foreign compounds is usually carried out in two stages, namely phase I and phase II. Phase I includes oxidation, reduction and hydrolytic reactions, and phase II involves the conjugation of phase I products and similar compounds with molecules endogenous to the body.

Phase I reactions

Oxidation reactions include epoxidation, hydroxylation, oxidative dealkylation, oxidation at N, S or P, and the replacement of S by O. These reactions have been summarised by Williams (1978), and are shown in Figure 1.4. Similarly, there are a number of different reduction reactions, such as azo reduction, nitro reduction, ketone, aldehydic and carbon-carbon double bond reduction, reduction of penta-valent to trivalent arsenic, reduction of disulphide bonds to sulphydryl groups, and of sulphoxides and N-oxides to the corresponding divalent sulphur compounds and tertiary amines respectively - see Figure 1.5. Hydrolytic reactions include hydrolysis of carboxylic esters, carbamates, amides, phosphoric esters, thio esters and epoxides (see Figure 1.6).

Phase II reactions

These are synthetic reactions, involving the conjugation of phase I metabolites with glucuronic acid, glycine, cysteine, glutamine, methyl groups, glutathione, sulphate or acetyl moieties. The conjugates produced generally have a greater polarity than their precursors, and are therefore more readily excreted from the body via the kidneys. These conjugates are usually regarded as detoxification products. However, xenobiotic metabolism does not necessarily lead to the production of a less active molecule. For example, several sulphonamide drugs and their acetyl conjugates are highly insoluble in water, and can lead to kidney damage by crystallising in the renal tubules (see Williams, 1959). Similarly, the sulphate conjugate of 2-acetylamino-fluorene is believed to be the active metabolite of this carcinogen. In addition, certain intestinal bacteria possess the ability to deconjugate a number of phase II metabolites, releasing a more reactive, less polar phase I product.

(a) Phase I

(i) Oxidation reactions

1. The Mixed Function Oxidase System

In 1958, Klingenberg reported the detection of a cytochrome in rat liver homogenate which bound carbon monoxide, forming an absorption band at 450nm. This haemoprotein, termed cytochrome P450, was shown by Cooper et al. (1965) to be the terminal oxidase of the mixed function oxidase (MFO), or monooxygenase system of mammalian tissues. This oxidative enzyme system was at one time considered to be associated solely with the endoplasmic reticulum of liver cells, but now most tissues have been shown to possess MFO activity (Nebert and Gelboin,

1969). At least two protein components are involved; a flavoprotein, NADPH-cytochrome c reductase (or NADPH-cytochrome P450 reductase), and cytochrome P450, the former passing electrons to the latter. Cytochrome b5 may be responsible for the input of the second electron in some reactions. The cytochrome P450 complex is actually a group of isozymes rather than a single enzyme. The different isozymes possess overlapping substrate specificity and properties and exhibit differential induction by various drugs and organic compounds. Some are involved in metabolism of compounds prior to excretion, and some in the synthesis of necessary cellular metabolites.

Oxidation of substrates by the MFO system requires molecular O_2, and the cofactor NADPH. One atom of each molecule of oxygen is incorporated into the substrate, and the other is used to form a molecule of water. The overall reaction can be summarised as follows:

$$SH + NADPH + O_2 + H^+ \longrightarrow SOH + NADP^+ + H_2O$$

A schematic representation of this oxidation process is shown in Figure 1.7.

The microsomal drug oxidising system has been discussed at length in numerous books and reviews, for example, see Mannering (1971). Much work has been carried out since the discovery of cytochrome P450 to investigate its general properties, mechanism of action, and the nature of the "active oxygen" complex. A number of workers have discussed various stages of the cycle (Waterman et al., 1973; Estabrook et al., 1981; Nordblom et al., 1976; Hrycay et al.,

1975). Schenkman and Gibson (1981) have recently reviewed the cytochrome P450 cycle, describing the effects of substrate binding on the spin state of the oxidised form of the haemoprotein, the input of the two electrons, activation of bound oxygen, oxidation of substrate, and release of product to yield the oxidised form of cytochrome P450 once again (see Figure 1.7).

The stoichiometry of the MFO reaction is not consistent with the formation of one mole of oxidised product for each mole of NADPH and O_2 utilised, however. Both microsomes and reconstituted systems have been found to require amounts of O_2 and NADPH in excess of those expected from such a stoichiometry, and the formation of peroxide has been reported during NADPH oxidation by liver microsomes (Hildebrandt and Roots, 1975). Cytochrome P450 in hepatic microsomes has been shown to support peroxide-dependent substrate oxidation in the absence of NADPH-cytochrome c reductase, NADPH, or molecular O_2 (Hrycay and O'Brien, 1973; Kadlubar et al., 1973; Nordblom et al., 1976; Capdevila et al., 1980). Therefore cytochrome P450 can bring about the oxidation of a substrate by means of its NADPH-dependent oxygenase activity, or through a coupled oxidase reaction wherein the generation of lipid peroxides can bring about the peroxidative metabolism of the substrate. The existence of these different pathways of metabolism may be responsible, at least in part, for the variety of products observed during the oxidative metabolism of substrates such as benzo(a)pyrene, and such variations will be discussed below. A scheme showing the simultaneous operation of these pathways is shown in Figure 1.8.

2. Induction of cytochrome P450

In 1966, Imai and Sato reported that the two-banded Soret spectrum produced upon addition of ethyl isocyanide to dithionite-treated microsomes from rabbit liver arose from the possible presence of two forms of cytochrome P450, functionally identical, but spectrally different from each other, existing in an equilibrium state which was dependent upon pH. There is a main peak at 430nm, with a smaller, anomalous peak at 455nm; alteration of pH changes the relative heights of these two peaks. Hildebrandt et al. (1968) recorded the absorption characteristics of rabbit liver microsomal cytochromes after induction with phenobarbital (PB) or 3-methylcholanthrene (MC), a polycyclic aromatic hydrocarbon, and found the different inducers to produce forms of the cytochrome with different spectral properties.

Welton and Aust (1974 and 1974a) studied the effect of inducers on microsomal cytochromes of rat liver by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The pattern of proteins of molecular weight around 50,000 was altered by the inducers; 3 haemoproteins were separated, with molecular weights of 44,000, 50,000 and 53,000. In control rats, the protein of molecular weight 50,000 predominated, whereas induction with PB increased the lower molecular weight cytochrome, and MC that of molecular weight 53,000. A study of the kinetics of substrate hydroxylation by rat liver homogenate after pretreatment of the animals with PB or MC also reveals qualitative, not merely quantitative changes in the cytochromes present (Alvares et al., 1968). A quantitative difference in the cytochrome fraction would be expected to increase Vmax for benzo(a)pyrene hydroxylation, whilst leaving the K_M unaltered. This was the case for PB induced animals, but MC altered the K_M for the reaction, indicating a qualitative difference

in the cytochromes present after pretreatment with this inducer.

Alvares et al. (1967) have also shown that the haemoprotein induced by MC in rat liver has an absorption maximum of 448nm, rather than 450nm as for the PB-induced cytochrome. Haemoproteins induced by Arochlor 1254, a mixture of polychlorinated biphenyls (PCB), appear to be a mixture of cytochromes P448 and P450 (Alvares et al., 1973). Alvares and Siekevitz (1973) confirmed the induction of cytochromes of differing molecular weights after treatment of rats with MC, PB or PCB, and suggested that the effect may be due to the induction of different subunits of the haemoprotein, having varying degrees of affinity for either substrate or haem group.

Lu et al. (1972) demonstrated that the hydroxylation of benz(a)pyrene was much greater using cytochrome P448 prepared from MC-induced rats than with P450 from PB-induced animals. The reverse was true for the N-demethylation of benzphetamine, again indicating varying substrate specificity of the different forms of the haemoprotein.

Wiebel et al. (1975) purified 3 cytochromes from PB-induced rabbit liver microsomes; these forms are classified by their relative electrophoretic mobilities, and are termed P450LM1,7, P450LM2 and P450LM4. They exhibit different rates of substrate oxidation, and also vary in the relative proportions of the products they form. Coon et al. (1977) also reported the occurrence of multiple forms of the cytochrome in the same system. Cytochrome P450LM2 was present in rabbit liver microsomes in significant amounts only after induction by drugs, whereas P450LM4 was present in microsomes from both normal and induced animals, and is induced with 5,6-benzoflavone. Estabrook and Werringloer (1979) have reported that there may be at least 6 different cytochromes. Therefore evidence for the existence of a number of isozymes of cytochrome P450 has been accumulating for over 15 years.

More recently, Kitchin and Woods (1978) reported that the increased aryl hydrocarbon hydroxylase (AHH) activity in rat liver associated with induction by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), was accompanied by de novo synthesis of the protein component of cytochrome P448; haem synthesis did not appear to be influenced by this inducer. Previously, Chhabra et al. (1976) had reported that TCDD and MC induce AHH by the same mechanism, but in mouse liver the increased AHH activity they observed was not accompanied by the production of cytochrome P448, as measured by absorption spectra. They suggested, however, that native cytochrome P448 may have been present in this tissue at a suitable concentration to allow for increased AHH activity over the time course of the experiment (12h).

Tissue, strain and species differences in the type of haemoprotein induced have been noted by a number of groups (Nebert and Gelboin,1969; Burki et al., 1973; Thomas et al., 1972; Cram et al., 1965; Diwan et al., 1975). These variations lead to the production of different profiles of metabolites from the same substrate; some examples are shown in Figure 1.9. In 1977, Hundley and Freudenthal compared the metabolites of benzo(a)pyrene produced by the microsomal fraction from Rhesus liver and lung. Qualitatively, the metabolite profiles were very similar; benzo(a)pyrene 9,10-, 7,8- and 4,5-dihydro-diols, 1,6-, 3,6- and 6,12-quinones and 9- and 3-hydroxybenzo(a)pyrene were formed by both tissues, as were 3 unidentified metabolites, but the liver produced two additional unknown metabolites. Quantitative differences were much greater. Liver activity was higher than lung activity for all metabolites formed, and the ratio of the metabolites also differed between the tissues. The predominant metabolite formed by the liver microsomes was 3-hydroxybenzo(a)pyrene (3OHBP), whereas quinones were formed to a greater extent by lung microsomes. The

metabolite ratios for the dihydrodiols were very similar for both tissues. These workers went on to compare BP metabolism by liver and lung from Rhesus monkeys and rats after treatment of the animals with MC. Lung activity in both species increased to a greater degree than did liver activity on treatment with the inducer, and rat lung and liver activity increased to a greater extent than activity in the tissues from Rhesus monkeys. MC treatment caused shifts in metabolite ratios; the 9,10- and 7,8-dihydrodiol fractions formed by rat liver and Rhesus lung increased to a greater degree than other fractions. In rat lung, MC treatment increased 1,6-quinone production by more than 60 times, and 9OHBP by over 50 times (Hundley and Freudenthal, 1977a). Kimura et al. (1977) also reported differences in the patterns of BP metabolites formed by rat liver and lung homogenates.

Mammary tissue has also been shown to produce a different metabolite profile from liver, with 7,12-dimethylbenz(a)anthracene (DMBA) substrate. Rat liver preparations produced 7-hydroxymethyl-12-methylbenz(a)anthracene (7OHM12MBA), 12-hydroxymethyl-7-methyl-benz(a)anthracene (12OHM7MBA) and 7,12-dihydroxymethylbenz(a)anthracene (7,12-diOHMBA), whereas mammary tissue formed only the monohydroxy compounds. MC treatment again affected the tissues differently; liver produced more polar compounds from all 3 metabolites, but mammary gland showed a reduction in 7OHM12MBA formation and an increase in production of 12OHM7MBA (Tamulski et al., 1973).

Prough et al. (1977) analysed the metabolites of BP formed on incubation with rat and human lung microsomes; the latter produced a higher percentage of the 7,8- and 9,10-dihydrodiols compared with rat lung microsomes. Selkirk et al. (1975) reported that the ratio of BP phenols formed by human liver microsomes and lymphocytes are different, and different again from the BP metabolite pattern from rat liver

microsomes.

In 1981, Wang tested the effects of PB, MC and β-naphthoflavone (BNF) on liver microsomal enzymes and the metabolite profiles of BP in three strains of mice. PB pretreatment induced five protein bands as separated by SDS-PAGE, and increased the yield of BP metabolites 1.5 to 3-fold. MC and BNF treatment increased enzyme levels and metabolite profiles in only 2 of the strains of mice; the yield of 7,8-dihydrodiol was increased 4 to 10 times, depending upon inducer and strain of mouse, and phenols 2.5 to 5 times. Therefore the induced haemoproteins may activate different carbon atoms of the BP molecule, and consequently result in different metabolite profiles.

Holder et al. (1975) noted species differences in BP metabolism by liver microsomes from rats and mice; those from rats produced a greater proportion of dihydrodiols. MC pretreatment increased the formation of phenols, quinones and diols in only one of the two mouse strains tested. Some of this data concerning tissue and species differences in PAH metabolism is summarised in Table 1.4.

It is therefore apparent that a number of different forms of cytochrome P450 exist, influenced differently by inducers of various types, and present to varying degrees depending upon species, strain and tissue under examination. The proportions of these enzymes present in a given tissue will thus influence the pattern of metabolites formed from a substrate by that tissue. The most frequently studied PAH is benzo(a)pyrene, and its metabolism will be discussed in greater detail in a later section.

(ii) Epoxide hydrase

The activity of epoxide hydrase has been measured in various organs of the rat and guinea pig. Oesch (1973) found the activity to be highest in liver, undetectable in brain, heart, spleen and muscle, and to have low activity in the kidney, lung and intestine. Subsequent studies using more sensitive assays have revealed the presence of the enzyme in human liver (Oesch et al., 1974).

Epoxide hydrase was originally reported as being an exclusively microsomal enzyme (Oesch et al., 1971; Oesch, 1973; Oesch et al., 1974), located on both rough and smooth endoplasmic reticulum, but more recently the activity has been found to be associated with the mitochondrial fraction of mouse liver (Gill and Hammock, 1981), the nuclear membrane of rat liver nuclei (Jernstom et al., 1976; Mukhtar et al., 1979), and also as a cytosolic enzyme in mouse liver and kidney (Hammock et al., 1976; Gill and Hammock, 1979). Different forms of the enzyme exist (Oesch et al., 1971a; Oesch, 1973; Ota and Hammock, 1980; Guengerich et al., 1979).

There are indications that epoxide hydrase may somehow be coupled to the MFO system (Oesch, 1973; Oesch and Daly, 1972; Guengerich and Davidson, 1982), as large quantities of dihydrodiols are present in the urine of animals treated with PAH despite the fact that arene oxides rearrange to phenols very easily in aqueous media. Epoxide hydrase activity is not induced to the same extent as is AHH. It is inducible by PB, but not to any great degree by hydrocarbons (Leutz and Gelboin, 1975). Under certain conditions, MC induces the MFOs but has no effect on epoxide hydrase. Oesch et al. (1971) reported that both PB and MC induced rat liver epoxide hydrase activity, but no significant increase was observed in rabbits or guinea pigs after pretreatment of

animals with these compounds. In some cases where both the AHH and epoxide hydrase activities are induced, the AHH is induced to a much greater extent, thus altering the metabolite profile observed after induction. Despite the fact that the MFO and epoxide hydrase may be linked in the endoplasmic reticulum, the two seem to be under separate genetic control (Oesch, 1976; Nebert et al., 1972). The hydrase is stereoselective in the hydration of racemic BP epoxides (Yang et al., 1977; Yang et al., 1977a).

(b) Phase II

(i) Glutathione S-transferase

Glutathione S-transferase has been found in a wide variety of tissues, including liver, kidney, lung and intestinal mucosa of a number of species (rats, rabbits, guinea pigs, sheep and other vertebrates; Grover and Sims, 1964; Wit and Snel, 1968; James et al., 1976; Hayakawa et al., 1974; Hook and Bend, 1976), as well as insects (Cohen et al., 1964). A number of different forms have been isolated, each showing unique specificity towards BP 4,5-oxide. Reeve et al. (1981) have reported the presence of 4 proteins in rat liver which covalently bind BP metabolites and possess glutathione S-transferase activity. The isozymes are predominantly cytoplasmic enzymes, although some microsomal and mitochondrial activity has been reported in rat liver (Friedberg et al., 1979; Wahllander et al., 1979). They catalyse phase II conjugation reactions of a wide variety of electrophilic compounds with glutathione.

The transferases are inducible. A number of cytoplasmic binding proteins, such as ligandin, have been reported to be induced by PB and PAH (Reyes et al., 1969; Reyes et al., 1971; Habig et al., 1974;

Litwack et al., 1971; Kaplowitz et al., 1975). Habig et al. (1974) reported that ligandin appears to be identical with glutathione S-transferase B, although there now seems to be some doubt about this; it has been suggested that the term ligandin be dropped, and that the glutathione S-transferase enzymes should be referred to on the basis of their sub-unit structure (Hayes et al., 1980). Enzyme structure has been discussed recently by Grover (1982), and Chasseaud (1979) has reviewed the glutathione S-transferases.

The specificity and quantity of each form of the enzyme present in a given tissue, as well as the intracellular glutathione concentration, plays an important role in the detoxification of activated metabolites of chemical carcinogens, drugs and other xenobiotics.

(ii) Sulphotransferase

Sulphotransferase enzymes are also soluble proteins catalysing phase II conjugation reactions. Sulphate conjugates of various monohydroxy metabolites of BP (3OH-, 7OH- and 9OHBP) have been reported to be formed by hamster, rat and human lung cultures (Cohen et al., 1976; Cohen et al. 1977), and the sulphate conjugate of 3OHBP by rat liver (Nemoto and Takayama, 1977). Nemoto et al. (1977) reported the formation of these water-soluble conjugates from five phenols, two quinones and three dihydrodiols of BP, with the enzymes from rat liver catalysing the reactions.

(iii) UDP-glucuronyl transferase

Nemoto and Gelboin (1976) reported the ability of a number of

BP metabolites, including phenols, dihydrodiols and epoxides to form glucuronide conjugates when incubated with UDP-glucuronic acid and rat liver microsomes. The 7,8-dihydrodiol is conjugated to a lesser extent than the phenol metabolites and the 4,5-diol in this system. Glucuronide conjugates have been found in the bile and urine of rats and rabbits (Falk et al., 1962; Plummer et al., 1980; Chipman et al., 1982). Rat liver microsomes also conjugate UDP-glucuronic acid with BP-3,6-quinone (Bock et al., 1980) and naphthalene dihydrodiol (Bock et al., 1976). This transferase activity has also been reported in hamster embryo cells (Baird et al., 1977) and rat intestine (Hietanen, 1980).

(c) Benzo(a)pyrene metabolism

As with the majority of chemical carcinogens, BP requires metabolic activation before it can exert its effect. This activation is carried out by the MFO system. The first stage in the process is the production of simple epoxide molecules – the 9,10-, 7,8- and 4,5-oxides of BP. These epoxides can then undergo a number of alternative transformations. They may rearrange non-enzymatically to form phenols, or alternatively can be converted to the corresponding trans-dihydrodiols by epoxide hydrase. They may also be conjugated with glutathione by the action of glutathione-S-transferase (Nemoto and Gelboin, 1975), or react with proteins and nucleic acids.

Five phenols (1OH-, 3OH-, 6OH-, 7OH- and 9OHBP) have been isolated (references cited in Gelboin, 1980). These can be converted into quinones; 1,6-, 3,6- and 6,12-quinones have been found, and these are thought to be formed from 6-hydroxyBP. 6-hydroxymethylBP and 6-oxyBP, an intermediate in 6OHBP formation, have also been reported (Sloane, 1975; Flesher and Sydnor, 1973; Lorentzen et al., 1975; Lesko

et al., 1975).

The phenols and dihydrodiols can be conjugated with sulphate or glucuronide to yield more water soluble metabolites, or undergo further oxidation by the MFO system. Further action of the monooxygenase system on BP 7,8- and 9,10-dihydrodiols produces diol-epoxide molecules. These metabolites can exist in two stereoisomeric forms, with the epoxide group on either the same side of the cyclohexane ring as, or on the opposite side of the ring to, the benzylic hydroxyl group (the syn and anti isomers respectively). Since the trans-dihyrodiols can exist as (+) or (-) optical enantiomers, four isomeric forms of each diol-epoxide are possible; one syn and one anti isomer of each optically active enantiomer. These structures are shown in figure 1.10, together with some of the nomenclature used by various workers. More detailed explanations of the nomenclature may be found in Gelboin (1980).

Each diol-epoxide can be hydrolysed to two tetrols, and reduced to one triol. They are also highly electrophilic and interact with nucleophilic groups on cellular macromolecules such as proteins, RNA and DNA. Furthermore, like the simple epoxides formed initially, they may undergo phase II conjugation reactions, forming glucuronide, sulphate and thio-ether conjugates through the action of UDP-glucuronyl transferase, sulphotransferase and glutathione-S-transferase respectively. A general scheme outlining these pathways of BP metabolism is shown in Figure 1.11.

(d) DNA binding

It is now widely accepted, based on the work of Miller and Miller, that chemical carcinogens which are not themselves chemically reactive initiate the carcinogenic process by the interaction of an

activated, electrophilic metabolite with nucleophilic groups in cellular macromolecules (for a review, see Miller, 1978). Much interest has therefore been focussed on such interactions (Baird and Brookes, 1973; Wang et al., 1972; Duncan and Brookes, 1970; Brookes et al., 1975; Autrup et al., 1977; Meehan et al., 1977; King et al., 1977; Jeffrey et al., 1977; Dock et al., 1978; Pezzuto et al., 1978; Boobis et al., 1979; Allen and Coombs, 1980; Boroujerdi et al., 1981).

Up until the mid 1960's, work carried out to study the binding of carcinogens to cellular macromolecules concentrated on protein rather than the nucleic acid component of cells, but Brookes and Lawley (1964) investigated the binding of carcinogenic hydrocarbons to DNA, RNA and protein of mouse skin and observed a positive correlation between the carcinogenic potency of the hydrocarbons and the extent of their binding to DNA. No such correlation was found with the binding to RNA or protein. Huberman and Sachs (1977) obtained similar results with the binding of carcinogens of differing potency to macromolecules in hamster cells. Interactions between chemical carcinogens and DNA have been discussed in a number of reviews (Heidelberger, 1975; Miller, 1978; Gelboin, 1980; Margison and O'Connor, 1979; Culvenor and Jago, 1979; Garner and Martin, 1979; Kriek and Westra, 1979; Phillips and Sims, 1979).

Sims et al. (1974) have shown that DNA treated with the 7,8-diol-9,10-oxide of BP in solution chromatographed, after hydrolysis, with the same characteristics as a peak in the hydrolysate of DNA obtained from hamster cells after treatment with BP, and Daudel et al. (1975) found that the fluorescence spectral characteristics of DNA from BP-treated mouse skin corresponded to those of DNA treated in solution with 7,8-dihydroxyBP-9,10-oxide. The 7,8-dihydrodiol-9,10-oxide metabolite of BP is therefore thought to be an ultimate carcinogenic

form of this hydrocarbon (Yang and Gelboin, 1976). The _anti_ isomers appear to be the major metabolic products of hydrocarbons, and the principal nucleic acid adducts formed in tissues treated with BP arise through reactions of the _anti_ isomer of the 7,8-diol-9,10-oxide, although products involving the _syn_ isomer have also been detected to a lesser extent (see Phillips and Sims, 1979).

1.6 Summary and choice of model

In view of the ubiquitous nature of the carcinogen benzo(a)pyrene, the presence of this PAH as a contaminant of many foodstuffs, and its ability to induce cancer at the site of application, the possibility of its playing a role in the aetiology of colon cancer would doubtless have been considered long ago were it not for its high lipid solubility. This property has resulted in BP being disregarded as a possible colon carcinogen, due to its absorption from the upper intestine i.e. the duodenum, jejunum and ileum.

However, oxidation products of BP and their polar conjugates are readily excreted in the bile and can thus reach the lower intestine. In the absence of intestinal bacteria, these detoxification products would be eliminated from the body in the faeces. But gut flora have been shown to carry out deconjugation reactions (see Chapter 4 for details), and the ability of certain strains of intestinal micro-organism to produce the parent hydrocarbon from the inactive biliary metabolites of benzo(a)pyrene has also been demonstrated (Renwick and Drasar, 1976). Potential carcinogens can thus be released locally within the colon, and may be activated by further action of the gut flora, or by the MFO system of the colonic mucosa.

Since a high incidence of colon cancer has been associated with

a high intake of dietary fat in epidemiological studies, the interaction of such a diet with a lipid soluble carcinogen such as BP could provide an interesting insight into the aetiology of the disease. Therefore, the aim of this research was to investigate how a high fat diet, or a high fat + high cholesterol diet may alter the metabolism of BP in the body in such a way as to predispose to colon cancer. Such diets may predispose to the disease in the presence of BP by influencing any of the following factors:

(1) increased excretion of cholesterol (or neutral sterols) and bile acids which could be converted to carcinogenic, co-carcinogenic or promoter compounds by the gut flora

(2) increased excretion of biliary metabolites of BP

(3) increased solubilisation of active lipid soluble compounds by an increased bile acid concentration

(4) increased activity of intestinal microflora, releasing more active metabolites in the gut lumen

(5) increased activity of MFO enzymes in colonic mucosal cells

(6) decreased activity of detoxifying enzymes such as UDP-glucuronyl transferase, sulphotransferase and glutathione S-transferase.

The male guinea pig was chosen as the animal model to investigate these interactions. The biliary threshold of the guinea pig is 400 $\pm$ 50, whereas that of the rat is 325 $\pm$ 50, and that of man, 500

$\pm$ 50 (Hirom et al., 1972; Smith and Millburn, 1975). The rabbit has a biliary threshold of 475 $\pm$ 50, and from that point of view would be a better model in which to study biliary metabolites of BP as it is closer to the threshold measured in man. However, the threshold reported for guinea pigs is biased by the data for a single compound. If adipylsulphathiazole, were excluded from the calculations, the remaining compounds studied would yield a value of 450 $\pm$ 50 for the biliary threshold of the guinea pig.

Furthermore, the bile salts produced by the guinea pig are closer in composition to those produced by man (Haslewood, 1967), and as the quantity of fat consumed influences the production of bile salts, then the guinea pig would seem to be the more appropriate model. In man, the main bile acids produced are glycocholate (GC), taurocholate (TC), glycochenodeoxycholate (GCDC), taurochenodeoxycholate (TCDC), glycodeoxycholate (GDC) and taurodeoxycholate (TDC). The ratio of glycine-conjugated to taurine-conjugated bile salts varies, but generally lies between 1 and 6 in normal subjects (see Heaton, 1972). The ratio of cholic to chenodeoxycholic to deoxycholic (C:CDC:DC) acids is also variable, typical values being 1.1:1:0.6. Expressing these values as a percentage of total bile salts in human bile, the average figures obtained are 5% TDC, 9% TCDC, 10% TC, 31% GC, 28% GCDC and 17% GDC. The guinea pig produces taurine and glycine conjugates of bile acids (C, CDC, 3 -hydroxy-7-oxo-, 7 -hydroxy-3-oxo- and 3,7-dioxocholanic acids and lithocholic acid), whilst the rabbit produces glycine conjugates of C, DC, lithocholic acid and allodeoxycholic acid (Haslewood, 1967).

The guinea pig was therefore chosen to study the possible

interactions of the lipid soluble environmental pollutant, benzo(a)-pyrene, with a high fat or high fat plus high cholesterol diet.

Table 1.1   Promoting effects of bile acids and neutral sterols in
experimental colon carcinogenesis in rats

Bile acid or sterol	Route of administration and carcinogen	Effect	Reference
Cholic acid	i.r. MNNG	+	a
Chenodeoxycholic acid	i.r. MNNG	+	a
Lithocholic acid	i.r. MNNG	+	b
Lithocholic acid	i.r. MNNG	+	c
Taurodeoxycholic acid	i.r. MNNG	+	b
Deoxycholic acid	i.r. MNNG	+	d
Cholesterol	s.c. DMH	+	e
Cholesterol	i.r. MNNG	−	c
Cholesterol epoxide	i.r. MNNG	−	c
Cholestane-3β,5α,6β-triol	i.r. MNNG	−	c

+ promotion
− no promotion
i.r. intrarectal administration
s.c. subcutaneous administration

MNNG N-methyl-N'-nitro-N-nitrosoguanidine
DMH   dimethylhydrazine

References

a. Reddy et al. (1977b)
b. Narisawa et al. (1974)
c. Reddy and Watanabe (1979)
d. Reddy et al. (1976b)
e. Cruse et al. (1978)

Table 1.2   Distribution of intestinal bacteria in
                 populations at different risk for colon cancer

Bacteria	High risk group	Low risk group	Comment	Ref.
Bacteroides	English	Ugandan		a
	English/Scot/ U.S.A.	Uganda/S. India/ Japan	Greater numbers in high risk	b
	High beef diet	Normal diet	groups	c
	Copenhagen	Kuopio		d
Clostridium paraputrificum	Britain/ U.S.A.	Hong Kong/ Uganda/Japan	as above	e
C. septicum	Non-7th-Day	7th-Day		f
C. tertium	Adventists	Adventists	as above	f
C. perfringens	(mixed diet)	(vegetarian)		f,g
Streptococci	English	Ugandan	Greater numbers in	a
S. faecalis	Japanese on western diet	Japanese on Japanese diet	low risk groups	g
Enterococci	English	Ugandan	as above	a
	English/Scot/ U.S.A.	Uganda/S. India/ Japan	as above	b
	Copenhagen	Kuopio	as above	d
Eubacteria	Copenhagen	Kuopio	as above	d
Lactobacilli	English	Ugandan	as above	a
Yeasts	English	Ugandan	as above	a
Sarcinae	British on mixed western diet	British vegetarians	only in low risk group	h

References
a. Aries et al. (1969)   e. Drasar et al. (1976)
b. Hill et al. (1971)   f. Finegold et al. (1977)
c. Maier et al. (1974)   g. Finegold et al. (1974)
d. IARC (1977)   h. Aries et al. (1971a)

Table 1.3   Levels of three polycyclic aromatic hydrocarbons
in various foods

Food	Benzo(a)pyrene	Benz(a)anthracene	Chrysene
Smoked meat or fish	0.2-107	0.02-189	0.3-123
Broiled meat or fish	0.2-11.2	0.2-31	0.5-25.4
Refined oils and fats	0.9-15	0.5-13.5	0.5-129
Spinach	7.4	16.1	28.0
Tomatoes	0.22	0.3	0.5
Roasted coffee	0.1-4	0.5-14.2	0.6-19.1
Tea	3.9-21.3	-	4.6-6.3
Cereals	0.25-0.84	0.4-6.8	0.8-14.5

Values are given as µg/kg dried material.
From Baum (1978).

Table 1.4   Tissue and species differences in PAH metabolism

Substrate	Species and tissue	Products	Ref.
BP	Rhesus liver	Diols, quinones and phenols- mainly 3OHBP.	
	Rhesus lung	Diols, quinones and phenols- mainly quinones.	a
BP, after MC induction	Rhesus lung	Increased % of 9,10- and 7,8-diols.	
	Rat liver	As above.	b
	Rat lung	Increased 1,6-quin- one and 9OHBP.	
DMBA	Rat liver	7OHM12MBA 12OHM7MBA 7,12-diOHMBA	
DMBA, after MC induction	Rat liver	More polar compounds from all 3 metab- olites.	c
DMBA	Rat mammary tissue	7OHM12MBA 12OHM7MBA	
DMBA, after MC induction	Rat mammary tissue	Increased 12OHM7MBA	c
BP	Human lung	Greater % of 7,8- and 9,10-diols than rat lung.	d
BP	Mouse liver	Smaller % of diols than rat liver.	e

BP	Benzo(a)pyrene
MC	3-Methylcholanthrene
DMBA	7,12-Dimethylbenz(a)anthracene
7OHM12MBA	7-Hydroxymethyl-12-methylbenz(a)anthracene
12OHM7MBA	12-Hydroxymethyl-7-methylbenz(a)anthracene
7,12-diOHMBA	7,12-Dihydroxymethylbenz(a)anthracene

References

a. Hundley and Freudenthal (1977)
b. Hundley and Freudenthal (1977a)
c. Tamulski et al. (1973)
d. Prough et al. (1977)
e. Holder et al. (1975)

Figure 1.1   The chemical conversion of deoxycholic acid to
the carcinogen 3-methylcholanthrene

Deoxycholic acid

Dehydronorcholene

3-Methylcholanthrene

From Haddow (1958)

Figure 1.2   Production of polycyclic aromatic compounds

from bile salts by human intestinal bacteria

The diagram shows 4 types of nuclear dehydrogenation reaction.

a. Aries et al. (1971)

b. Goddard and Hill (1972)

c. Goddard and Hill (1973)

Figure 1.3   Proposed relationship between bacterial degradation

of a compound, and transit time

From Hill (1974)

Figure 1.4   Examples of oxidation   reactions

$$-HC=CH- \longrightarrow -HC\underset{O}{\overset{}{\diagdown\diagup}}CH-$$ Epoxidation

ArH $\longrightarrow$ ArOH   Hydroxylation of aromatic ring

RH $\longrightarrow$ ROH   Alkyl hydroxylation

R'OR $\longrightarrow$ R'OH ⎤
R'NHR $\longrightarrow$ R'NH ⎬ Oxidative dealkylation
R'SR $\longrightarrow$ R'SH ⎦

R'NH$_2$ $\longrightarrow$ R'NHOH ⎫
R$_2$NH $\longrightarrow$ R$_2$NOH ⎬ Oxidation at N
R$_3$N $\longrightarrow$ R$_3$NO ⎭

$\diagdown$S$\diagup$ $\longrightarrow$ $\diagdown$SO$\diagup$ $\rightarrow$ $\diagdown$SO$_2\diagup$ Oxidation at S

$\diagdown$-P$\diagup$ $\longrightarrow$ $\diagdown$-PO$\diagup$ Oxidation at P

$\diagdown$C=S$\diagup$ $\longrightarrow$ $\diagdown$C=O$\diagup$ ⎫
⎬ Replacement of S with O
$\diagdown$-P=S$\diagup$ $\longrightarrow$ $\diagdown$-P=O$\diagup$ ⎭

Figure 1.5   Examples of reduction reactions

Azo reduction

$-N=N-$ $\longrightarrow$ $-NH-NH-$ $\longrightarrow$ $-NH_2 + -NH_2$

Nitro reduction

$-NO_2$ $\longrightarrow$ $-NO$ $\longrightarrow$ $-NHOH$ $\longrightarrow$ $-NH_2$

R_2CO $\longrightarrow$	R_2CHOH	Ketone
$RCHO$ $\longrightarrow$	RCH_2OH	Aldehyde
$-HC=CH-$ $\longrightarrow$	$-H_2C-CH_2-$	Double bond
$AsO.OH$ $\longrightarrow$	AsO	Pentavalent to trivalent
$RS-SR$ $\longrightarrow$	$2RSH$	Disulphide to sulphydryl
R_2SO $\longrightarrow$	R_2S	Sulphoxide to divalent S
R_3NO $\longrightarrow$	R_3N	N-Oxide to tertiary amine

Figure 1.6   Examples of hydrolytic reactions

$RCOO\ R' \longrightarrow RCOOH + R'OH$ Carboxylic esters

$RNHCOOR' \longrightarrow RNHCOOH + R'OH$ Carbamates

$RCONHR' \longrightarrow RCOOH + NH_2R'$ Amides

$(RO)_2POOR \longrightarrow (RO)_2POOH + ROH$ Phosphoric esters

Epoxides

Figure 1.7   Substrate oxidation by the cytochrome P450 monooxygenase
system

Figure 1.8   Proposed scheme for the multiple pathways of
oxygen activation by the cytochrome P450 system
of mammalian liver microsomes

S   Substrate
X   Endogenous membrane lipid

From Estabrook et al. (1981).

Figure 1.9

| | Substrate | Cytochrome P448 | Cytochrome P450 |

Hydroxylation of various substrates by different

forms of cytochrome P450

Different products formed in vitro by liver microsomes after treatment

of rats with 3-methylcholanthrene (P448) or phenobarbitone (P450).

From Atlas and Nebert (1977).

Figure 1.10   Stereoisomeric forms of
               BP 7,8-dihydrodiol-9,10-oxide

Nomenclature of these compounds varies depending on author.

A. (-)-7β,8α-diol-9β,10β-epoxy-1
    (-)-1
    (-)-diol-epoxide 1
    (-)-syn isomer
    diol-epoxide II

B. (+)-7α,8β-diol-9α,10α-epoxy-1
    (+)-1
    (+)-diol-epoxide 1
    (+)-syn isomer
    diol-epoxide II

C. (-)-7α,8β-diol-9β,10β-epoxy-2
    (-)-2
    (-)-diol-epoxide 2
    (-)-anti isomer
    diol-epoxide I

D. (+)-7β,8α-diol-9α,10α-epoxy-2
    (+)-2
    (+)-diol-epoxide 2
    (+)-anti isomer
    diol-epoxide I

References

Buening et al. (1978)   Wood et al. (1976)
Koreeda et al. (1978)   Yagi et al. (1977)
Meehan and Straub (1979)   Slaga et al. (1979)
King et al. (1976)   Jennette et al. (1977)
Harvey and Cho (1977)

Stereoisomeric forms of Benzo(a)pyrene-7,8-diol-9,10-epoxide

A.

B.

C.

D.

Figure 1.11

The Metabolic Activation of Benzo(a)pyrene

Chapter 2

MATERIALS AND METHODS

2.1 <u>Animals</u>

Male Dunkin Hartley guinea pigs were housed, 4 per cage, in metal metabolism cages. They were maintained on normal laboratory diet, high fat diet, or high fat/high cholesterol diet <u>ad</u> <u>libitum</u> for a period of at least 15 weeks from weaning. For each group of animals used, controls, high fat and high cholesterol-fed animals were put on diet simultaneously to give age-matched animals.

2.2 <u>Diets</u>

(a) <u>High fat diet</u>

The high fat diet was prepared from a method used by the Nutrition Group (ProfessorT.G.Taylor) in the School of Biochemistry and Physiology, University of Southampton. The diet was made up in batches of 12kg, and the recipe used is given in Table 2.1. The recipes for the vitamin and mineral mixes used in the diet are given in Tables 2.2 and 2.3 respectively.

(b) <u>High cholesterol diet</u>

The cholesterol in the "high cholesterol" diet was given at a concentration of 0.1% of the diet, as the more usual level of 1% given to rabbits is reported to cause fatal haemolytic anaemia in guinea pigs (Traber and Ostwald, 1978). Green <u>et</u> <u>al</u>. (1976) reported that a 15-17 week dietary adaptation period is necessary for a 0.1% cholesterol diet, and so the animals were maintained on their appropriate diets for this length of time.

The diet was made up as for high fat diet, but with 172g/kg of coconut oil, and 1g/kg cholesterol. The cholesterol was dissolved in the oil before addition to the remaining ingredients.

2.3 Reference metabolites

Reference standards of benzo(a)pyrene metabolites were obtained from the IIT Research Institute, Chemical Repository (Kansas City, Missouri). These metabolites were:-

benzo(a)pyrene phenols: 1-, 3-, 6-, 7- and 9-hydroxybenzo(a)pyrene

benzo(a)pyrene quinones: 3,6- and 6,12-quinone

benzo(a)pyrene dihydrodiols: 7,8-, 9,10- and 4,5-diol

sulphate conjugates: potassium salts of sulphates of 3-, 7- and 9-hydroxybenzo(a)pyrene

glucuronide conjugates: 3-, 7- and 9-benzo(a)pyrenyl-beta-D-glucopyranosiduronic acid

2.4 General reagents

General laboratory reagents were analytical or hplc grade, and were obtained from BDH Chemicals Ltd. (Poole, Dorset).

2.5 Radiochemical techniques

The total tritium content of samples was determined by addition of a known aliquot (0.1ml-0.5ml) to Beckman Ready Solv EP scintillation fluid, or a scintillation cocktail prepared as described by Renwick et al. (1978), and counting in a Packard 3255 Tricarb Scintillation

Counter with correction for counting efficiency. Urine and DNA samples, hplc eluate fractions and petroleum ether and ethyl acetate extracts from epoxide hydrase assays were counted without prior treatment, whilst samples of faeces were bleached with alkaline aqueous hydrogen peroxide, and neutralised prior to scintillation counting.

The ^{14}C content of fractions of eluate from high-performance liquid chromatography (hplc) was similarly determined after the addition of liquid scintillation solution. Radioactivity associated with peaks detected on thin layer chromatography (tlc) plates was determined by removing the gel and liquid scintillation counting after addition of methanol (0.5ml).

Table 2.1

High fat diet

INGREDIENT	CONCENTRATION (g/kg)
Promosoy 100 (Prolux)	405.0
Bran	200.0
Coconut oil	173.0
Maize starch (cornflour)	49.5
Sucrose	100.0
Mineral mix	54.0
Vitamin mix	10.0
Methionine	4.2
NaCl	4.32
Vitamin C	6.0

The ingredients were mixed together, moistened with water, pelleted and dried.

Table 2.2

Vitamin mix

VITAMIN	WEIGHT (mg)
Biotin	26.4
Folic acid	264.0
Inositol	176.0
Nicotinic acid	5280.0
Calcium pantothenate	2640.0
Pyridoxine HCl (B6)	880.0
Vitamin A Mix	1000.0
Vitamin D Mix	200.0
Rovimix E25	30980.0
Menaphthone K	35.2
Vitamin B12	3.5
Riboflavin	880.0
Thiamine HCl (B1)	352.0
Choline chloride	176000.0

These vitamins were made up to a total of 1kg by the addition of 781.3g of cornflour. The vitamin B12 was added to the mixture as a solution in 3.0ml ethanol.

Table 2.3

Mineral mix

MINERAL	WEIGHT (g)
KIO_3	0.843
$CuSO_4 \cdot 5H_2O$	6.09
$MnSO_4 \cdot 4H_2O$	53.77
$ZnSO_4 \cdot 7H_2O$	61.95
$Na_2MoO_4 \cdot 2H_2O$	0.449
$FeSO_4 \cdot 7H_2O$	87.613
$CrK(SO_4)_2 \cdot 12H_2O$	0.960
$Na_2SeO_4 \cdot 10H_2O$	0.466
$Mg(OH)_2$	190.2
$CaCO_3$	879.0
KCl	1234.0
$CaHPO_4$	2338.0
$CaH_4(PO_4)_2 \cdot H_2O$	501.0
Limestone flour	501.0

Crystals were first ground to powder form using a pestle and mortar, then all powdered compounds mixed together to form the mineral mix.

Chapter 3

BILIARY EXCRETION OF BENZO(a)PYRENE METABOLITES

3.1 Introduction

Biliary and urinary excretion are complementary pathways for the elimination of xenobiotic compounds from the body (Hirom et al., 1976). Compounds of low molecular weight (below 350) are mainly eliminated in the urine, those with molecular weight above 450 are excreted largely in the bile in the rat, and compounds of intermediate molecular weight are eliminated extensively by both routes. Above the threshold molecular weight for biliary excretion, the extent of biliary elimination of compounds of similar molecular weight is very variable. These variations can be attributed to the chemical structure of the compound under examination (Hirom et al., 1972a). The molecular structure may influence lipid solubility of the compound, which in turn can influence the extent to which the substance is eliminated in the bile. The conjugation of xenobiotics with sulphate, glucuronic acid or glutathione increases the molecular weight of the compounds by approximately 100 to 300, and therefore increases the possibility of biliary excretion. The molecular weight of BP is 252 whilst the phenolic sulphates and glucuronides have molecular weights of 348 and 444 respectively. Thus the bile is likely to be the major route for elimination of BP metabolites.

In 1970, Levine investigated the effect of inducers of the MFO system (PB, MC and BP) on the biliary excretion of BP in the rat. The induction of these enzymes greatly enhanced the rate of biliary excretion as well as the rate of metabolism of BP. The injection of BP metabolites resulted in a higher rate of biliary excretion which was not increased further by treatment with inducing agents. He concluded that the rate-limiting step in the biliary elimination of BP metabolites was their formation from the parent hydrocarbon in the

liver, and went on to propose a scheme for the uptake of BP by the liver, and biliary excretion of its metabolites (Levine and Singer, 1972). The hydrocarbon is rapidly taken up from the blood by the liver, where it is oxidised by the MFO system of the endoplasmic reticulum. The products of these oxidation reactions are transferred to the cytosol where they may undergo phase II conjugation reactions and are rapidly excreted in the bile.

The biliary metabolites of BP produced by normal guinea pigs after administration of [^{14}C]-benzo(a)pyrene were therefore studied, as the molecular weight threshold may be more relevant to man than that of rats. The effect of high fat and high cholesterol diet on the biliary elimination of BP metabolites was also examined.

3.2 <u>Materials and Methods</u>

(a) <u>[7,10-^{14}C]Benzo(a)pyrene</u>

$$(116.9\mu Ci/mg)$$

[7,10- ^{14}C]Benzo(a)pyrene $\bigwedge$ was obtained from Amersham International plc, Amersham, Bucks., as a dilute toluene solution. Aliquots (250μl; 25μCi) were removed as required, the toluene evaporated in a stream of nitrogen and the solute dissolved in dimethyl sulphoxide (DMSO; 50μl). Doses were stored at -20°C for up to 24h until required.

(b) <u>Enzymes</u>

β-Glucuronidase was supplied by Sigma Chemical Company Ltd., Poole, Dorset, and β-glucuronidase/aryl sulphatase by Boehringer Mannheim GmbH, Mannheim, West Germany.

(c) <u>Collection of bile</u>

Guinea pigs were maintained on the appropriate diet for a period of 30-46 weeks after weaning.

Animals were anaesthetised by intraperitoneal injection of 25% urethane (0.5ml/100g), further quantities (0.1ml to 0.5ml) being administered as necessary to maintain anaesthesia. The bile duct was cannulated and the cystic duct tied off so that only hepatic bile was collected (Abou-El-Makarem <u>et</u> <u>al</u>., 1967). [7,10-^{14}C]Benzo(a)pyrene (25μCi in 50μl DMSO) was administered by injection into the external jugular vein via a cannula and the dose was flushed into the vein with saline. Bile samples were collected for up to 4h and saline was injected into the external jugular vein to maintain body fluid volume.

At the end of the experiment the animal was sacrificed, urine was taken from the bladder, and the caecum was removed and weighed. A 25% suspension of caecal contents in 0.01M phosphate buffer, pH 7.4, was prepared and gassed with nitrogen to maintain the anaerobic environment.

(d) <u>Incubations and extractions</u>

Bile samples (0.5ml) were incubated and extracted essentially as described by Chipman <u>et</u> <u>al</u>. (1981). Incubations were set up with 0.1M acetate buffer, pH 5.0 (0.5ml), β-glucuronidase (Ketodase, 10,000 units/ml), β-glucuronidase plus aryl sulphatase (500 units/ml) or a 25% suspension of caecal contents in 0.01M phosphate buffer, pH 7.4 (0.5ml). Tubes were gassed with nitrogen and incubated overnight at 37°C in the dark, with shaking. Tubes containing caecal content incubations were centrifuged in an MSE bench centrifuge for 15min, the

supernatant removed and adjusted to pH 7 by the addition of 0.1M sodium acetate buffer, pH 5.0. The remaining incubates were adjusted to pH 7 if necessary with 0.2M NaOH, and aliquots (20µl to 40µl) removed for thin layer chromatography. The remainder of the neutralised incubates and supernatant were extracted with ethyl acetate (3x5ml). Samples of non-incubated bile (0.2ml) were extracted directly with ethyl acetate (3x2ml). The ethyl acetate was evaporated to dryness with nitrogen at 35°C and the extracts dissolved in methanol (200µl).

(e) Thin layer chromatography (tlc)

Biliary metabolites of [^{14}C]benzo(a)pyrene were separated by thin layer chromatography using TLC-Ready Plastic Sheets (Schleicher and Schüll, F1500, Silica Gel) with a benzene:ethanol:2M aq. HCl (30:75:3) solvent system. Analyses were carried out on non-incubated bile, incubated bile prior to extraction with ethyl acetate, and also post-extraction β-glucuronidase, sulphatase and caecal content incubates. Plates were scanned using a Packard Model 7201 radiochromatogram scanner.

(f) High-performance liquid chromatography (hplc)

The method used for the separation of benzo(a)pyrene metabolites by reversed phase hplc was similar to that described by Chipman et al. (1981). The hplc system comprised a Waters Model 660 solvent programmer, 2 Model 6000A solvent delivery systems, a Model 440 uv absorbance detector (254nm), and the eluent was also monitored using an Aminco-Bowman spectrophotofluorimeter, with excitation and emission wavelengths of 290nm and 420nm respectively. Separation was effected

with a μBondapak C18 column (39cm x 1.8cm) using a linear gradient of 60-85% methanol in water over 40min, at a flow rate of 1.0ml/min. Final conditions were maintained for a further 10min at the end of the programme, and fractions of eluent were collected at 30sec intervals throughout the 50min run. The samples (50μl-150μl) were injected onto the column using a Waters U6K injector, as methanolic solutions derived from ethyl acetate extracts.

Fractions were collected either directly into scintillation vial inserts (Hughes and Hughes Ltd., Romford, Essex), using a 2112 Redirac fraction collector (LKB, S.Croydon, Surrey) or a Pharmacia FRAC-100 fraction collector (Pharmacia Fine Chemicals), or transferred to scintillation vial inserts after collection using a Gilson microcol TDC 80 fraction collector. The latter fraction collector required the flow to be stopped after 40min to allow the tubes on the collector to be changed. All tubes were rinsed with 0.5ml methanol, and the rinsings were added to the fractions in the scintillation vial inserts.

3.3 Results and Discussion

(a) Biliary excretion of [^{14}C]benzo(a)pyrene metabolites

An initial study of the metabolism of benzo(a)pyrene involved the administration of a dose of [7,10-^{14}C]benzo(a)pyrene (25μCi) to an anaesthetised guinea pig by intraperitoneal injection. The animal died 3h after dosing and only 9.9% of the dose was recovered in the bile in this time, with 0.02% in the urine. Subsequent animals were dosed intravenously, which resulted in increased recovery of radioactivity in the bile (see Table 3.1).

Bile from a normal guinea pig was collected at 30min intervals

after an intravenous dose of [^{14}C]benzo(a)pyrene (25μCi) and the ethyl

acetate extracts from the resulting incubates analysed by hplc with

fractions of eluate collected every minute for 50min. The elution

profiles showed no change in the pattern of radioactive peaks with time

(a typical elution profile is shown in Figure 3.1). The values for the

quantity of radioactivity in each of the four major peaks expressed as

a percentage of the radioactivity in the bile are given in Table 3.2.

The percentages indicate that over a 2h period of bile collection there

is no consistent change in the proportion of radioactivity in any of

the peaks with time. Bile taken from subsequent animals was, therefore,

collected at intervals of 2h. Some guinea pigs survived for less than

4h after administration of the radioactive benzo(a)pyrene, in which

case only the 0-2h bile samples collected from these animals were

included in the results. Bile was collected for a maximum period of 4h

after dosing, as much of the excreted dose was eliminated within this

time (see Figure 3.2). Some of the incubates from the original guinea

pig were extracted with 4 x 5ml ethyl acetate to determine whether

three extractions were sufficient to remove all extractable metabolites

from the incubation mixture. The fourth extract was dried down

separately, redissolved in methanol (200μl) and analysed by hplc. Only

3% of the extracted ^{14}C was recovered in the fourth extract, and hplc

analysis of this showed that only very polar metabolites remained in

the incubation mixture after three extractions with ethyl acetate.

Therefore subsequent bile samples were extracted only 3 times with

ethyl acetate.

To test whether the extracted BP metabolites could be detected

by uv absorption fluoresence, a sample of pre-dose bile was extracted

with ethyl acetate (3 x 2ml) and the solvent evaporated. The extract

was redissolved in methanol and analysed by hplc as described in the

Methods. The resulting u.v. and fluorescence traces of the eluate from the column showed a number of peaks which corresponded to those in the bile of animals given benzo(a)pyrene (see Figures 3.3 and 3.4). This indicated that the u.v.-absorbing and fluorescent material was largely intrinsic to the bile, rather than a product of benzo(a)pyrene metabolism, and analysis of the eluate was, therefore, carried out using the radioactivity elution profiles only.

(b) Biliary metabolites of [^{14}C]benzo(a)pyrene in guinea pigs fed various diets

The percentages of an intravenous dose of [^{14}C]benzo(a)pyrene which were excreted in the bile and urine of guinea pigs fed normal, high fat, or high cholesterol diets are shown in Table 3.1. The bile was the principal route of excretion in all groups, with about one-third of the dose recovered in 4h compared with less than 1% in the urine (see Table 3.1). There was no significant difference in the percentage dose in the bile between the dietary groups over a 4h period of bile collection, but high cholesterol animals showed significantly less ($p < 0.05$) radioactivity in their urine than normal guinea pigs after this time. This is probably a reflection of variable volumes of urine in the bladder at sacrifice.

Incubations of bile were performed as described in the Methods and analysed by hplc and tlc. Centrifugation of the caecal content incubates, and subsequent scintillation counting of the supernatant and resuspended pellet showed that 11-30% of the radioactivity in the incubate was present in the supernatant (see Table 3.3). Only 11.2% of the radioactivity was present in the supernatant of the caecal incubation of 0-2h bile from animals fed high cholesterol diet, which

was significantly lower than the 27.4% recovered in supernatant of caecal incubates of bile from normal animals. This suggests the presence either of more reactive or more lipid soluble benzo(a)pyrene metabolites in bile from high cholesterol-fed animals, which were bound to macromolecules retained in the pellet rather than remaining in the soluble fraction.

(i) <u>Tlc</u>

Tlc analysis of an incubate of guinea pig bile showed the presence of 3 peaks of radioactivity. A typical radiochromatogram scan is shown in Figure 3.5. The percentages of radioactivity remaining at the origin and running in peaks 1 and 2 in each of the incubates are given in Tables 3.4 and 3.5.

Tlc data for 0-2h bile from normal animals show that 30% of the radioactivity runs at the origin, 46% in peak 1, and 1% in peak 2 (see Table 3.4). Incubation of this bile with buffer (B; Table 3.4) resulted in a reduction in the amount of material in peak 1, and an increase in radioactivity remaining at the origin. Thus peak 1 contains some labile material which is hydrolysed non-enzymatically to yield highly polar products. A similar pattern is seen in 2-4h bile (see Table 3.5).

Unincubated bile from animals fed high fat and high cholesterol diets showed a greater proportion of radioactivity in peaks 1 and 2 than did normal guinea pigs, and the values were significantly higher in some cases (see Tables 3.4 and 3.5, A). Control incubation led to a shift of radioactivity from peak 1 back to the origin, as in normal animals, but a greater quantity of radioactivity remained in peak 1 (about 50% in 0-2h bile, compared with 33% in normals); therefore the extra material present in peak 1 in unincubated bile of high fat and high cholesterol fed animals appears to be stable to non-enzymatic

incubation.

Incubation of 0-2h bile from normal guinea pigs with β-glucuronidase (C) increased the percentage of radioactivity running in peak 2 from 4% to 13%. For animals fed high fat or high cholesterol diet, the increase in peak 2 material after β-glucuronidase incubation was significantly greater, so that 23-29% of the radioactivity on the plate was present in peak 2 (see Table 3.4). The quantity of radioactivity in peak 1 decreased after incubation with β-glucuronidase, and therefore peak 1 is probably the source of the increased peak 2 radioactivity. However, the lability of the peak 1 material prevents a definite conclusion.

Incubations of bile with β-glucuronidase/aryl sulphatase (D) showed similar results to the incubations with β-glucuronidase alone. The percentage of radioactivity in peak 2 of 0-2h bile from normal animals increased from 4% to 13%, whereas the values for high fat and high cholesterol fed animals (20-30%) were significantly higher (see Tables 3.4 and 3.5). Again the increase in radioactivity in peak 2 was accompanied by a decrease in peak 1.

Ethyl acetate extracted peak 2 almost completely, and partially extracted peak 1 material in all dietary groups (see Tables 3.4 and 3.5, F, G and H). Approximately 10-16% of the radioactivity remained in peak 1 after extraction.

Hydrolysis of peak 1 to peak 2 material may occur in vivo as part of the enterohepatic circulation of benzo(a)pyrene. Bile from high fat and high cholesterol fed animals contained significantly more ethyl acetate-extractable material after incubation with β-glucuronidase and β-glucuronidase/aryl sulphatase than did normal bile (see Table 3.6), the extra material running in peak 2 in tlc. This indicates the presence of more glucuronide, and possibly sulphate, conjugates in bile

of animals fed high fat and high cholesterol diet. Analysis of the 2-4h bile samples gave similar results to the 0-2h values but due to wider variation and smaller numbers for some of the analyses the treatment-related effects did not always achieve the same statistical significance.

(ii) Hplc

Fractions of hplc eluates were collected every 30sec for 50min, and the radioactivity profile of a typical eluate is shown in Figure 3.6. The fluorescence profile of the eluate is given in Figure 3.4. The radioactivity in each of the 8 peaks indicated in Figure 3.6 was calculated as a percentage of the total radioactivity in the bile for 0-2h and 2-4h bile samples, under each of the incubation conditions described in the Methods section (Tables 3.7-3.14). Values for radioactivity in incubates with caecal contents (E in Tables 3.7-3.14), are given as the percentage of radioactivity in the supernatant, and not the total caecal incubate (see Table 3.3). Values of percentage radioactivity in the supernatant, and total caecal incubations which are extractable by ethyl acetate are given in Table 3.6 for comparison.

In some incubates, predominantly the control incubations, a ninth peak was evident with a retention time 2min greater than that of peak 8. The radioactivity in this peak as a percentage of the total radioactivity in the bile did not show any dietary differences, and averaged less than 1% for each of the dietary treatment groups.

Peaks were identified by co-chromatography with authentic standards (see Chapter 2).

Peak 1 (see Table 3.7), consisted of highly polar material which eluted rapidly from the column. In unincubated 0-2h bile of

normal animals, this peak represented 6.8% of the radioactivity in the bile. Incubation with enzymes, especially sulphatase, reduced the amount of radioactivity in this peak, indicating the presence of ethyl acetate-extractable conjugates of BP metabolites. High fat or high cholesterol diet had little effect on the quantity of material detected in peak 1, and no definite trends towards increase or decrease were evident.

Peak 2 (see Table 3.8) eluted shortly after peak 1, and contained a smaller proportion of the total radioactivity in the bile (2.4% in unincubated, 0-2h bile from normal animals). Incubation with β-glucuronidase increased the proportion of radioactivity in this peak slightly, but sulphatase and microbial incubations did not result in an increase. No dietary differences were observed in the amount of radioactivity in peak 2.

Peak 3 (see Table 3.9), represented less than 1% of the radioactivity in unincubated bile. This peak had a retention time similar to that of the 9,10-dihydrodiol of benzo(a)pyrene (Figure 3.6). Control incubation increased the amount of radioactivity in this peak to 1-5%, but incubation with hydrolytic enzymes produced an even greater increase. β-glucuronidase alone had a more pronounced effect than β-glucuronidase/aryl sulphatase or microbial enzymes, resulting in 2-8% of the radioactivity in the bile running in peak 3. The bile of high fat and high cholesterol fed animals contained significantly more peak 3 material after incubation with β-glucuronidase than did that of normal animals (4-8%; $p < 0.01$ for 0-2h bile).

Peak 4 was identified as benzo(a)pyrene-4,5-dihydrodiol since it co-chromatographed with this metabolite when bile extract and the reference compound were injected simultaneously. It represented less than 1% of the radioactivity in unincubated bile (see Table 3.10).

Incubation with both buffer and enzymes increased the proportion of radioactivity running in this peak to 1-3% in 0-2h bile from guinea pigs fed normal diet. Incubation of 0-2h bile from animals fed high fat or high cholesterol diet produced a significantly greater increase, to about 13% after β-glucuronidase incubation, 11% after sulphatase, and 8% after microbial incubation.

Peak 5, corresponding to benzo(a)pyrene-7,8-dihydrodiol, was almost undetectable in unincubated and control incubated bile. Incubation of 0-2h bile from normal guinea pigs with β-glucuronidase increased the radioactivity in peak 5 to 4%, and in 2-4h bile, to 3% (see Table 3.11). The increases were lower after sulphatase and microbial incubations. Bile from animals fed high fat or high cholesterol diet contained more peak 5 material than that from guinea pigs fed normal diet after enzymatic incubations, but the differences were not statistically significant.

Peaks 6 and 7 (see Tables 3.12 and 3.13 respectively), probably represent quinone metabolites of benzo(a)pyrene. Each peak contained less than 1% of the radioactivity in unincubated bile, and this percentage increased to 1-5% for peak 6, and 1-3% for peak 7 after incubation with hydrolytic enzymes. No dietary differences were apparent.

Peak 8 represented a major constituent of the bile, its retention time suggesting that it was an hydroxylated metabolite of benzo(a)pyrene. However, on co-chromatography its retention time did not correspond exactly with either 3-hydroxy- or 9-hydroxy-benzo(a)pyrene, and the presence of interfering peaks in pre-dose bile prevented analysis by stopped flow fluoresence spectrophotometry. As was found for peaks 3 to 7, unincubated bile contained less than 1% of peak 8 material, but incubation with β-glucuronidase enzyme increased

this to 4% in normal 0-2h bile, and 6% in 2-4h bile (see Table 3.14). Increases in bile from animals fed high fat or high cholesterol diet were greater, especially after sulphatase incubation, though in most cases not statistically different from the normal values.

Most of these hplc peaks contained less radioactivity after incubation with sulphatase and caecal contents than after β-glucuronidase incubation. This suggests either a decreased hydrolysis of glucuronide conjugates, or an increased decomposition of the aglycone in the sulphatase and microbial incubates.

3.4 Summary

(a) Normal animals

To summarise the chromatography data, approximately 30% of the intravenous dose of [^{14}C]benzo(a)pyrene was excreted in the bile of guinea pigs during a 4h collection period, most of this in the 0-2h bile. Only 12-15% of the radioactivity in unincubated bile was extracted with ethyl acetate, but this percentage increased to 23-36% after incubation of bile with β-glucuronidase, aryl sulphatase or microorganisms. Thus, most of the radioactivity in unincubated bile consists of polar, non-extractable metabolites of benzo(a)pyrene. The material running at the origin on tlc appears not to be hydrolysed by incubation with enzymes; on the contrary, the percentage of radioactivity at the origin increased after incubation, probably due to decomposition of labile metabolites running in peak 1 on tlc. Incubation of bile with hydrolytic enzymes increased the amount of radioactivity in tlc peak 2 to 20-30% (from 2-7%).

Hplc analysis revealed 8 peaks of radioactivity. Peaks 1 and 2, representing the most polar of the extractable benzo(a)pyrene

metabolites, accounted for the largest percentage of the extractable material (approximately 6% and 3% of the radioactivity in the bile respectively). Incubation with hydrolytic enzymes had little effect on the material in these peaks.

Peaks 3, 4 and 5, the benzo(a)pyrene dihydrodiol peaks, each represented less than 1% of the radioactivity in unincubated bile, but increased to 2-5% after enzyme hydrolysis of normal guinea pig bile. Peaks 6 and 7, possibly quinone metabolites, also contained less than 1% of the radioactivity in unincubated bile, but incubation with enzymes increased this to approximately 2-3%.

Peak 8, a phenolic metabolite, increased from less than 1% in unincubated bile to 4-6% in normal bile after incubation with β-glucuronidase. Recently published data on the biliary elimination of benzo(a)pyrene metabolites in species other than the guinea pig will be discussed in Chapter 7.

(b) Effects of diet

Bile from animals fed high fat and high cholesterol diets had a higher percentage of radioactivity running in peak 1 after tlc (60-70%) than did normal guinea pigs (46-54%). This extra ^{14}C was found in peak 2 after treatment with hydrolytic enzymes and caecal contents.

Hplc analysis of the ethyl acetate extracts showed that the β-glucuronidase incubates of bile from animals fed high fat and high cholesterol diets contained more of the dihydrodiols (peaks 3, 4 and 5) than did bile from normal animals, and also more peak 8, although the differences in peak 8 were not significant.

The percentage dose recovered in the bile of animals fed high fat and high cholesterol diet was not different from the recovery in

normal bile, suggesting that the rate of epoxidation is unaltered by the fat or cholesterol content of the diet. This indicates either an increase in epoxide hydrase activity, or a decrease in activity of competing pathways such as glutathione conjugation.

Whatever the cause of the altered metabolism, high fat and high cholesterol diet in the guinea pig model system lead to an increased biliary excretion of glucuronide conjugates of benzo(a)pyrene dihydrodiols.

Table 3.1   Excretion of ^{14}C by guinea pigs after i.v. administration
of [^{14}C]benzo(a)pyrene

Diet	% dose in bile within 4h	% dose in urine within 4h
Normal	33.2 (12.5)	0.8 (0.5)
High fat	26.7 (13.4)	0.4 (0.5)
High cholesterol	36.5 (14.5)	0.1 (0.1)*

Male guinea pigs were maintained on the appropriate diet for 30-46 weeks from weaning. [^{14}C]Benzo(a)pyrene (25µCi/animal) was administered by cannula into the external jugular vein. Values are the means for 4 or 5 observations, with standard deviation in parentheses.

* $p < 0.05$ by Student's t-test when compared with animals fed normal diet.

Table 3.2

Biliary excretion of ¹⁴C after i.v. administration of a [¹⁴C]benzo(a)pyrene dose to a normal guinea pig, and distribution of radioactivity after hplc of extracts of bile incubates

¹⁴C in peak as percentage of radioactivity in bile

TIME (h)	CUMUL-ATIVE % DOSE	UNINCUBATED BILE				CONTROL INCUBATION				KETODASE INCUBATION				SULPHATASE INCUBATION			
		PEAK 1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
0-0.5	15.5	2.0	0.2	0.3	0.4	3.3	0.4	0.7	1.6	9.4	6.7	4.4	3.7	6.2	3.0	3.7	3.1
0.5-1	23.3	3.8	0.2	0.3	0.4	3.7	0.4	1.4	2.9	4.4	3.5	3.3	1.9	6.8	3.0	5.4	4.6
1-1.5	27.2	-	-	-	-	6.8	1.4	2.6	0.7	13.2	4.7	4.9	7.6	9.4	5.7	5.8	6.1
1.5-2	30.0	4.7	0.4	0.2	0.1	8.4	1.5	1.4	2.8	7.3	6.2	2.4	10.3	12.6	3.7	7.4	7.8
2-2.5	32.6	5.5	0.1	0.2	0.2	8.3	0.7	1.0	1.4	15.1	4.0	3.2	5.2	12.4	4.7	4.9	7.1
2.5-3	34.8	-	-	-	-	10.2	0.6	0.8	0.5	16.5	3.9	2.8	7.6	11.9	5.5	5.2	4.5
3-3.5	36.8	7.6	0.3	0.4	0.3	-	-	-	-	15.4	3.8	2.9	7.3	12.0	5.9	3.9	5.0
3.5-4	38.3	8.9	0.2	0.1	0.1	14.2	0.6	1.0	1.6	19.9	4.2	2.6	7.0	15.2	6.6	4.2	6.3
4-4.5	39.7	9.7	0.3	0.2	0.1	19.3	0.7	0.9	0.1	22.7	5.6	3.0	9.1	15.1	5.4	5.2	6.4
4.5-5	41.0	10.3	0.4	-	0.2	18.8	1.1	1.1	0.6	21.7	4.0	2.5	6.0	17.4	7.6	4.7	5.2
5-5.5	41.6	10.8	-	0.3	0.4	19.8	1.3	1.7	1.5	28.0	6.1	4.5	6.9	19.7	4.2	7.2	5.8

PEAK 1: polar metabolites   PEAK 2: diol   PEAK 3: quinone   PEAK 4: hydroxylated metabolites

Bile was collected at 30min intervals following an i.v. dose of [¹⁴C]benzo(a)pyrene (25µCi) to a normal guinea pig. Bile samples were incubated overnight at 37°C with buffer or hydrolytic enzymes, and ethyl acetate extracts of these incubates analysed by hplc. Peaks 1 - 4 in the table are those labelled as shown in Figure 3.1.

Table 3.3   Percentage of radioactivity in supernatant
             after centrifugation of caecal contents incubates.

Diet	0-2h bile	2-4h bile
Normal Diet	27.4 (10.2)	30.7 (11.7)
High Fat Diet	28.2 (24.4)	29.7 (18.3)
High Cholesterol Diet	11.2 (5.0)*	16.0

* p<0.05 by Student's t-test when compared with animals fed normal diet

Bile samples were collected from bile duct-cannulated guinea pigs
following i.v. administration of [^{14}C]benzo(a)pyrene (25µCi/animal).
Aliquots (0.5ml) of the samples were incubated overnight at 37°C with
homogenates of caecal contents taken from the animals at the end of the
experiment. The incubates were centrifuged, and the radioactivity in
the supernatant and in the resuspended pellet determined. The table
shows the percentage of the radioactivity in the total incubate which
was present in the supernatant. Values are the means for 2-5
observations, with standard deviation in parentheses.

Table 3.4   Radioactivity peaks in 0-2h guinea pig bile
after thin layer chromatography

	Peak		A	B	C	D	E	F	G	H
			Radioactivity in tlc peak as % on tlc plate					^{14}C in peak as % of total in bile after extraction		
Normal Diet	0	Mean	29.7	45.3	37.6	53.1	39.0	38.4	56.1	44.7
		SD	10.4	5.6	2.8	6.1	3.8	8.0	7.8	3.7
	1	Mean	45.9	32.7	29.2	21.4	24.6	16.2	12.9	14.2
		SD	8.6	6.0	3.9	4.3	2.9	4.5	3.2	8.3
	2	Mean	0.9	4.0	13.2	12.9	17.8	0.3	0.0	0.0
		SD	2.1	3.2	7.8	2.4	11.2	0.7	0.0	0.0
High Fat Diet	0	Mean	16.7	26.7*	31.8	36.5*	23.7	26.5*	31.2**	42.3
		SD	8.9	12.9	10.4	12.1	15.8	5.5	9.4	8.3
	1	Mean	63.5*	49.6**	26.6	26.2	19.1	11.9	10.6	13.4
		SD	10.5	4.1	4.7	4.6	13.2	1.8	2.1	9.6
	2	Mean	1.4	1.6	29.1**	27.6$$	14.8	0.0	0.0	0.0
		SD	2.7	2.1	4.2	3.7	17.2	0.0	0.0	0.0
High Cholesterol Diet	0	Mean	19.6	28.0$$	28.8$$	37.3**	31.8	21.8*	30.8*	31.9
		SD	2.5	4.4	2.3	7.3	7.0	7.7	18.1	13.7
	1	Mean	66.4**	48.0**	28.6	25.4	19.6	14.2	11.2	13.8
		SD	6.6	5.9	1.4	1.9	11.0	5.6	6.4	9.5
	2	Mean	5.2*	6.6	23.3*	25.6**	21.0	0.9	0.8	0.1
		SD	3.4	9.7	3.0	5.8	13.0	1.5	1.3	0.3

    * $p < 0.05$ ⎫
   ** $p < 0.01$ ⎬ when compared with normal diet
  $$ $p < 0.001$ ⎭

A unincubated bile
B control incubation
C β-glucuronidase incubation
D β-glucuronidase + sulphatase incubation
E caecal contents incubation
F C ⎫
G D ⎬ after ethyl acetate extraction
H E ⎭

Table 3.5   Radioactivity peaks in 2-4h guinea pig bile
                   after thin layer chromatography

	Peak		A	B	C	D	E	F	G	H

		Peak	A	B	C	D	E	F	G	H
	0	Mean	18.2	36.3	36.4	49.2	23.6	35.0	49.3	34.2
		SD	6.5	5.9	5.9	4.2	15.9	5.3	4.6	5.0
Normal Diet	1	Mean	53.5	32.7	22.2	20.9	5.3	12.9	13.9	12.1
		SD	9.4	6.2	15.5	2.5	10.7	8.8	2.0	10.5
	2	Mean	1.8	0.0	6.6	11.8	12.0	0.0	0.0	0.0
		SD	3.6	0.0	13.2	8.1	13.9	0.0	0.0	0.0
	0	Mean	16.3	40.0	30.5	42.2	17.7	27.4	35.8*	27.8
		SD	7.4	7.7	8.1	7.9	14.4	7.7	8.6	18.7
High Fat Diet	1	Mean	62.0	41.9	29.3	27.1	11.8	14.1	9.8*	12.6
		SD	16.2	7.1	4.6	8.5	14.0	2.1	2.0	15.9
	2	Mean	2.7	0.6	19.6	20.6	7.1	0.0	0.0	0.0
		SD	3.0	1.1	10.7	5.2	14.2	0.0	0.0	0.0
	0	Mean	5.4	15.9*	26.0*	29.8**	14.7	15.4*	24.3	20.6
		SD	9.4	14.1	3.4	5.8	25.5	9.6	21.6	19.9
High Cholesterol Diet	1	Mean	71.1*	41.6	32.2	25.1	0.0	10.6	8.2	9.2
		SD	5.3	6.9	2.9	3.6	0.0	7.2	7.2	15.9
	2	Mean	6.5	10.8	18.1	29.7*	0.0	0.0	0.7	0.0
		SD	5.8	9.8	16.2	9.9	0.0	0.0	1.2	0.0

Column group headers: A–E under "Radioactivity in tlc peak as % on tlc plate"; F–H under "^{14}C in peak as % of total in bile after extraction"

* $p < 0.05$ ⎫
** $p < 0.01$ ⎬ when compared with normal diet

A unincubated bile
B control incubation
C β-glucuronidase incubation
D β-glucuronidase + sulphatase incubation
E caecal contents incubation
F C ⎫
G D ⎬ after ethyl acetate extraction
H E ⎭

Table 3.6
Percentage of radioactivity in incubates extracted by ethyl acetate

Diet and Bile		A	B	C	D	Es	Et
Normal;	Mean	14.7	12.0	35.5	24.0	24.4	6.5
0-2h	SD	7.9	6.4	6.1	6.9	5.6	2.3
High fat;	Mean	10.6	20.9	53.5**	51.9**	32.7	4.0
0-2h	SD	6.0	16.2	4.6	10.9	22.7	1.9
High Choles- terol; 0-2h	Mean	11.0	27.5	56.4*	40.9	41.4	3.7
	SD	2.3	28.0	14.7	8.5	19.1	0.3
Normal;	Mean	12.3	11.1	36.1	22.6	30.1	9.2
2-4h	SD	2.8	3.3	1.9	5.4	6.6	3.9
High fat;	Mean	9.2	16.7	47.0*	47.4**	27.8	5.6
2-4h	SD	2.7	7.4	8.7	8.7	16.1	4.0
High Choles- terol; 2-4h	Mean	18.4	32.9	66.8*	43.0	37.2	3.6
	SD	7.0	31.5	21.5		20.5	

* $p < 0.05$ ⎤ by Student's t-test
** $p < 0.01$ ⎦ when compared with normal diet

A unincubated bile
B control incubation
C β-glucuronidase incubation
D β-glucuronidase + aryl sulphatase incubation
Es supernatant from caecal contents incubation
Et caecal contents incubation

Table 3.7   Peak 1 in radioactivity profile of hplc eluate

Diet and bile sample	Radioactivity in hplc peak as % of total in bile				
	A	B	C	D	E
N; 0-2h	6.8(2.0)	3.1(1.9)	5.1(2.2)	2.2(1.0)	2.8(1.1)
HF; 0-2h	6.2(4.2)	7.3(4.3)	4.6(1.7)	3.3(1.0)	4.7(4.4)
HC; 0-2h	4.7(0.3)	5.5(3.8)	4.5(2.6)	2.1(1.0)	4.5(1.8)
N; 2-4h	5.3(1.6)	5.9(4.5)	4.8(2.2)	2.2(1.1)	3.2(1.6)
HF; 2-4h	4.1(1.4)	4.7(2.8)	4.0(0.9)	4.3(1.2)*	3.9(2.7)
HC; 2-4h	7.7(3.4)	5.9(4.1)	13.2(14.0)	2.5	4.7(1.2)

* $p < 0.05$ by Student's t-test when compared
            with animals fed normal diet

N, HF and HC: Normal, high fat and high cholesterol diets.

A Unincubated bile
B Control incubation
C β-Glucuronidase incubation
D β-Glucuronidase/aryl sulphatase incubation
E Caecal contents incubation

Values given are means of 3-5 observations, with standard deviation
in parentheses

Table 3.8   Peak 2 in radioactivity profile of hplc eluate

Diet and bile sample	Radioactivity in hplc peak as % of total in bile				
	A	B	C	D	E
N; 0-2h	2.4(2.8)	0.9(0.6)	3.1(3.2)	2.5(1.3)	2.0(1.3)
HF; 0-2h	0.9(1.3)	1.9(2.3)	2.5(1.8)	1.8(2.4)	1.3(1.2)
HC; 0-2h	2.2(1.8)	2.3(3.3)	3.0(2.0)	1.0(1.5)	1.6(1.7)
N; 2-4h	3.4(0.6)	1.6(1.2)	6.5(1.4)	2.9(1.3)	2.5(1.0)
HF; 2-4h	2.0(1.7)	3.2(2.4)	4.6(2.4)	4.0(1.6)	3.4(2.7)
HC; 2-4h	4.2(2.6)	4.1(2.9)	3.5(3.0)	3.3	2.8(3.9)

N, HF and HC: Normal, high fat and high cholesterol diets.

A   Unincubated bile
B   Control incubation
C β-Glucuronidase incubation
D β-Glucuronidase/aryl sulphatase incubation
E   Caecal contents incubation

Values given are means of 3-5 observations, with standard deviation in parentheses

Table 3.9   Peak 3 in radioactivity profile of hplc eluate

Diet and bile sample	Radioactivity in hplc peak as % of total in bile				
	A	B	C	D	E
N; 0-2h	0.8(0.6)	0.7(0.7)	2.8(0.5)	1.5(1.1)	1.8(0.7)
HF; 0-2h	0.3(0.3)	1.4(1.3)	4.9(0.9)**	2.6(3.7)	2.8(2.0)
HC; 0-2h	0.8(0.5)	4.5(4.2)	6.3(1.8)**	3.7(2.8)	3.7(1.9)
N; 2-4h	0.2(0.2)	0.5(0.2)	2.0(1.1)	1.2(1.1)	1.5(1.0)
HF; 2-4h	0.3(0.2)	1.5(1.2)	3.5(1.1)	3.8(1.4)*	1.8(1.2)
HC; 2-4h	0.5(0.5)	3.0(2.8)	8.3(3.0)*	5.9	1.8(3.1)

* $p < 0.05$ by Student's t-test when compared
** $p < 0.01$ with animals fed normal diet

N, HF and HC: Normal, high fat and high cholesterol diets.

A Unincubated bile
B Control incubation
C β-Glucuronidase incubation
D β-Glucuronidase/aryl sulphatase incubation
E Caecal contents incubation

Values given are means of 3-5 observations, with standard deviation in parentheses

Table 3.10   Peak 4 in radioactivity profile of hplc eluate

Diet and bile sample	Radioactivity in hplc peak as % of total in bile				
	A	B	C	D	E
N; 0-2h	0.8(1.0)	1.1(1.1)	2.4(0.9)	3.0(2.7)	2.5(2.1)
HF; 0-2h	0.2(0.2)	2.1(3.1)	13.5(4.1)$$	11.2(10.7)	6.3(5.5)
HC; 0-2h	0.2(0.2)	2.8(4.0)	11.6(3.4)$$	11.8(3.2)**	12.1(8.9)*
N; 2-4h	0.3(0.3)	0.5(0.2)	2.4(0.7)	4.7(0.7)	3.2(1.3)
HF; 2-4h	0.2(0.2)	0.7(0.4)	2.8(1.2)	2.5(0.5)**	1.8(1.5)
HC; 2-4h	0.2(0.2)	1.4(0.9)	2.9(2.5)	5.3	2.8(2.3)

* $p < 0.05$ ⎞ by Student's t-test when compared
** $p < 0.01$ ⎬   with animals fed normal diet
\$\$ $p < 0.001$ ⎠

N, HF and HC: Normal, high fat and high cholesterol diets.

A   Unincubated bile
B   Control incubation
C β-Glucuronidase incubation
D β-Glucuronidase/aryl sulphatase incubation
E   Caecal contents incubation

Values given are means of 3-5 observations, with standard deviation in parentheses

Table 3.11   Peak 5 in radioactivity profile of hplc eluate

| Diet and bile sample | Radioactivity in hplc peak as % of total in bile | | | | |
	A	B	C	D	E
N; 0-2h	0.1(0.2)	0.0(0.1)	3.7(2.2)	2.6(1.6)	1.6(1.5)
HF; 0-2h	0.3(0.6)	0.7(0.6)*	4.6(0.6)	4.3(2.9)	2.5(1.7)
HC; 0-2h	0.3(0.2)	1.7(2.2)	5.6(2.3)	3.6(2.4)	3.0(1.3)
N; 2-4h	0.0(0.0)	0.2(0.1)	2.7(0.9)	1.2(2.4)	1.2(2.4)
HF; 2-4h	0.0(0.1)	0.0(0.0)	4.2(2.2)	5.6(1.7)*	1.7(1.0)
HC; 2-4h	0.0(0.0)	1.2(1.8)	10.3(10.0)	4.7	2.2(1.0)

* $p < 0.05$ by Student's t-test when compared
        with animals fed normal diet

N, HF and HC: Normal, high fat and high cholesterol diets.

A   Unincubated bile
B   Control incubation
C β-Glucuronidase incubation
D β-Glucuronidase/aryl sulphatase incubation
E   Caecal contents incubation

Values given are means of 3-5 observations, with standard deviation in parentheses

Table 3.12   Peak 6 in radioactivity profile of hplc eluate

Diet and bile sample	Radioactivity in hplc peak as % of total in bile				
	A	B	C	D	E
N; 0-2h	0.5(0.4)	0.7(0.5)	2.3(1.1)	2.8(1.8)	2.4(1.3)
HF; 0-2h	0.2(0.3)	1.0(0.9)	2.9(1.0)	3.1(3.0)	2.8(2.5)
HC; 0-2h	0.2(0.2)	1.5(1.0)	2.7(0.6)	4.5(2.3)	2.7(1.5)
N; 2-4h	0.3(0.4)	0.3(0.2)	3.4(1.7)	1.7(1.5)	2.4(1.8)
HF; 2-4h	0.2(0.3)	0.6(0.4)	4.0(3.7)	3.5(1.3)	1.8(1.9)
HC; 2-4h	0.4(0.5)	1.3(1.3)	1.3(1.1)	2.4	1.5(0.8)

N, HF and HC: Normal, high fat and high cholesterol diets.

A   Unincubated bile
B   Control incubation
C β-Glucuronidase incubation
D β-Glucuronidase/aryl sulphatase incubation
E   Caecal contents incubation

Values given are means of 3-5 observations, with standard deviation in parentheses

Table 3.13   Peak 7 in radioactivity profile of hplc eluate

Diet and bile sample	Radioactivity in hplc peak as % of total in bile				
	A	B	C	D	E
N; 0-2h	0.2(0.3)	0.7(0.4)	2.0(1.3)	1.6(1.5)	0.4(0.7)
HF; 0-2h	0.1(0.1)	0.8(1.0)	3.3(1.7)	0.9(1.7)	0.4(0.8)
HC; 0-2h	0.3(0.2)	1.2(1.3)	3.0(0.9)	1.4(1.6)	0.4(1.0)
N; 2-4h	0.0(0.1)	0.3(0.3)	0.3(0.6)	1.3(1.5)	0.0(0.0)
HF; 2-4h	0.0(0.0)	0.5(0.6)	1.6(1.1)	3.0(2.1)	0.9(1.0)
HC; 2-4h	0.0(0.0)	1.1(1.0)	1.6(1.5)	2.6	0.9(1.0)

N, HF and HC: Normal, high fat and high cholesterol diets.

A   Unincubated bile
B   Control incubation
C β-Glucuronidase incubation
D β-Glucuronidase/aryl sulphatase incubation
E   Caecal contents incubation

Values given are means of 3-5 observations, with standard deviation in parentheses

Table 3.14   Peak 8 in radioactivity profile of hplc eluate

Diet and bile sample	Radioactivity in hplc peak as % of total in bile				
	A	B	C	D	E
N; 0-2h	0.4(0.5)	1.1(1.5)	4.2(1.8)	2.0(1.4)	4.2(1.6)
HF; 0-2h	0.1(0.2)	1.5(2.4)	4.4(2.5)	4.5(3.9)	3.4(2.9)
HC; 0-2h	0.4(0.2)	2.6(3.6)	5.0(2.6)	3.3(2.0)	5.4(2.3)
N; 2-4h	0.1(0.1)	0.4(0.2)	6.3(3.4)	2.1(2.1)	7.0(6.1)
HF; 2-4h	0.2(0.2)	0.9(0.8)	8.6(6.2)	9.9(5.1)*	7.4(7.0)
HC; 2-4h	0.3(0.4)	1.4(0.9)	6.8(5.9)	9.6	9.4(7.4)

* $p < 0.05$ by Student's t-test when compared
        with animals fed normal diet.

N, HF and HC: Normal, high fat and high cholesterol diets.

A   Unincubated bile
B   Control incubation
C β-Glucuronidase incubation
D β-Glucuronidase/aryl sulphatase incubation
E   Caecal contents incubation

Values given are means of 3-5 observations, with standard deviation
in parentheses

Figure 3.1

Hplc profile of [^{14}C]benzo(a)pyrene

metabolites in guinea pig bile (30min sample)

Bile was collected at 30min intervals following adminis-

tration of an intravenous dose of [^{14}C]benzo(a)pyrene (25μCi)

to a bile duct–cannulated guinea pig (normal diet). Bile

samples were incubated overnight at 37°C with hydrolytic

enzymes, and ethyl acetate extracts of these incubates

analysed by hplc. Fractions of eluate were collected every

minute for 50min.

HPLC Profile of [¹⁴C]Benzo(a)pyrene Metabolites in Guinea Pig Bile (30min Sample)

Figure 3.2

Biliary elimination of ^{14}C after i.v.

administration of [^{14}C]benzo(a)pyrene

[^{14}C]Benzo(a)pyrene (25μCi) was administered intravenously to a bile duct-cannulated guinea pig (normal diet). The proportion of the dose eliminated in the bile within 5.5h of administration of the dose is shown.

Biliary Elimination of ^{14}C after i.v. Administration of [^{14}C]Benzo(a)pyrene

% dose in bile

Time after dosing (h)

Figure 3.3

Fluorescence profile of an extract of

pre-dose bile after HPLC

A sample of bile was taken from a bile duct-cannulated guinea

pig prior to administration of an i.v. dose of [^{14}C]benzo-

(a)pyrene. The bile sample (0.2ml) was extracted with ethyl

acetate, the solvent removed, and the extract redissolved in

methanol (200µl). The methanolic solution was analysed by

hplc, and the eluate monitored fluorimetrically.

Fluorescence Profile of an Extract of Pre-dose Bile after HPLC

Figure 3.4

Fluorescence profile of an extract of guinea pig

bile incubate after HPLC

Bile samples (2h) were collected from bile duct-cannulated guinea pigs after i.v. administration of [^{14}C]benzo(a)pyrene (25µCi), and incubated with buffer or hydrolytic enzymes. Ethyl acetate extracts of the incubates were analysed by hplc. A typical fluorescence profile of an hplc eluate is shown.

Fluorescence Profile of an Extract of Guinea Pig Bile Incubate after HPLC

Figure 3.5

Typical radiochromatogram scan of thin layer plate

after tlc of guinea pig bile incubate

Bile samples (2h) were collected from bile duct-cannulated guinea pigs following intravenous administration of [^{14} C]-benzo(a)pyrene (25μCi), and incubated overnight with buffer or hydrolytic enzymes, at 37°C. Samples (20-40μl) were analysed by tlc. Tlc analysis was also carried out on the incubates after ethyl acetate extraction.

Typical Radiochromatogram Scan of Thin Layer Plate after TLC
of Guinea Pig Bile Incubate

Figure 3.6

Hplc profile of [^{14}C]benzo(a)pyrene

metabolites in guinea pig bile

Bile samples (2h) were collected from bile duct-cannulated guinea pigs following intravenous administration of [^{14}C]-benzo(a)pyrene (25μCi). Samples were incubated at 37°C overnight with hydrolytic enzymes, and ethyl acetate extracts of these incubates were analysed by hplc. Fractions of eluate were collected every 30sec for 50min.
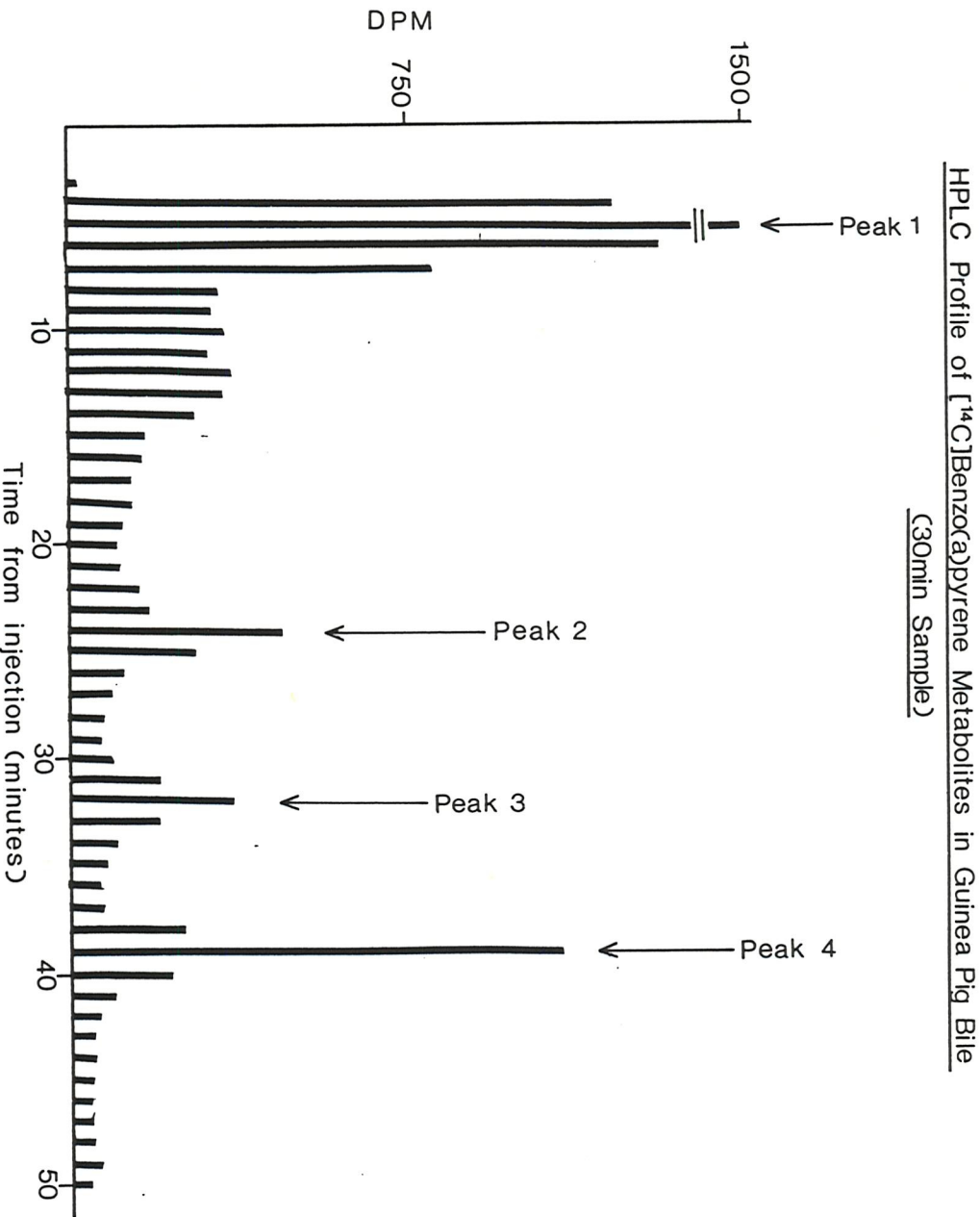
HPLC Profile of (^{14}C)Benzo(a)pyrene Metabolites in Guinea Pig Bile

Chapter 4

ACTIVITY OF INTESTINAL BACTERIA

4.1 Introduction

Quantitative changes in the intestinal microflora due to dietary alterations have been discussed in Chapter 1. Differences in the activity of certain bacterial enzymes have been noted in faecal samples from rats and from volunteers consuming different diets, and also between colon cancer patients and controls, and between high and low risk populations.

Enzyme activities which are commonly measured include β-glucuronidase and 7α-dehydroxylase. Reddy et al. (1977d) compared the intestinal mucosal and bacterial glucuronidase activity in rats fed high fat and high protein diets with those in animals fed diets containing normal levels of fat and protein. Animals fed a high fat (20% corn oil or 20% lard) diet had a higher β-glucuronidase activity in the caecal and colonic contents than did rats fed a 5% corn oil or 5% lard diet; however the type of fat in the diet did not affect the enzyme activity. Colonic mucosal β-glucuronidase activity was not influenced by diet, but animals fed high levels of fat and protein had significantly higher enzyme activity in the small intestinal mucosa than did the other groups. Goldin et al. (1978) reported an increase in faecal β-glucuronidase activity in rats after transfer from a grain to a high beef diet. The activities of bacterial azoreductase and nitroreductase also increased with this dietary change. In addition to rat faecal bacterial enzymes, these workers examined faecal β-glucuronidase, azoreductase and nitroreductase activity in human volunteers who normally consumed a mixed western diet, and were given dietary supplements of bran or wheat germ. These fibre supplements did not significantly alter the activity of any of the three enzymes.

Cummings et al. (1978a) studied the effect of high fat diet on

the faecal flora of male volunteers. They found no alteration in either the number of nuclear dehydrogenating clostridia or faecal β-glucuronidase activity. Reddy et al. (1974) did, however, find a difference in faecal β-glucuronidase activity in human volunteers consuming different diets. Those on a mixed western, high meat diet had significantly higher activity in their faeces than did volunteers consuming a non-meat diet.

Aries et al. (1971a) compared faecal flora of strict vegetarians and non-vegetarians. The flora of the group on the mixed western diet were similar to those of the strict vegetarians but the strains isolated from the former group were more active in degrading bile salts and acids in vivo. Only 20% of the cultures of Bacteroides spp. isolated from the faeces of strict vegetarians possessed 7α-dehydroxylase activity compared with 44% of the cultures isolated from the mixed diet group. The dehydroxylase enzyme produces secondary bile acids from the di- and tri- substituted primary cholanic acids in the bile (see Figure 4.1). One half of the faecal bile acids of the non-vegetarian group (49%) were mono- or unsubstituted, whereas only 30% of those of the strict vegetarians had been degraded.

MacDonald et al. (1978) measured 3α-, 7α- and 12α-hydroxy-steroid dehydrogenase (which oxidise the 3α-, 7α- and 12α-hydroxyl groups of bile salts) activities of faecal bacteria from Seventh-Day Adventists, control subjects eating a mixed diet, and patients with cancer of the large bowel. Cancer patients were found to have significantly more NAD- and NADP-dependent 7α-hydroxysteroid dehydrogenase activity than did control subjects, who in turn showed more activity than the vegetarian Seventh-Day Adventists. The other enzyme activities were not significantly different between the three groups.

Reddy et al. (1975) compared faecal 7α-dehydroxylase activity of colon cancer patients with control subjects, and reported activity to be significantly higher in the former group. A comparison of faecal flora obtained from high and low risk populations (English and Ugandan, respectively), revealed a greater number of Bacteroides in the high risk population, but no activity differences in the strains isolated from the two groups (Aries et al., 1969). However, in a later study (Hill et al., 1971), it was shown that although there were no large differences in the proportion of English and Ugandan strains producing hydrolases (which deconjugate bile salts to release the free bile acid) and dehydrogenases, very few Ugandan strains had 7α-dehydroxylase but approximately 40% of the English Bacteroides spp., Clostridium spp. and Bifidobacterium spp. possessed this activity. Furthermore, very few anaerobic strains isolated from Indian subjects (at low risk) showed 7α-dehydroxylase activity, whereas 40-50% of those from high risk American and Scottish populations were able to carry out this reaction.

Therefore there are not only increases in the numbers of the most active bacterial strains, Bacteroides and Clostridia, in high risk populations (see Chapter 1), but there are also differences in the activity of certain bacterial enzymes present in these strains. The promotional activity of various bile acids in experimental carcinogenesis in rats has been discussed in Chapter 1. The intestinal microflora convert the primary bile acids, cholic and chenodeoxycholic acid, to the secondary bile acids, deoxycholic and lithocholic acid respectively. Greater 7α-dehydroxylase activity will thus increase the conversion of these primary bile acids to the secondary forms, and result in a greater proportion of the latter in the bile, after entero-hepatic circulation. Cholecystectomy patients do have an increased concentration of secondary bile acids in their bile, and show an

increased risk for colon cancer (see Chapter 1). Van der Werf et al.
(1982) examined the absorption of deoxycholate from the colon in
adenomatous polyp patients and matched controls, and reported the
former group to have significantly greater absorption, and also a
greater proportion of secondary bile acids in the bile. The results
suggest that secondary bile acids are involved in the adenoma-carcinoma
sequence of the colon.

Hydrolysis of conjugates in bile by the gut flora and the
consequent release of the aglycone within the intestine increases the
possibility of enterohepatic circulation of a compound, and so prolongs
its presence in the body. If the aglycone is more toxic or carcinogenic
than the conjugate, then deconjugation by the gut bacteria has a very
important bearing on the toxicology of xenobiotics which have been
detoxified by the liver and excreted as phase II metabolites in the
bile. Factors which increase the activity of deconjugating enzymes of
the intestinal microflora, or the proportion of bacterial strains
capable of carrying out these reactions can therefore indirectly
influence the fate of a xenobiotic molecule within the body. Since
diets high in fat and animal protein can lead to an increase in faecal
β-glucuronidase activity, then individuals consuming such diets are
more likely to be capable of carrying out the hydrolysis of glucuronide
conjugates excreted in the bile.

The gut flora play an essential role in the carcinogenicity of
cycasin (methylazoxymethyl beta-D-glucoside), a compound present in
cycad nuts. This is hydrolysed to a carcinogenic aglycone (methyl-
azoxymethanol) by the gut flora, and is carcinogenic in rats only when
administered orally, and is not carcinogenic in germ-free rats when
administered by this route (Laqueur et al., 1967). The aglycone is
carcinogenic when administered by any route in both germ-free and

conventional rats, thus indicating the importance of the gut bacteria in the metabolism of this compound. A possible role for the gut flora in the generation of active metabolites of BP was studied by in vitro incubation using species common to control subjects and patients with colonic polyps (Finegold et al., 1975).

4.2 Materials and Methods

(a) Bacterial strains

The following pure bacterial strains were obtained from the Bacterial Metabolism Research Unit, Public Health Laboratories, Porton Down, Wiltshire. The bacteria were supplied as viable cultures in nutrient medium (Todd-Hewitt broth).

(i) Anaerobes

Clostridium perfringens 696, 697, 699, 700, 683 and 685, Clostridium bifermentans 672 and 726(1), Peptococcus 488, Bifidobacteria H Bif 4, T Bif 7 and H Bif 8, and Bacteroides thetaiotaomicron 503(1).

(ii) Aerobes

Escherichia coli 752, 80.5 and 72.6.

(b) Benzo(a)pyrene metabolites

(i) [³H]BP-7,8-dihydrodiol and [³H]3OHBP

[G³H]Benzo(a)pyrene-7,8-dihydrodiol (395 mCi/mmole) and [³H]3-hydroxybenzo(a)pyrene (258 mCi/mmole) were obtained from the NCI Radiochemical Repository, Midwest Research Institute, Kansas City, Missouri. 10μl of each solution in a tetrahydrofuran/triethyl-amine/benzene mixture was dried down under nitrogen, dissolved in DMSO (0.5ml), and 10μl aliquots of these solutions (25μCi/ml for the 7,8-dihydrodiol; 12.2μCi/ml for 3OHBP) used in the incubations.

(ii) Unlabelled benzo(a)pyrene reference compounds

A selection of those obtained from the Chemical Repository, IIT Research Institue, and listed in Chapter 2, were used in the incubations.

(c) Incubations and extractions

An aliquot of the appropriate bacterial culture (0.5ml) was added to 10μl of substrate solution (1mg/ml in DMSO) in a sterilised tube, and the anaerobic strains gassed with nitrogen. Tubes were incubated in the dark at 37°C for approximately 40h. Blanks were set up containing substrate solution (10μl) and anaerobic or aerobic medium (0.5ml) as appropriate, and incubated as for the samples. Substrate blanks containing bacteria but no substrate were also analysed.

Incubates were adjusted to pH 7, where necessary, with 0.2M NaOH. All incubates were extracted with ethyl acetate (3.0ml),

evaporated to dryness under nitrogen at $35°C$, and the extracts taken up in 200μl methanol. Solutions were stored in the dark at $-70°C$.

(d) Hplc

Extracts of the incubates in methanol ($50-75\mu$l) were analysed using a Waters Associates hplc system with Model 660 solvent programmer, Model 6000A solvent delivery systems, Model 440 (or 441) uv absorbance detector (254nm), and Model U6K injector, with a μBondapak C18 column (39cm x 1.8cm). A Waters Associates WISP 710B was used for automatic injection of some of the samples. The eluent was monitored fluorimetrically using an Aminco-Bowman spectrophotofluorimeter with the excitation and emission wavelengths set at 290nm and 420nm respectively, connected to an Infotronics Model 308/9 computing integrator. Metabolites were eluted from the column with a linear gradient of 80-100% methanol in water over 15min (for incubations with BP phenols as substrates), or 70-100% methanol in water over 15min (for incubations with BP-9,10-diol, [^{3}H]BP-7,8-diol, 3,6-quinone, and the sulphate and glucuronide conjugates of 9OH-, 3OH- and 7OHBP as substrates), at a flow rate of 1.0ml/min. Retention times are given in Table 4.1. For extracts from the incubations with radioactive substrates, fractions of eluate were collected every 30sec (for 30min), using a Pharmacia FRAC-100 fraction collector (Pharmacia Fine Chemicals, Hounslow, Middx.).

4.3 Results and Discussion

A fluorescence profile of standard BP metabolites, separated using a 70-100% methanol gradient as described in the Methods, is shown in Figure 4.2. The bacterial strains were incubated with a number of different BP metabolites as described in the Methods. Tables 4.2 and 4.3 show the incubations which were carried out, and whether or not any deconjugation or dehydroxylation took place. Radioactivity profiles of hplc eluates from the incubations with tritiated substrates ([^{3}H]3OHBP and [^{3}H]7,8-dihydrodiol) are shown in Figure 4.3. None of the primary BP metabolites, namely 9OH-, 3OH-, 7OH-, 6OH- and 1OHBP, BP-9,10-diol and BP-7,8-diol, underwent detectable metabolism by any of the strains tested. However, some of the bacterial incubates did result in deconjugation of glucuronide and, to a lesser extent, sulphate conjugates of BP metabolites. Figure 4.4 shows the fluorescence profiles of hplc eluates after separation of extracts of (a) 3-benzo(a)pyrenyl beta-D-glucopyranosiduronic acid (3 glc) incubated with anaerobic medium, and (b), 3 glc incubated with a strain of Clostridium perfingens. This strain resulted in deconjugation of the glucuronide to release the aglycone, 3-hydroxybenzo(a)pyrene. Table 4.4 shows the proportions of the strains tested which were able to carry out deconjugation reactions. 9-Benzo(a)pyrenyl beta-D-glucopyranosiduronic acid (9glc in Table 4.4) was deconjugated to differing extents by 3 strains of Clostridium and one of E.coli to release 9-hydroxyBP, and 3glc was hydrolysed by 2 strains of Clostridium, one each of Peptococcus and E.coli and by Bacteroides thetaiotaomicron to form 3-hydroxyBP. 9-Hydroxybenzo(a)pyrene sulphate was deconjugated by only 1 strain (E.coli 72.6). None of the strains tested was capable of releasing the aglycone from 3-hydroxybenzo(a)pyrene sulphate, or from

7-benzo(a)pyrenyl beta-D-glucopyranosiduronic acid (7glc). The 7-hydroxybenzo(a)pyrene sulphate underwent spontaneous deconjugation in all incubates, including the medium blank.

The percentages of the total fluorescence which corresponded to the conjugate, and to the primary metabolite released from the conjugate by the bacteria, are shown in Table 4.5. The differences in the percentage of the total fluorescence representing the aglycones is not necessarily a measure of activity differences between the strains, as the number of viable organisms in the cultures was not determined. The numbers indicate whether or not any deconjugation has occurred over and above that found in the blank, and therefore whether that particular strain of microorganism is capable of producing the primary metabolites of BP from the conjugates which are eliminated in the bile.

From these experiments, it is apparent that some strains of bacteria commonly occurring in the human gut are capable of hydrolysing biliary conjugates of BP, releasing the primary phenolic metabolites. These aglycones, and other primary metabolites used as substrates, appear to be stable to further degradation by the strains tested. The production of BP from conjugates in rat bile on incubation with homogenates of rat and human faeces, and certain pure cultures of bacteria reported by Renwick and Drasar (1976) was not observed with the strains and substrates tested in these experiments. However, deconjugation of phase II compounds by gut bacteria in the colon will increase the possibility of reabsorption of these metabolites, and prolong their presence in the body. Once in the mucosal cells, they may be metabolised to the highly reactive electrophilic compounds by the MFO system of the colonic mucosa and become bound to DNA unless they are conjugated once more by the action of transferase enzymes.

The activities of the bacterial enzymes are dependent upon the

physiological conditions within the gut, and these conditions can be altered by the diet. Many bacterial enzymes have pH optima close to pH 7, and consequently a lowering of colonic pH may reduce enzyme activity. Ugandans living on matoke only rarely carry C. paraputrificum, and the pH of their faeces is more acidic (pH 5-6) than that of people on a western diet, who have a neutral faecal pH. Hill and Aries (1971) reported that the faeces from Ugandan subjects showed a low level of faecal steroid degradation, and this could be due to the acidity of their colonic contents. Breast-fed babies also have an acid faecal pH compared with the almost neutral pH of faeces from bottle-fed babies; the former group rarely carry Clostridia before weaning, but these microorganisms are common in the gut of babies fed by bottle (Willis et al., 1973). Bowel cancer patients were reported to produce faeces with significantly higher pH than a group of vegetarian Seventh-Day Adventists (MacDonald et al., 1978).

Therefore a diet which increases colonic pH could increase bacterial enzyme activity in the gut. Colonic redox potential (Eh) is another factor influencing activity of gut bacteria. A more reducing environment favours the overgrowth of strictly anaerobic strains. If the ratio of faecal anaerobic/aerobic bacteria is an index of the reducing conditions in the gut, then people living in high risk areas for colon cancer and consuming a high fat diet have a lower Eh, and so favour the production of carcinogens from bile acids (Hill, 1974).

In the experiments described above, generation of primary metabolites of benzo(a)pyrene from glucuronides and sulphates was investigated. However, recently another pathway of xenobiotic metabolism, methylthiolation, has been described, which may also be important for BP (see Stillwell, 1981). This involves the incorporation of an $-SCH_3$ group into a molecule. One proposed mechanism for this

reaction is the cleavage of glutathione conjugates of xenobiotic molecules which are excreted in the bile, by C-S lyase enzymes of the gut microflora or mucosal cells. Experiments with germ-free and antibiotic-treated animals have shown that the presence of the gut flora is required for the formation of methylsulphonyl metabolites of the herbicide propachlor (2-chloro-N-isopropylacetanilide; Bakke et al., 1980), and methylthio metabolites of naphthalene, in vivo. Bakke et al. (1981) have shown that 2-acetamido-4-chloromethylthiazole (see Figure 4.5,I), is metabolised to the 4-methylthiomethyl-, 4-methylsulphinylmethyl- and 4-methylsulphonylmethyl derivatives (III, IV and V, respectively) by conventional rats, but not by germ-free animals. Chatfield and Hunter (1973) have shown the mercapturic acid analogue (II) to be a precursor of 2-acetamido-4-methylthiomethylthiazole, and the corresponding sulphoxide and sulphone (see Figure 4.5). The gut flora are required for the conversion of II to III.

Bakke et al. (1981) proposed two possible pathways for these reactions, both involving the gut bacteria (see Figure 4.6). One pathway involves the excretion of the mercapturate or its precursors in the bile, production of the sulphide (compound VI in Figure 4.6) by the action of microfloral C-S lyase on the mercapturate, and subsequent methylation. A thiol S-methyltransferase which could catalyse this reaction has been shown to be present in the intestinal mucosa in rats, and the highest concentration of the enzyme in the gut occurs in the large bowel (Weisiger et al., 1980). The second proposed pathway involves the production of compound VI in the liver by a tissue C-S lyase. This sulphide is then conjugated with glucuronic acid, and the conjugate excreted in the bile, to be hydrolysed in the gut by bacterial glucuronidase. The sulphide can then be methylated as in the first pathway. It is possible that the generation of a thiomethyl

derivative of benzo(a)pyrene could explain the apparent detection of BP from rat bile (Renwick and Drasar, 1976) since separation was dependent on simple tlc which may not have differentiated between BP and its thiomethyl derivative.

Table 4.1   Retention Times of Benzo(a)pyrene Reference Compounds
after HPLC

Retention Time (min)

Compound	80-100% Methanol Programme	70-100% methanol Programme
BP	16.5	18.7
3OHBP	12.7	15.4
[³H]3OHBP	12.2	—
9OHBP	14.6	15.4
7OHBP	15.2	15.4
1OHBP	15.9	—
9,10-Diol	—	6.7
9glc	—	3.2
9sulph	—	3.5
3glc	—	3.2
3sulph	—	3.5
7glc	—	3.4
7sulph	—	3.0
[³H]7,8-Diol	—	10.3

BP   Benzo(a)pyrene

3OHBP
9OHBP   3-, 9-, 7- and 1-Hydroxybenzo(a)-
7OHBP   pyrene
1OHBP

9,10-Diol   Benzo(a)pyrene 9,10- and 7,8-
7,8-Diol   dihydrodiols

glc   Glucuronide and sulphate conjugates
sulph   of 9-, 3- or 7-hydroxybenzo(a)pyrene

See Methods for details of hplc programmes.

Table 4.2   Effect of Various Clostridia spp. on Metabolites of
Benzo(a)pyrene

Substrate	Clostridium perfringens						C. bifermentans	
	696	697	699	700	683	685	672	726(1)
9OHBP	−	−		−			−	−
9glc	+	+		−			−	+
9sulph	−	−		−			−	−
3OHBP	−	−	−	−	−	−		
3glc	+	+	−	−	−	−		
3sulph	−	−	−	−	−	−		
7OHBP			?		−	−	−	−
7glc		−			−	−	−	−
7sulph			+		+	+	+	+
1OHBP	?	−	−	−	−	−	−	−
6OHBP	−		−	−	−	−	−	−
9,10-diol	−	−		−		−	−	
3,6-quinone		ND	ND	ND	ND		ND	ND
[³H]7,8-diol	−	−	−	−	−	−	−	−

+ indicates deconjugation or dehydroxylation reaction
− indicates no conversion of substrate by bacterial strain
ND No detectable fluorescence
Spaces indicate that no corresponding incubation was carried out.

See Table 4.1 for key to substrates used.

Table 4.3   Effect of Various Pure Bacterial Strains on Metabolites
of Benzo(a)pyrene

Substrate	ANAEROBES					AEROBES		
	488	H 4	T 7	H 8	BT	752	80.5	72.6
9OHBP								−
9glc								+
9sulph								+
3glc	+	−		−	+	+		
3sulph			−	−			−	
6OHBP			−					
9,10-diol	−							
3,6-quinone	ND	ND						
[^{3}H]7,8-diol	−	−	−	−	−	−	−	−
[^{3}H]3OHBP	−	−	−	−	−	−	−	−

+ indicates deconjugation or dehydroxylation reaction
− indicates no conversion of substrate by bacterial strain
ND No detectable fluorescence
Spaces indicate that no corresponding reaction was carried out.

Key to bacterial strains

488   Peptococcus
H 4 ⎤
T 7 ⎬   Bifidobacteria
H 8 ⎦
BT   Bacteriodes thetaiotaomicron
752 ⎤
80.5 ⎬ Escherichia coli
72.6 ⎦

See Table 4.1 for key to substrates used.

Table 4.4   Proportion of bacterial strains deconjugating
glucuronides and sulphates of benzo(a)pyrene phenols

BACTERIA	SUBSTRATE					
	9glc	9s	3glc	3s	7glc	7s*
Clostridium perfringens (6 strains)	2/3	0/3	2/6	0/6	0/3	3/3
Clostridium bifermentans (2 strains)	1/2	0/2	–	–	0/2	2/2
Peptococcus (1 strain)	–	–	1/1	–	–	–
Bifidobacteria (3 strains)	–	–	0/2	0/1	–	–
Bacteroides thetaio-taomicron (1 strain)	–	–	1/1	0/1	–	–
E. coli (3 strains)	1/1	1/1	1/1	0/1	–	–

* Spontaneous deconjugation in blank
- No corresponding incubation

Glc Glucuronides of 9-, 3- or 7-hydroxybenzo(a)pyrene
S   Sulphates   of 9-, 3- or 7-hydroxybenzo(a)pyrene

Table 4.5   Deconjugation of Benzo(a)pyrene Metabolites by Pure
                            Bacterial Strains

| Metabolite | Strain | % Fluorescence | |
		Conjugate	Aglycone
9OHBP-glucuronide	Blank	90	<1
	696	31	66
	697	6	85
	726(1)	78	19
	72.6	18	74
9OHBP-sulphate	Blank	95	1
	72.6	57	6
3OHBP-glucuronide	Blank	87	<1
	696	<1	90
	697	22	72
	488	12	80
	BT	8	55
	752	30	62
3OHBP-sulphate	None of the strains tested		
	deconjugated 3OHBP sulphate		
7OHBP-glucuronide	or 7OHBP glucuronide.		
7OHBP-sulphate	Blank*	32	48
	699	55	38
	683	37	57
	685	36	55
	672	36	52
	726(1)	32	56

Bacterial Strains

Clostridia
696, 697, 699, 683, 685, 672 and 726(1)

488 Peptococcus

BT   Bacteroides thetaiotaomicron

752 and 72.6   Escherichia coli

* Spontaneous deconjugation in blank.

Figure 4.1   Conversion of Primary to Secondary Bile Acids by Gut Bacteria

From Heaton(1972)

Figure 4.2

Fluorescence Profile of Standard BP Metabolites after HPLC

Separation was carried out using a linear gradient of 70-100% methanol in water over 15min at a flow rate of 1.0ml/min.

30HBP   3-Hydroxybenzo(a)pyrene
BP   Benzo(a)pyrene

Figure 4.3   Radioactivity Profile of HPLC Eluate after

Incubation of BP Metabolites with Bacteria

(a) [^{3}H]BP-7,8-Dihydrodiol

Substrate was incubated with a strain of Clostridium. Separation
was carried out using a linear gradient of 70-100% methanol in
water over 15min at a flow rate of 1.0ml/min.

(b) [^{3}H]3-Hydroxybenzo(a)pyrene

Substrate was incubated with a strain of Bifidobacterium.
Separation was carried out using a linear gradient of 80-100%
methanol in water over 15min at a flow rate of 1.0ml/min.

In both eluates, the major radioactive peak represents the primary
metabolite of benzo(a)pyrene. Neither was degraded by the strain
of bacteria used in the incubation.

Radioactivity Profile of HPLC Eluate after Incubation of BP Metabolites with Bacteria

Figure 4.4   Fluorescence Profiles of Incubations

using 3OHBP Glucuronide Substrate after HPLC

(a) Anaerobic medium

(b) Clostridium perfringens

3OHBP   3-Hydroxybenzo(a)pyrene

(a)

(b)

Figure 4.5

The Metabolism of
2-Acetamido-4-chloromethylthiazole
by Conventional Rats

2-acetamido-4-chloromethyl-
thiazole (I)

mercapturic acid
analogue (II)

4-methylthiomethyl
analogue (III)

4-methylsulphinyl-
methyl analogue (IV)

4-methylsulponyl-
methyl analogue (V)

From Chatfield and Hunter (1973)

Figure 4.6   Proposed pathways for the conversion of

2-acetamido-4-chloromethylthiazole to its methylthio

derivatives by the gut flora

2-acetamido-4-chloromethylthiazole

From Bakke et al (1981). See also Figure 4.5.

Chapter 5

TISSUE LEVELS OF XENOBIOTIC METABOLISING ENZYMES
AND GLUTATHIONE

5.1 Introduction

The properties of the cytochrome P450 complex, epoxide hydrase, UDP-glucuronyl transferase, sulphotransferase and glutathione S-transferase have been described in Chapter 1. Their sub-cellular and tissue distributions have also been reviewed. The profile of biliary metabolites produced from a xenobiotic compound is dependent upon the relative activities of these various enzymes in the liver and hepatic levels of their substrates and cofactors. Induction or repression of activity by exposure to environmental factors such as dietary inducers may alter this profile.

If polycyclic aromatic hydrocarbons are important in colon carcinogenesis, then the enzyme activities in the target organ will be of major importance. These enzymes can bring about the reactivation or detoxification of aglycones which have been released from biliary conjugates by the action of gut microfloral enzymes. The location of these enzymes renders them susceptible to inducing compounds present in the diet. Wattenberg et al. (1962) reported that in normal rats, benzo(a)pyrene hydroxylase (BP hydroxylase; AHH) activity was found in the mucosa of the small intestine, but in no other sections of the gut. Oral administration of 1,2-benzanthracene induced activity unevenly throughout the gut, whereas starvation or a diet low in fat decreased the activity of the enzyme system. AHH activity has also been detected in the duodenum of several other species, including mouse, rabbit, guinea pig, hamster, dog, monkey, baboon and man. In man, AHH was undetectable in the stomach and colon. Wattenberg (1971) also studied the effects of dietary manipulation on AHH activity in the small intestine of the rat, concluding that most, and possibly all, of the AHH activity at this site was attributable to exogenous inducers

present in the diet. A number of vegetables, particularly Brassicas such as cabbage, broccoli and cauliflower, were found to possess inducing activity. A number of different classes of compound can induce AHH, including flavones (Wattenberg et al., 1968), and polycyclic aromatic hydrocarbons.

The influence of dietary lipids and proteins on cytochrome P450 and AHH has also been studied. Stohs et al. (1976) reported that corn oil had no effect on AHH activity in intestinal microsomes from rats which were fed adequately, but increased that in microsomes from fasted rats. However, this increased level was still below the level observed in intestinal microsomes from fed rats. Olive oil and herring oil, fed to rats as 5 or 10% of the diet, have been reported to enhance hepatic and kidney AHH activity (Paine and McLean, 1973). Marshall and McLean (1971) reported that the liver microsomal cytochrome P450 content of rats fed on purified synthetic diets was much lower than that in rats consuming stock pellets; both protein and fat were needed to bring concentrations up to the levels found in stock pellet-fed animals. Phenobarbitone did not induce P450 in rats fed coconut oil in the diet, but did if unsaturated oils such as herring oil and linoleic acid were used in place of coconut oil. The potentiation by fats of cytochrome P450 induction in the liver by PB may depend upon their degree of unsaturation and peroxidation (Marshall and McLean, 1971). The polyunsaturated compounds are not themselves inducers (Marshall and McLean, 1971). Conversely, diets containing 24-34% olive oil, cacao butter or cholesterol were found to decrease intestinal and hepatic drug hydroxylation and glucuronidation (Hietanen et al., 1975). Lipid-rich diets can alter the structure and composition of both hepatic and intestinal microsomes (Laitinen et al., 1975). Such modifications may account for observed decreases in the metabolism of xenobiotics.

Hietanen and Hanninen (1974) measured BP hydroxylase and UDP-glucuronyl transferase activity in rat liver and intestinal mucosa in four groups of rats which had been fed on different commercially prepared pellets. Hepatic enzyme activities were very similar between the groups, but small intestinal mucosa showed marked variations in BP hydroxylase activity. The highest activity was noted in the group fed a diet containing the highest proportion of fat and certain vitamins.

Starvation reduced the activity of several drug-metabolising enzymes in rat liver and small intestinal mucosa, but enhanced the inducibility of several of these enzymes by phenobarbitone (Marselos and Laitinen, 1975).

Stimulation of cytochrome P450-dependent drug metabolism in rat colonic microsomes by gastrointestinal hormones has been reported (Fang and Strobel, 1981). Pentagastrin, cholecystokinin and secretin all increase colonic cytochrome P450 content, albeit to different extents. These hormones also increase the hydroxylation of various substrates to different degrees; cholecystokinin had the most marked effect on BP hydroxylation, producing a 2-fold increase. The hydroxylating activity of hepatic enzymes is also increased by pretreatment with these hormones, although to a lesser extent than colonic activity.

Therefore intestinal xenobiotic-metabolising enzymes appear to be more susceptible to the presence of inducers in the diet than those of the liver. Inducers include compounds such as flavones occurring naturally in the diet, as well as lipid components of the diet and contaminants such as BP, (although this occurs in some foodstuffs naturally to a small degree). In view of the changed pattern of biliary metabolites of BP in guinea pigs fed high fat and high cholesterol diet, the enzyme complement of the colonic mucosa is of major importance. The levels of enzyme activity were measured in animals fed

normal, high fat or high cholesterol diet, and also in animals pretreated with BP to investigate the possibility of an interaction between the high fat diet and the lipid soluble carcinogen.

5.2 Materials and Methods

(a) [7(n)-^{3}H]Styrene oxide

[7(n)-^{3}H]Styrene oxide (72mCi/mmol) was obtained from Amersham International plc, Amersham, Bucks., in liquid form, and dissolved in hexane (5.0ml). The solution was divided into 0.5ml aliquots and stored at -70°C until required. Each aliquot was purified before use, according to the method of Seidegard et al. (1977). The [^{3}H]styrene oxide solution (0.5ml; appoximately 200μCi) was washed 3 times with 100μl Tris buffer, pH 7.5, then extracted 3 times from the hexane with 0.5ml acetonitrile. Approximately 60% of the radioactivity was recovered in the combined acetonitrile extracts, and cold styrene oxide was added to give an 87mM substrate solution, with specific radioactivity of 3-4mCi/mmole. Substrate solutions were stored at -70°C overnight prior to use.

(b) Enzymes and Reagents

Glyoxalase I and glutathione reductase, both from yeast, methylglyoxal, oxidised glutathione and reduced nicotinamide adenine dinucleotide phosphate (NADPH) were supplied by Sigma Chemical Company Ltd., Poole, Dorset. Reduced glutathione and Ellman's reagent (5,5'-dithiobis(2-nitrobenzoic acid), DTNB) were obtained from BDH Chemicals Ltd., Poole, Dorset.

(c) Dosing of animals

Guinea pigs in each dietary group (normal,N; high fat, HF; high cholesterol, HC) were given 6 doses (each 3mg/kg) of BP orally over a 3 week period prior to sacrifice. Normal guinea pigs were dosed with BP in dimethyl sulphoxide (DMSO, 5mg/ml) and HF and HC animals with BP in coconut oil (5mg/ml). Animals were maintained on the appropriate diet throughout the dosing period, and sacrificed 16 weeks after weaning.

(d) Preparation of microsomes

The animals were killed and immediately removed to a cold room (4°C). The liver, small intestine, colon and rectum were removed and placed in ice-cold potassium chloride solution (1.15% KCl in 0.01M phosphate buffer, pH 7.4) as quickly as possible. The gut tissues were washed briefly with running cold tap water and rinsed with the ice-cold KCl solution. The mucosa was removed by scraping with a spatula onto a glass plate at 4°C. Part of the liver was chopped finely in KCl solution which was decanted off. The mucosa and liver preparations were homogenised in 3 volumes of ice-cold sucrose solution (0.25M in 0.01M phosphate buffer, pH 7.4) using 6 passes with a glass-teflon homogeniser. The suspensions were centrifuged at 12,000g for 20min at 4°C in a Beckman Model J2-21 centrifuge, and the resulting supernatants at 100,000g for 60min at 4°C in an MSE Prepspin 50 to prepare the microsomes. The microsomal pellets (from approximately 12g of liver, 12g of small intestinal mucosa, 2g of colonic mucosa and 3g of rectal mucosa) were resuspended in 0.052M Tris buffer, pH 7.5 (10.0ml for liver, 5.0ml for gut mucosae), and aliquots (2.5ml) removed for AHH assay. The remainder was recentrifuged at 100,000g for 60min, the

supernatant discarded and the microsomal pellet frozen (overlayered with 0.05M phosphate buffer containing 1mM EDTA, pH 7.6) for later determination of cytochromes P450 and b5.

(e) Determination of cytochrome P450 and cytochrome b5

These were determined essentially as described by Mazel (1971). Frozen microsomal pellets were allowed to thaw,and were resuspended in 0.05M phosphate buffer, pH 7.6, containing 1mM EDTA. The cytochrome b5 spectrum was recorded from 500 to 400nm using a Beckman Model 25 Spectrophotometer and Beckman recorder, by adding sodium dithionite to the sample cuvette. The cytochrome P450 spectrum was recorded similarly from the same microsomal suspensions, after addition of sodium dithioinite to the reference, and bubbling CO through the sample cuvette.

(f) Determination of aryl hydrocarbon hydroxylase

The assay procedure was essentially that described by Nebert and Gelboin (1968). Assay mixtures contained NADPH (0.36μmoles), Tris chloride buffer, pH 7.5 (50μmoles), MgCl (3μmoles), microsomal suspension (100μl) and benzo(a)pyrene (80nmoles). The reaction was stopped after 30min by addition of ice-cold acetone (1.0ml) and the reaction products extracted into hexane (3.25ml) by shaking for 10min at 37°C and standing at room temperature in the dark overnight. Acetone was added to blanks before incubation. The hydroxylated metabolites were recovered by extracting an aliquot (1.0ml) of the organic phase with 1M NaOH (3.0ml) in the dark at room temperature for 10min. The fluorescence of the alkaline solution was determined at the

activation maximum (396nm) and emission maximum (522nm) using an Aminco-Bowman spectrophotofluorimeter. Values were related to the fluorescence of a standard 3-hydroxybenzo(a)pyrene solution, using quinine sulphate (0.3µg/ml) at its maxima (activation 352nm; emission 452nm), as a working reference standard.

(g) Preparation of liver for glutathione determination

The tissue was prepared largely as described by Akerboom and Sies (1981). The animals were killed, and a portion of their liver dropped immediately into liquid nitrogen. The frozen liver was then pulverised, an aliquot (0.5g) of the powdered tissue weighed, and homogenised in 5 volumes of ice-cold 1M perchloric acid, containing 2mM EDTA. The extracts were centrifuged in a bench centrifuge for 5min to separate the precipitated protein, and the supernatants neutralised with a solution containing 2M KOH and 0.3M N-morpholinopropanesulphonic acid (MOPS). The extracts were then centrifuged once more to remove the precipitate, and the supernatant assayed immediately.

(h) Determination of reduced glutathione

Reduced glutathione (GSH) was assayed as described by Akerboom and Sies (1981), based on the method described by Racker (1951). The assay involves the first stage of a two-step reaction in which methylglyoxal is converted to lactic acid. The first stage of this reaction is the condensation of methylglyoxal with GSH, catalysed by glyoxalase I. The condensation product, S-lactoyl-GSH, is hydrolysed to glutathione and lactic acid in the presence of glyoxalase II. The formation of S-lactoyl-GSH was followed spectrophotometrically at 240nm

using a Beckman Model 25 spectrophotometer. Cuvettes contained 2.0ml 0.05M potassium phosphate buffer, pH 7.0, 2-100μl 1mM GSH standard (in 1M perchloric acid/2mM EDTA neutralised with 2M KOH/0.3M MOPS), or neutralised liver extract (undiluted, or diluted 10 times in 0.05M phosphate buffer), and 100μl glyoxalase I enzyme (37.5U/ml). After a period of equilibration at 37°C, the reaction was started by the addition of 110mM methylglyoxal (40μl) to the sample cuvette. A further 40μl 110mM methylglyoxal was added at the end of the reaction to determine the blank absorbance.

(i) Determination of total glutathione

The sum of reduced (GSH) and oxidised glutathione (GSSG) was determined by a method from Owens and Belcher (1965) and Tietze (1969), described by Akerboom and Sies (1981). Reactions were again carried out at 37°C in a Beckman Model 25 spectrophotometer. Cuvettes contained 2.0ml 0.1M phosphate buffer, pH 7.0, containing 1mM EDTA, 200μl GSH standard (0-20μM in buffer) or neutralised extract diluted 50 or 100 times in buffer, 40μl DTNB (1.5mg/ml in 0.5% NaHCO$_3$) and 40μl glutathione reductase (6U/ml). After equilibration for 3min, the reaction was started by the addition of 100μl NADPH (4mg/ml in 0.5% NaHCO$_3$). The absorbance of the 5-thio-2-nitrobenzoate (TNB) formed was followed at 412nm.

(j) Epoxide hydrase assay

Epoxide hydrase in liver was determined essentially by the method developed by Oesch et al. (1971), and modified by Seidegard et al. (1977). Portions of liver from those animals used for assay of

glutathione levels were dropped immediately into ice-cold 0.25M sucrose, blotted with filter paper, weighed, returned to the same solution, and washed several times with 0.25M sucrose solution. The samples were then chopped finely before homogenisation in a glass/teflon homogeniser. The volume was made up with 0.25M sucrose to give approximately 100mg fresh liver/ml. The homogenate was centrifuged at 10,000g for 10min and the supernatant used in the epoxide hydrase determinations.

Assays were set up in duplicate, with a blank containing 0.25M sucrose in place of supernatant. Incubations contained 0.25ml 0.5M Tris chloride buffer, pH 7.5 and 0.75ml 10,000g supernatant, and were pre-incubated at 37°C for 2min before addition of purified [^{3}H]styrene oxide (20µl; 2.9-3.3mCi/mmole). After 45min incubation at 37°C, reactions were stopped by the addition of ice-cold petroleum ether (b.p. 40-60°; 5.0ml). The mixtures were vortexed immediately, and unused substrate removed by shaking for 5min, centrifuging for 5min in a bench centrifuge, and freezing. The petroleum ether layer was removed, and this extraction repeated. [^{3}H]Styrene glycol product was similarly extracted into ethyl acetate (5.0ml), omitting the freezing step. Aliquots of the combined petroleum ether extracts (200µl) and ethyl acetate extract (500µl) were counted as described in Chapter 2. Aliquots of the 10,000g supernatant were used to determine protein content.

(k) Protein determination

Both microsomal and 10,000g supernatant protein were determined as described by Mazel (1971), based on the original method by Lowry et al. (1951), as modified by Miller (1959). All assays were carried out

in duplicate, and the absorbance read at 540nm. Bovine serum albumin
was used as the standard.

5.3 Results and Discussion

(a) Cytochrome P450 levels in guinea pig tissues, and effects of oral administration of benzo(a)pyrene

The levels of cytochrome P450 in liver, small intestine, colon
and rectum of guinea pigs in each dietary group before and after
administration of benzo(a)pyrene orally are given in Table 5.1. The
enzyme was undetectable in colon and rectum in all 6 groups of
animals. The cytochrome P450 content of small intestinal mucosal cells
was unaltered by the high fat or high cholesterol diets, by benzo(a)-
pyrene pretreatment, or by a combination of diet and dosing.
Examination of the amount of cytochrome P450 in the liver again showed
pretreatment with BP to have no effect at the level used. In this
tissue, however, dietary differences were observed; in both dosed and
undosed groups of guinea pigs, the feeding of high fat and high
cholesterol diets significantly reduced the quantity of cytochrome P450
in relation to the amount of microsomal protein in the tissue. When
expressed as nmoles/g liver, however, the quantity of cytochrome P450
was no longer significantly different between the dietary groups of
undosed animals, but again showed a reduction in the dosed high fat and
dosed high cholesterol groups when compared to dosed, normal animals
(see Table 5.2). The high fat and high cholesterol diets increased the
quantity of microsomal protein per gram liver in undosed animals,
although the difference was not significant for the high fat group

(see Table 5.3). Therefore, although the quantity of cytochrome P450 per mg microsomal protein was reduced in animals fed high fat or high cholesterol diet, the elevation in total microsomal protein in these same animals resulted in similar levels of the haemoprotein in all groups when expressed as nmoles per gram tissue. Hence, no difference was observed in the extent of epoxidation of a dose of [^{14}C]benzo-(a)pyrene (this is consistent with the previous finding that the percentage of the dose eliminated in the bile within 4h was not significantly different between the three dietary groups; see Chapter 3, Table 3.1).

Pretreatment of guinea pigs with benzo(a)pyrene increased the quantity of microsomal protein per gram liver in normal animals (see Table 5.3), but did not enhance the inducing effect of the high fat and high cholesterol diets; thus dosed high fat and dosed high cholesterol groups had comparable levels of microsomal protein per gram liver to normal, dosed animals.

Small intestinal microsomal protein content was not altered by diet or BP pre-treatment (Table 5.3), and cytochrome P450 levels consequently remained at a similar value in all 6 groups when expressed as nmoles per gram tissue (see Table 5.2).

(b) Cytochrome b5 levels in guinea pig tissues, and the effects of oral administration of benzo(a)pyrene

The cytochrome b5 levels detected in guinea pig tissues are shown in Table 5.4. In the colon, cytochrome b5 was detected in only one animal (undosed, normal group). In the rectum it was detected in only three animals - one in each of the three groups of dosed animals. These levels were low, the highest values being 0.06 nmoles cytochrome

0.5

b5/mg microsomal protein, and have not been included in Table 5.4.

As described for cytochrome P450, the quantity of cytochrome b5 in the small intestinal mucosa was unaffected by diet or BP pretreatment. In the liver, BP pretreatment again had no effect, but high cholesterol diet significantly reduced the level of cytochrome b5 in undosed animals ($p < 0.05$) when expressed as nmoles per mg microsomal protein. Values expressing the quantity of cytochrome b5 in liver as nmoles per gram tissue are given in Table 5.5. The difference between high cholesterol-fed and normal animals was no longer significant, due to the increase in microsomal protein in the former animals. Pretreatment of normal animals with BP increased b5 in liver, from 2.7 $\pm$ 0.9 to 4.2 $\pm$ 1.0 nmoles/0.5 g liver ($p < 0.05$); no such induction was seen in high fat or high cholesterol fed animals.

The effects of diet upon this haemoprotein are therefore less marked than in the case of cytochrome P450, with only one group showing a statistically significant difference from the appropriate control group. The difference was no longer evident when increased microsomal protein content was taken into account.

(c) <u>AHH levels in guinea pig tissues, and effects of oral administration of benzo(a)pyrene</u>

AHH levels in guinea pig liver, small intestine, colon and rectum are given in Table 5.6. There was no significant difference in the quantity of enzyme in liver, colon or rectum when comparing dietary groups of dosed or undosed animals, and pretreatment with BP did not significantly alter AHH levels in these tissues. Administration of high fat and high cholesterol diets did not alter AHH activity in small intestinal mucosa when compared with that in normal, undosed animals.

However, the hydroxylase was found to be inducible in the small intestinal mucosa of normal guinea pigs by pretreatment with BP ($p < 0.05$). Conversely, BP pretreatment did not induce AHH activity in the small intestinal mucosa of animals fed high fat and high cholesterol diets. A significant difference was observed between the activity of the enzyme in the small intestinal mucosa of BP-dosed animals fed high fat and high cholesterol diets when compared with BP-treated guinea pigs fed normal diet ($p < 0.01$). This presumably reflects the increased level of AHH in the latter group, and the lack of induction by BP in the high fat and high cholesterol groups.

The aryl hydrocarbon hydroxylase of small intestinal mucosa is therefore more susceptible to induction by oral administration of BP than the enzyme system of the liver, or mucosa of the lower gut. The small intestine is the site of absorption for lipid-soluble substances such as BP, and this may account for the greater sensitivity of this tissue towards the inducing effects of this compound. A diet rich in fat or fat and cholesterol may be expected to increase the solubility of BP, and thus potentially enhance its inducing effects by increasing the quantity of inducer present within the tissue. However, these experiments show that the high levels of fat or fat + 0.1% cholesterol in the diet are counteracting the inductive effects of BP pretreatment observed in control animals. This may be due to alterations in the structure of the endoplasmic reticulum of the small intestinal mucosa, brought about by the dietary fat or cholesterol, rendering the hydroxylase less susceptible to induction by BP, or possibly due to an increased absorption of BP with lipid in the lacteal system.

Wills (1983) has studied the effects of various dietary lipids on the metabolism of BP in liver and small intestine of rats. The lipids chosen contained different proportions of saturated and poly-

unsaturated fatty acids. The results of the experiments showed that the dietary fats caused marked variations in the fatty acid composition of the phospholipids of the endoplasmic reticulum, especially linoleic acid (C18:2), and two ω-3 fatty acids, eicospentaenoic acid (C20:5) and docosahexaenoic acid (C22:6). The phospholipid composition of the membrane reflected the fatty acid composition of the administered lipid. Thus, feeding a corn oil diet results in a greater proportion of linoleic acid being incorporated into the membrane than does feeding a lard or coconut oil diet which contain less of this fatty acid. Fish oils contain little linoleic acid, but higher proportions of eicospentaenoic and docosahexaenoic acids, and feeding fish oil diets leads to a reduction in linoleic acid content, but a marked increase in the incorporation of the ω-3 fatty acids into phospholipids. These alterations in membrane composition resulted in significant changes in the rate of BP metabolism by these tissues, the rate increasing with increased proportion of unsaturated fatty acids in the diet.

Gower and Wills (1982) reported that increasing quantities of coconut oil in the diet increased BP hydroxylase activity in rat small intestinal mucosa by as much as 2.5 times over the levels in rats fed fat-free diet. Guinea pig intestinal mucosa did not show similar increases in AHH activity in the experiments reported in this thesis, when comparing animals fed a diet containing 17% coconut oil and animals on a normal diet.

(d) Epoxide hydrase levels in liver

The quantities of epoxide hydrase in liver extracts from animals fed normal, high fat and high cholesterol diets are shown in

Table 5.7. Table 5.7(a) shows values only for age-matched animals, but other guinea pigs which were not age-matched are included in Table 5.7(b). The inclusion of these non-age-matched animals does not alter the significance of the results; hepatic epoxide hydrase levels did not change with diet.

Oesch and Bentley (1976) have reported that the styrene oxide and BP 4,5-oxide hydrases co-purify with the same ratio of activities and susceptibilities to various inducers. In the experiments in this thesis, 10,000g supernatant was used with styrene oxide substrate, so both cytosolic and microsomal epoxide hydrases were present. Ota and Hammock (1980) examined epoxide hydrase activities towards three substrates in hepatic microsomes and 100,000g supernatant from rats, mice and guinea pigs. Substrates used were styrene oxide, trans-β-methylstyrene oxide and allylbenzene oxide (see Figure 5.1). Cytosolic activity towards styrene oxide was undetectable in all three species, but this compound was a good substrate for the microsomal enzyme. Microsomal activity towards methylstyrene oxide was undetectable; allylbenzene oxide was a suitable substrate for both cytosolic and microsomal enzymes.

An increase in epoxide hydrase activity in the liver of high fat and high cholesterol fed animals could have, at least in part, explained the observation that an increased proportion of dihydrodiol metabolites was eliminated in the bile of these animals (see Chapter 3). However, no increases were observed, so the cause of the differences in biliary metabolite profiles must be sought elsewhere. If epoxidation of benzo(a)pyrene by the liver is unaltered by diet, and dihydrodiol formation is increased in high fat and high cholesterol fed animals without an increase in epoxide hydrase activity, then there must be a reduction in the latter groups of animals of an enzyme or

substrate involved in a pathway competing with epoxide hydrase for the phase I metabolites of BP in the liver. A reduction in either quantity or activity of the transferase enzymes, or in hepatic levels of glutathione, UDP-glucuronic acid, or inorganic sulphate could result in increased dihydrodiol formation in the liver by high fat and high cholesterol fed animals.

(e) Glutathione levels in guinea pig liver

Levels of reduced, and reduced plus oxidised glutathione are shown in Table 5.8, together with the percentage of total glutathione which was in the reduced form. Dietary alterations did not significantly change any of these levels in age-matched animals (Table 5.8). This was also observed when non-age-matched animals were included in the study. The proportion of reduced glutathione was surprisingly low in many of the animals. Reports in the literature estimate the percentage of reduced glutathione to be about 95% or more of the total glutathione present (Akerboom and Sies, 1981; Sies et al., 1980). Values obtained in these experiments were as low as 40% in some cases. The tissue was frozen immediately upon removal from the animal by dropping it into liquid nitrogen. This could have trapped a layer of air around the tissue, delaying the freezing process (Williamson and Corkey, 1969), and consequently autooxidation of glutathione will have occurred to an unknown extent. However, there were no differences between the dietary groups, and therefore altered glutathione levels cannot explain increased dihydrodiol production in the high fat and high cholesterol groups. The sulphate conjugates represent only a small proportion of biliary metabolites of BP in the guinea pig, so it seems unlikely that alterations in sulphotransferase activity or the quantity

of the enzyme present in the liver of animals fed high fat or high cholesterol diets have occurred which could explain the increased dihydrodiol production in these animals. Glucuronide and (probable) thio-ether conjugates are the main polar biliary metabolites of BP, and therefore changes in the UDP-glucuronyl transferase or glutathione S-transferase enzymes are more likely to be responsible for the alteration in profile of biliary BP metabolites. These possibilities will be discussed in Chapter 7.

5.4 Summary

Although guinea pig liver and small intestinal mucosa are susceptible to BP pretreatment or the administration of high fat and high cholesterol diets to varying extents, the colon in this animal possesses undetectable levels of cytochrome P450 and AHH activity. Furthermore, the administration of repeated oral doses of BP and/or high fat and high cholesterol diets does not induce either P450 or AHH to levels at which they become detectable. Therefore, negligible activation of BP metabolites to carcinogenic compounds can be expected to occur in this tissue, mediated by the mixed function oxidase system.

Table 5.1   Cytochrome P450 levels in guinea pig tissues
(nmoles/mg microsomal protein) $\swarrow$ 0.5

Tissue	Treat-ment	Normal Diet	High Fat Diet	High Cholest-erol Diet
Liver	UD	0.89(0.08)	0.60(0.10)**	0.51(0.01)$$
	D	0.73(0.14)	0.50(0.07)**	0.53(0.19)*
S. Int-estine	UD	0.12(0.04)	0.07(0.03)	0.07(0.03)
	D	0.10(0.03)	0.10(0.06)	0.11(0.05)
Colon	UD	←————————— ND —————————→		
	D	←————————— ND —————————→		
Rectum	UD	←————————— ND —————————→		
	D	←————————— ND —————————→		

Values are given as nmoles/mg microsomal protein, and are the means of 3-8 observations, with standard deviation in parentheses.

ND Not detectable
UD Undosed animals
D   Animals pretreated with BP (6 oral doses, each 3mg/kg, over a 3 week period prior to sacrifice)

Animals were maintained on the appropriate diet for 16 weeks after weaning.

* $p < 0.05$ ⎫
** $p < 0.01$ ⎬ By Student's t-test when compared with the appropriate
\$\$ $p < 0.001$ ⎭   control group.

Table 5.2   Cytochrome P450 levels in guinea pig tissues
(nmoles/g tissue)   / 0·5

Tissue	Treat-ment	Normal Diet	High Fat Diet	High Cholest-erol Diet
Liver	UD	6.57(1.88)	6.64(0.79)	5.63(0.57)
	D	8.55(1.29)	6.40(1.11)**	5.47(1.80)**
S. Int-estine	UD	0.44(0.21)	0.21(0.05)	0.33(0.14)
	D	0.31(0.11)	0.38(0.21)	0.39(0.27)

Values (nmoles/g tissue) are the means of 3-8 observations, with standard deviation in parentheses.

UD Undosed animals
D   Animals pretreated with BP (6 oral doses, each 3mg/kg, over a 3 week period prior to sacrifice)

Animals were maintained on the appropriate diet for 16 weeks after weaning.

** $p < 0.01$ by Student's t-test when compared with the appropriate control group.

Table 5.3   Microsomal protein content of livers of guinea pigs used
             in cytochrome P450 and cytochrome b5 assays

Tissue	Treat-ment	Normal Diet	High Fat Diet	High Cholest-erol Diet
Liver	UD	7.4 (2.4)	11.4 (2.7)	11.1 (1.3)*
	D	12.1 (2.9)*	13.0 (3.0)	10.8 (3.0)
S. Int-estine	UD	3.8 (1.4)	3.1 (0.8)	4.6 (0.8)
	D	3.1 (0.9)	3.6 (0.5)	3.5 (1.3)

Values (mg microsomal protein/g tissue) are the means of 3-8 observations, with standard deviation in parentheses.

UD   Undosed animals
D   Animals pretreated with BP (6 oral doses, each 3mg/kg, over a 3 week period prior to sacrifice)

Animals were maintained on the appropriate diet for 16 weeks after weaning.

* $p < 0.05$ by Student's t-test when compared with the appropriate control group.

Table 5.4   Cytochrome b5 levels in guinea pig tissues
             (nmoles/mg microsomal protein)   ⟨ 0·5

Tissue	Treat-ment	Normal Diet	High Fat Diet	High Cholest-erol Diet
Liver	UD	0.38(0.03)	0.34(0.05)	0.31(0.02)*
	D	0.36(0.06)	0.31(0.01)	0.35(0.07)
S. Int-estine	UD	0.08(0.03)	0.05(0.01)	0.07(0.03)
	D	0.08(0.03)	0.08(0.04)	0.10(0.04)

Values (nmoles/mg microsomal protein) are the means of 3-8 observations, with standard deviation in parentheses.

UD   Undosed animals
D   Animals pretreated with BP (6 oral doses, each 3mg/kg, over a 3 week period prior to sacrifice)

Animals were maintained on the appropriate diet for 16 weeks after weaning.

* $p < 0.05$ by Student's t-test when compared with the appropriate control group.

Table 5.5   Cytochrome b5 levels in guinea pig tissues
(nmoles/g tissue)   /0.5

Tissue	Treat-ment	Normal Diet	High Fat Diet	High Cholest-erol Diet
Liver	UD	2.74(0.85)	3.75(0.68)	3.43(0.24)
	D	4.23(0.97)*	4.05(0.90)	3.68(0.90)
S. Int-estine	UD	0.28(0.08)	0.16(0.01)*	0.29(0.09)
	D	0.23(0.08)	0.28(0.15)	0.34(0.20)

Values (nmoles/g tissue) are the means of 3-8 observations, with standard deviation in parentheses.

UD Undosed animals
D   Animals pretreated with BP (6 oral doses, each 3mg/kg, over a 3 week period prior to sacrifice)

Animals were maintained on the appropriate diet for 16 weeks after weaning.

* $p < 0.05$ by Student's t-test when compared with the appropriate control group.

Table 5.6   AHH levels in guinea pig tissues

Tissue	Treatment	Normal Diet	High Fat Diet	High Cholesterol Diet
Liver	UD	880 (590)	500 (100)	340 (280)
	D	1310 (710)	800 (690)	750 (820)
S. Intestine	UD	70 (34)	25 (38)	27 (32)
	D	325 (190)*	41 (35)**	51 (78)**
Colon	UD	65 (89)	0 (1)	0 (0)
	D	3 (6)	5 (4)	6 (7)
Rectum	UD	0 (1)	0 (1)	0 (0)
	D	538 (965)	5 (6)	3 (4)

Values (pmoles/mg microsomal protein/h) are the means of 3-8 observations, with standard deviation in parentheses.

UD Undosed animals
D   Animals pretreated with BP (6 oral doses, each 3mg/kg, over a 3 week period prior to sacrifice)

Animals were maintained on the appropriate diet for 16 weeks after weaning.

* $p < 0.05$ by Student's t-test when compared with the appropriate
** $p < 0.01$ control group.

Table 5.7   Epoxide hydrase levels in livers of guinea pigs
fed various diets

(a) Age-matched animals

Normal Diet	High Fat Diet	High Cholesterol Diet
2.9 (0.5)	2.9 (0.6)	2.7 (0.4)

Values are expressed as nmoles styrene glycol formed/mg protein/min and are the means of 3 or 4 observations. Standard deviations are shown in parentheses.

Animals were maintained on the appropriate diet for 21 weeks from weaning.

(b) Non-age-matched animals

Normal Diet	High Fat Diet	High Cholesterol Diet
2.7 (1.1)	2.6 (0.7)	2.4 (0.7)

Values are expressed as nmoles styrene glycol formed/mg protein/min and are the means of 6 or 8 observations. Standard deviations are shown in parentheses.

Animals included those age-matched guinea pigs in Table 5.7(a) above, and other animals which had been fed the appropriate diet for up to 20 months from weaning.

Table 5.8   Hepatic glutathione levels in guinea pigs
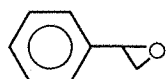   fed various diets

	Normal Diet	High Fat Diet	High Cholesterol Diet
Total Glutathione	8.1 (1.0)	8.6 (2.3)	8.5 (2.4)
Reduced Glutathione	6.7 (1.4)	6.2 (3.2)	6.4 (0.9)
% Reduced Glutathione	82.5 (9.0)	68.3(31.7)	79.0(18.9)

Values are the means of 4 observations and total and reduced gluta-
thione are expressed as μmoles /g liver. Standard deviations are shown
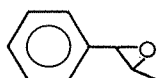in parentheses.

Animals were maintained on the appropriate diet for a period of 21
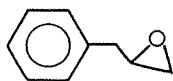weeks from weaning.

Figure 5.1   Substrates for Epoxide Hydrase

Styrene Oxide

trans-ß-Methylstyrene Oxide

Allylbenzene Oxide

Chapter 6

DNA BINDING IN VIVO AND
EXCRETION OF TRITIUM AFTER [^{3}H]BENZO(a)PYRENE
ADMINISTRATION

6.1 Introduction

Although a considerable amount of time and effort has been
devoted to the problem, particularly during the last two decades, the
process of carcinogenesis is far from being completely understood. A
number of theories have been put forward which can be broadly
classified as either mutation or epigenetic theories. It seems likely
that the production of cancer involves not simply either genetic or
environmental factors, but an interaction of certain genetic
characteristics with particular environmental agents at certain stages
of development. Trosko and Chang (1979) have discussed the two-stage
theory of carcinogenesis, which involves initiation and promotion.
Initiation of tumour development results from chemical interaction with
DNA and subsequent promotion by other chemicals resulting in enhanced
tumour formation. Both initiation and promotion need not be
prerequisites for the carcinogenic process, however, and Trosko and
colleagues have proposed an "integrative theory" of carcinogenesis
to explain such examples (see Trosko and Chang, 1979). This theory
comprises mutation and epigenetic theories of carcinogenesis, the two-
stage theory, and Comings' general theory of cancer (Comings, 1973).

Chemically induced DNA damage appears to play a major role in
mutation fixation and carcinogenesis. There is a high correlation
between carcinogenicity and mutagenicity; 90% of 175 carcinogenic
compounds tested for mutagenicity in the Salmonella/microsome test gave
a positive result (McCann and Ames, 1976). Certain chemicals, such as
the sulphur mustards, are alkylating agents which react directly with
DNA, RNA and protein. However, the majority of chemical carcinogens
require metabolic activation to electrophilic metabolites before such
interactions can occur. For benzo(a)pyrene, these reactions have been

described in Chapter 1.

Brookes et al. (1975) reported that the polycyclic compounds 7-methylbenz(a)anthracene (7MBA) and BP, when incubated with mouse embryo cell DNA, reacted exclusively with the purine moieties of the nucleic acid. Similarly, Bennett et al. (1981) reported that the major urinary aflatoxin metabolite in aflatoxin B_1-treated rats was a guanine adduct, 2,3-dihydro-2-(N^7-guanyl)-3-hydroxyaflatoxin B_1. Dimethyl-nitrosamine (DMN) alkylates guanine residues of rat liver DNA (Pegg and Perry, 1981). King et al. (1977) examined 3-methylcholanthrene (MC) binding to the DNA of mouse embryo cells in culture. They concluded from chromatographic and spectrophotometric observations that the metabolites bound to DNA were likely to be dihydrodiols of MC, but not the 1,2- or 11,12-dihydrodiols reported by Sims (1966) to be the major metabolites formed by rat liver homogenate. They hypothesised that MC binding to DNA was mediated via a 7,8-epoxy-9,10-diol, possibly with some hydroxylation at positions 1, 2 or 3. This is consistent with the "bay region" theory of hydrocarbon carcinogenesis discussed by Jerina and Daly (1977), which predicts that the ultimate carcinogenic form of 3MC would be the carbonium ion shown in Figure 6.1. The proposed ultimate carcinogenic forms of a number of other compounds are also shown in Figure 6.1.

Following both oral and intravenous administration of DMN to rats, 10 times more 7-methylguanine was formed in hepatic tissue than in kidney (Pegg and Perry, 1981). At doses below 1mg/kg, the liver metabolised most of the DMN, with very little 7-methylguanine production by the kidney. O^6-methylguanine is rapidly removed from the liver, but more slowly from kidney. At low doses of DMN p.o. (less than 50μg/kg), very little renal O^6-methylguanine was formed. Therefore there appear to be route of administration differences in the

interaction of DMN with various organs; the alkylation of DNA bases in extrahepatic tissues may depend upon the dose, and rate of absorption of the carcinogen.

Covalent binding to cellular DNA has been found for almost all carcinogens, suggesting that such binding may be necessary for tumour induction. If a carcinogenic compound entering the body is to initiate cancer of the colon, then the ultimate carcinogenic form of this substance must somehow reach, or be produced within, the colonic mucosa, and interact with the DNA of this tissue. The tritium binding to DNA was therefore measured in animals which had received a single oral dose of [G-^{3}H]benzo(a)pyrene.

6.2 Materials and Methods

(a) [G-^{3}H]Benzo(a)pyrene

;50mg

[G-^{3}H]Benzo(a)pyrene (2Ci / in toluene:ethanol solvent) was obtained in impure form from Amersham International plc, Amersham, Bucks., as the product of a tritium exchange reaction. The solution was evaporated to dryness, dissolved in a small volume of benzene, and the resulting solution washed onto a neutral alumina column with 10% benzene in petroleum ether (b.p. 60-80°). The column was treated with about 150ml of petroleum ether:benzene in which the proportion of benzene was increased from 10 to 60%. At 60% benzene in petroleum ether (b.p. 60-80°), a purple/blue fluorescent band was visible under uv light, slowly moving down the column, followed by a thin yellow band. Brown impurities remained at the top of the column.

The purple/blue fluorescent band of [^{3}H]benzo(a)pyrene was

eluted with 70-80% benzene in petroleum ether (b.p. 60-80°), evaporated to dryness, and dissolved in 20ml benzene. The solution was divided into 1ml aliquots, gassed with nitrogen and stored at -70°C.

Thin layer chromatography in benzene:ethanol (95:5) revealed that the fraction of [^{3}H]benzo(a)pyrene recovered from the alumina column was radiochemically pure. The yield of tritium recovered as pure [^{3}H]benzo(a)pyrene was 62%. Prior to dosing of animals, an aliquot was allowed to thaw, and evaporated to dryness at room temperature with a stream of nitrogen. The [G-^{3}H]benzo(a)pyrene was dissolved in ethanol (1.2ml) and 0.4ml given to each animal. An aliquot was analysed by liquid scintillation counting to determine the dose administered.

(b) Tritiated water

Tritiated water (approximately 100mCi/ml; 0.5ml; Amersham International plc, Amersham, Bucks.) was obtained from the School of Biochemistry and Physiology, University of Southampton, and diluted to 5.0ml with unlabelled water to give approximately 10mCi/ml. Further tritiated water (1Ci; 5Ci/ml) was obtained direct from Amersham International plc, and diluted to 2ml with unlabelled water. An aliquot (0.1ml) of this dilution was further diluted with unlabelled water to give a dosing solution with specific activity of approximately 10mCi/ml.

(c) Dosing of animals with [G-^{3}H]benzo(a)pyrene

Initial experiments were carried out to establish the length of time after dosing when DNA binding was at a maximum. Adult animals, fed either normal or high fat diet, were given a single oral dose of

[³H]benzo(a)pyrene (3-5mCi) in ethanol and sacrificed up to 7 days after dosing. DNA was isolated and purified as described below, and binding determined by scintillation counting.

In subsequent experiments, age-matched animals were dosed orally with 2-4mCi of [G-³H]benzo(a)pyrene in ethanol (0.4ml). The animals were housed individually in wire metabolism cages after dosing and sacrificed 3 days later.

(d) Dosing of animals with tritiated water orally

Animals were dosed orally with 0.5ml of labelled water (equivalent to 4-6mCi) and housed individually after dosing. In an initial study, animals were sacrificed 1-7 days after dosing. For comparison with [G-³H]benzo(a)pyrene data the animals were sacrificed 3 days after the dose.

(e) Collection of urine and faeces

Urine and faeces were collected between the time of dosing and sacrifice and the tritium content determined as described in Chapter 2. The percentage of the tritium present as tritiated water in the urine was determined by evaporating an aliquot of each urine at room temperature under reduced pressure. The water vapour was collected onto a cold finger containing liquid nitrogen, and aliquots (0.5ml, after dilution, if necessary) were counted after thawing.

(f) DNA Binding

(i) Isolation and purification of DNA

The method used was that described by Baird and Brookes (1973), as modified from Diamond et al. (1967) and Kirby (1957), and based on further modifications suggested by Dr. P. Brookes. The reagents used are listed below.

1. KCl buffer: 1.15% w/v KCl in 0.01M phosphate buffer, pH 7.4

2. PAS reagent: 6% p-aminosalicylic acid sodium salt, 1% NaCl and 1% sodium dodecyl sulphate in water

3. Phenol reagent: phenol (500g), 8-hydroxyquinoline (0.25g), m-cresol (34ml) and water (27.5ml)

4. Ribonuclease A: 1mg/ml in 0.5M phosphate buffer, pH 7.4

5. CTAB solution: cetyltrimethylammonium bromide, 1% in water

Liver, small intestine, colon and rectum were removed from the animal and rinsed with cold tap water, followed by KCl buffer. The mucosa was scraped from the gut tissues, weighed, and finely chopped with scissors in PAS reagent (2 volumes). A fraction of the liver was weighed, finely chopped with scissors, and homogenised in PAS reagent (2 volumes) with 2 passes in a glass/teflon homogeniser. Phenol reagent (1 volume) was added to each tissue homogenate, the mixture stirred on ice for 30min, and centrifuged at 1200g in an MSE Model GF8

centrifuge for 20min.

The slightly turbid, pale yellow, upper (aqueous) layer was removed, placed in a beaker and kept on ice, and excess ice-cold 2-ethoxyethanol added. The DNA which was precipitated was removed gently with a glass spatula and transferred to a clean beaker. Excess liquid was drained from the DNA and was removed carefully with a Pasteur pipette. The isolated DNA was dissolved in the minimum possible volume of 0.01M phosphate buffer, pH 7.2 and RNA removed by incubation with bovine pancreatic ribonuclease solution ($10\mu l/ml$ DNA solution) at 37°C for 20min.

The DNA from the gut tissues was precipitated by the addition of excess ice-cold ethoxyethanol, removed and redissolved in the smallest volume of 0.01M phosphate buffer, pH 7.2. Further purification was achieved by repeated precipitation from buffer with an excess of ice-cold ethanol.

The DNA from the liver, after treatment with ribonuclease, was separated from carbohydrate by shaking with an equal volume of ice-cold 2.5M K_2HPO_4 solution and 0.05 volumes of ice-cold 33% H_3PO_4, followed by shaking with 1 volume of ice-cold 2-methoxyethanol. The lower glycogen-containing layer was allowed to settle out in a cold room overnight. The upper layer which contained the DNA was removed and the DNA precipitated by addition of ice-cold CTAB solution (0.5 volumes). The DNA was removed with a glass spatula, washed in 3x50ml distilled water (for 10min each) and converted to its sodium salt by soaking in 2x25ml 2% sodium acetate in 70% ethanol for 20min. At this stage the product, which shrunk, was removed and washed in ice-cold ethanol and then ether and warmed on a water bath to remove residual ether. This product was immediately dissolved in 0.01M phosphate buffer, pH 7.2, and treated with ice-cold ethoxyethanol and ethanol as

described above for the other tissues. The amount of DNA and tritium content were determined at all later purification stages.

(ii) Estimation of DNA

DNA was assayed by the diphenylamine colorimetric method (Dische, 1955). The solution containing DNA (50-500µg; 1 volume) was mixed with diphenylamine reagent (diphenylamine (1g), glacial acetic acid (100ml) and concentrated sulphuric acid (2.75ml); 2 volumes) and heated at 100°C for 10min. The absorbance at 595nm was determined using a Beckman Model 25 spectrophotometer, and compared with a standard curve prepared using calf thymus DNA.

6.3 Results and Discussion

(a) Body weights of guinea pigs fed various diets

Animals used in the [³H]benzo(a)pyrene-DNA binding study were weighed weekly until dosing, and weights are recorded in Table 6.1. High fat diet caused a small reduction in weight gain (5-10%) compared with controls. This was not statistically significant and the addition of cholesterol did not cause any further decrease.

(b) The elimination of tritium after administration of [³H]benzo(a)-pyrene

Figures for the elimination of tritium after an oral dose of [³H]benzo(a)pyrene are given in Table 6.2. Normal animals excreted an average of 42.8% of the dose during a 72h period after dosing, 81%

of the eliminated tritium being found in the faeces. Animals fed high cholesterol diet showed a similar total excretion of tritium in 72h (40.7%), but with a smaller proportion (65%) in the faeces. Animals fed high fat diet excreted only 28.7% of the dose in total, with 57% of eliminated tritium in the faeces. The percentage of the dose eliminated in the urine of animals fed high fat diet was found to be significantly higher than in normal animals ($p < 0.05$) and the percentage in the faeces significantly lower ($p < 0.01$). This result indicates that the animals fed high fat diet show increased absorption of metabolites from the gut when compared with normal guinea pigs. This suggests a more effective enterohepatic circulation of benzo(a)pyrene and increases the possibility of interaction of the metabolites with macro-molecules in the cells lining the gut lumen.

The quantities of tritium excreted in the urine of animals fed high cholesterol diet were comparable to the values for the high fat group, but due to the wide inter-individual differences, the results showed no significant difference from the normal values. The quantity of tritium excreted in the faeces of animals fed high cholesterol diet was lower than that excreted in the faeces of normal animals, although the difference was not significant.

The quantity of ^3H_2O in urine after administration of [^3H]benzo(a)pyrene orally was determined by the evaporation of an aliquot of urine as described in the Methods. The percentage of the dose which was excreted as ^3H_2O in the urine was very similar for each dietary group (on average, 2.7%; see Table 6.2), the percentage of urinary tritium attributable to ^3H_2O being 32% for normal animals, 24% for animals fed high fat diet and 19% for animals fed high cholesterol diet. These average figures include urine samples which had been evaporated to dryness and some which had not been fully evaporated, and

may therefore have shown preferential evaporation of ^3H_2O, while ^3HHO was concentrating in the urine. Using values only for urine samples which were evaporated to dryness, the ^3H_2O content represented 51% for normal, 26% for high fat and 23% for high cholesterol-fed animals (urine from only one normal animal was treated in this way; high fat and high cholesterol values are each an average for 4 animals).

Animals given a single oral dose of tritiated water excreted 11.1% of the dose as such in the urine within 72h (see Table 6.3). Using this figure, the average of 2.7% of the dose of [^3H]benzo(a)-pyrene recovered as ^3H_2O in urine within 72h indicated that 24% of the dose of [^3H]benzo(a)pyrene underwent tritium exchange to ^3H_2O. Using only the figures where the urine was totally evaporated, the average value for the percentage of the dose of [^3H]benzo(a)pyrene which was excreted as ^3H_2O in the urine within 72h was 4.1%, suggesting that 37% of the [^3H]benzo(a)pyrene dose underwent tritium exchange.

(c) Tritium binding to DNA after administration of [^3H]benzo(a)pyrene

The results of the initial time-course experiment for tritium binding to DNA after the administration of a single oral dose of [^3H]-benzo(a)pyrene are given in Table 6.4. There was a large inter-individual variation which led to a scatter of points masking any obvious peak in the extent of tritium binding. The tritium levels bound to liver were relatively constant throughout the 7 day period, but the small intestine, colon and rectum showed a slight increase in tritium levels associated with the DNA 2-3 days after dosing. This result seems consistent with the probable time required for the dose to be absorbed, metabolised, excreted in the bile and converted to active metabolites in the gut lumen.

As a result of these experiments, further studies of DNA binding were performed using age-matched male guinea pigs which were dosed with [^{3}H]benzo(a)pyrene 3 days before sacrifice. The results of these experiments are shown in Table 6.5. There were no significant differences in the level of tritium binding to DNA between the dietary groups in any of the tissues examined. The livers of animals fed high fat or high cholesterol diets were more friable than those of control animals, and were either generally lighter in colour than normal livers, or had distinct patches of apparently fat or cholesterol-rich tissue. These livers produced a poor yield of DNA; after centrifugation with phenol reagent there was no distinct top layer, and precipitation with ice-cold ethoxyethanol produced rather fragmented DNA.

The amounts of tritium present in the purified DNA were extremely low, and in many cases, as the yield of DNA was reduced during purification, the actual tritium counts obtained were only 2 or 3 times background levels. When this counting error was associated with a low yield of DNA the resulting errors in specific activity were large, such that "constant" specific activity was subject to an error of up to 30-40%. Large inter-individual variations were therefore observed, resulting in large standard deviations on statistical analysis.

(d) "Binding" of tritium to DNA after administration of tritiated water

After oral administration of a single dose of tritiated water (4.5mCi/animal), tritium was detected in the DNA isolated from both the liver and the gut mucosa. The levels of tritium associated with the DNA

of tritiated water-dosed animals varied little throughout a 7 day period (see Table 6.6).

Thus part of the tritium binding to DNA after administration of [^{3}H]benzo(a)pyrene may have resulted from the release of ^{3}H$_2$O during metabolism. In order to establish the extent of apparent covalent binding attributable to tritium exchange, levels of tritium associated with the DNA of liver, and mucosae of small intestine, colon and rectum were determined 3 days after administration of a single oral dose of tritiated water. The results of the DNA binding experiments are shown in Table 6.5.

The level of tritium bound to, or incorporated into, liver DNA of normal guinea pigs was 73 $\pm$ 34 dpm/mg DNA/mCi dose/kg. This is approximately 40% of the level observed in [^{3}H]benzo(a)pyrene-dosed animals (see Table 6.5). The proportion was even higher for the DNA of the gut mucosae. The excretion data indicate that 37% of the [^{3}H]benzo(a)pyrene dose underwent tritium exchange; therefore the apparent covalent binding of [^{3}H]benzo(a)pyrene metabolites to DNA of the tissues examined is in part due to tritium exchange during metabolism. Actual binding of radioactive benzo(a)pyrene metabolites to the DNA of liver and gut mucosa is therefore low, and unaltered by the feeding of a high fat or high fat/high cholesterol diet. The very low levels of DNA binding of BP metabolites precluded analysis of the nature of the adducts.

Table 6.1   Body weights of guinea pigs used in DNA-binding study

Time on diet (weeks)	Body weights (g)		
	Normal Diet	High Fat Diet	High Cholesterol Diet
1	–	243 (17)	238 (39)
2	363 (30)	308 (21)	298 (30)
3	420 (31)	377 (28)	366 (41)
4	472 (47)	428 (33)	397 (37)
5	544 (43)	475 (35)	466 (37)
6	588 (56)	523 (31)	524 (38)
7	629 (48)	582 (50)	577 (41)
8	694 (57)	613 (55)	599 (50)
9	697 (75)	645 (57)	660 (37)
10	741 (69)	703 (67)	695 (43)
11	761 (85)	716 (40)	712 (49)
12	826 (81)	745 (53)	745 (49)
13	841 (78)	759 (68)	766 (53)
14	848 (105)	790 (73)	764 (62)
15	880 (82)	828 (98)	806 (44)

Male guinea pigs were fed the appropriate diet from weaning. Values given are the mean body weight for 4-8 animals, with standard deviation in parentheses. The animals were given a single oral dose of [^{3}H]benzo-(a)pyrene (2-4mCi) after 15 weeks on diet.

Table 6.2   Excretion of ^{3}H by   guinea pigs dosed orally with
[3H]benzo(a)pyrene

| Diet | Weight (kg) | % dose in urine after 72h | | % dose in |
		Total urine	[3]H$_2$O in urine	faeces (72h)
N	0.880(0.082)	7.9(1.8)	2.5(1.7)	34.9(12.8)
HF	0.828(0.098)	12.4(3.6)*	3.0(0.7)	16.3(7.4)**
HC	0.806(0.044)	14.4(10.8)	2.7(2.8)	26.3(12.5)

Animals were maintained on the appropriate diet (normal, N; high fat,
HF; high cholesterol, HC) for 15 weeks after weaning, and were then
given a single oral dose of [^{3}H]benzo(a)pyrene (1.6-4.0mCi). The
tritium content of the urine and faeces produced between dosing and
sacrifice (3 days later) is given as a percentage of the dose
administered. Values are the means of 6-8 observations, with standard
deviation in parentheses.

*   p<0.05 ⎫
** p<0.01 ⎭ by Student's t-test, when compared with animals fed normal
diet.

Table 6.3   Excretion of ^{3}H by guinea pigs dosed orally
                           with tritiated water

| Weight(kg) | % dose in urine after 72h | | % dose in faeces (72h) |
	Total urine	^3H_2O in urine	
0.934(0.065)	13.1(5.4)	11.1(6.8)	0.45(0.28)

Animals were maintained on normal diet for at least 18 weeks from
weaning. They received a single oral dose of tritiated water (4.5 or
5.7mCi), and were sacrificed 3 days later. The tritium content of the
urine and faeces produced between dosing and sacrifice is given as a
percentage of the dose administered, and values reported are the means
of 6 observations, with standard deviation in parentheses.

Table 6.4   The binding of [^{3} H]benzo(a)pyrene to guinea pig DNA in vivo (non-age-matched animals) up to 7 days after administration of a single oral dose of [^{3}H]benzo(a)pyrene

Time from dosing to sacrifice	^{3}H binding to DNA (dpm/mg DNA/mCi)				
	Liver	S.Int.	Colon	Rectum	Diet
1 day	–	505	361	338	HF
	85	214	290	138	HF
	–	427	188	34	HF
Mean(SD)	85	382(151)	280(87)	170(155)	
2 days	36	302	–	118	HF
	–	385	722	212	N
	–	1322	495	246	N
Mean(SD)	36	670(566)	609	192(66)	
3 days	87	–	321	361	HF
	–	435	523	391	N
	43	365	236	491	N
	49	288	241	284	HF
	56	204	127	–	HF
	41	383	247	–	HF
Mean(SD)	55(18)	335(90)	283(133)	382(86)	
4 days	31	413	–	371	HF
5 days	21	120	150	–	HF
	22	92	188	–	HF
	63	271	–	–	HF
Mean(SD)	35(24)	161(96)	169		
7 days	–	241	252	44	HF
	66	101	–	–	HF
	123	128	110	326	N
	64	170	210	316	N
Mean(SD)	84(34)	160(61)	191(73)	229(160)	

Animals were fed normal (N) or high fat (HF) diet for a period of at least 3 months before dosing. They received a single oral dose of [^{3}H]-benzo(a)pyrene (3-5mCi) and were sacrificed up to 7 days later. Values shown are mean ^{3}H binding in dpm/mg DNA/mCi dose for two or more DNA purification stages for each tissue. Values for each individual guinea pig are shown, and means, with standard deviations in parentheses where applicable.

Table 6.5   The binding of ^{3}H to guinea pig DNA in vivo after oral
        administration of [^{3}H]benzo(a)pyrene or tritiated water

| Treatment | ^{3}H binding to DNA (dpm/mg DNA/mCi dose/kg) | | | |
	Liver	S.Intestine	Colon	Rectum
N; [^{3}H]BP	185(133)	399(185)	461(249)	485(313)
HF; [^{3}H]BP	112(50)	475(369)	236(76)	1008(1264)
HC; [^{3}H]BP	242(203)	354(94)	372(287)	625(234)
N; ^{3}H$_2$O	73(34)	186(41)	353(135)	963(414)

[^{3}H]Benzo(a)pyrene-dosed animals ([^{3}H]BP)

Animals were maintained on the appropriate diet (normal, N; high fat,
HF; high cholesterol, HC) for a period of 15 weeks from weaning, after
which they received a single oral dose of [^{3}H]benzo(a)pyrene (2-
4mCi/animal). All animals were sacrificed 3 days after dosing.

Tritiated water-dosed animals (^{3}H$_2$O)

Animals were fed normal diet for at least 18 weeks after weaning, and
sacrificed 3 days after a single oral dose of tritiated water (4-
6mCi/animal).

Values given are means of 4-8 observations (dpm/mg DNA/mCi dose/kg),
with standard deviation in parentheses.

Table 6.6   The binding of ^{3}H to guinea pig DNA in vivo
        (non-age-matched animals) at various times after administration
                of a single oral dose of tritiated water

Time from dosing to sacrifice	^{3}H binding to DNA (dpm/mg DNA/mCi)				
	L	S	C	R	Diet
1 day	1	151	13	431	N
	9	127	32	523	HF
Mean	5	139	23	477	
4 days	7	224	317	336	N
	82	165	0	495	HF
Mean	45	195	159	416	
7 days	–	109	63	–	N
	56	189	159	407	N
Mean	56	145	111	407	

Animals were fed normal (N) or high fat (HF) diet for a period of at
least 3 months before dosing. They received a single oral dose of
tritiated water (4.5mCi/animal), and were sacrificed up to 7 days
later.
Values shown are mean ^{3}H binding in dpm/mg DNA/mCi dose for two or more
DNA purification stages for each tissue (liver (L), small intestine
(S), colon (C) and rectum (R)). Values for each individual guinea pig
are shown, together with the mean values for each tissue. No standard
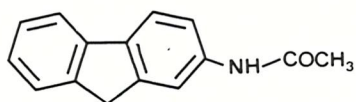deviations are given, as only two animals were studied at each time
point.

Figure 6.1

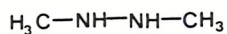## Some Chemical Carcinogens and their Ultimate Carcinogenic Forms
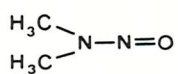
PARENT COMPOUND

ULTIMATE CARCINOGENIC FORM

Benzo(a)pyrene

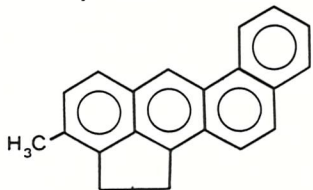7,8-Diol-9,10-epoxide

2-Acetylaminofluorene

$H_3C-NH-NH-CH_3$

1,2-Dimethylhydrazine

$H_3C-NH-NH-\overset{+}{C}H_3$

N,N-Dimethylnitrosamine

Aflatoxin B_1

Aflatoxin B_1-8,9-oxide

3-Methylcholanthrene

$Cl-CH=CH_2$

Vinyl chloride

$Cl-CH-CH_2$

Chapter 7

GENERAL DISCUSSION

In these studies, a number of avenues have been explored in an attempt to elucidate whether or not the lipid-soluble PAH benzo(a)pyrene could act as a carcinogen in the colon. Benzo(a)pyrene can enter the gastrointestinal tract as a food contaminant or to a lesser degree as a natural component of the diet. Its ability to interact with a high fat or high fat/high cholesterol diet was examined, in order to establish whether its metabolism was altered under these conditions with a resultant increase in events likely to result in the production of colon cancer.

7.1 Hepatic metabolism

A scheme showing the possible pathways of metabolism of a xenobiotic compound is shown in Figure 7.1. Following absorption from the gut, hepatic metabolism is the first step involved in the passage of a compound through the body (see Figure 7.2). Cytochrome P450, aryl hydrocarbon hydroxylase (AHH) and epoxide hydrase (EH) levels have been measured in the liver of guinea pigs fed normal diet.

Cytochrome P450 levels were 0.9 nmoles/mg microsomal protein, which is higher than values quoted for rat liver (Marselos and Laitinen, 1975; Fang and Strobel, 1981). High fat and high cholesterol diets reduced the quantity of cytochrome P450 in relation to microsomal protein in both undosed animals, and animals which had been pretreated with BP. However, these diets also increased the quantity of microsomal protein in relation to liver weight, and calculation of cytochrome P450 content in relation to liver weight revealed no dietary differences in undosed animals. Measurement of aryl hydrocarbon hydroxylase also revealed that hepatic activity was unaffected by diet or by BP pretreatment.

Hepatic epoxide hydrase activity (Table 5.7) was not altered by

the administration of high fat or high cholesterol diets. Similarly, hepatic glutathione levels were not different in animals fed high fat and high cholesterol diets when compared with controls (Table 5.8). Glutathione is a tripeptide (γ-glutamylcysteinylglycine) found in almost all living cells, and constitutes the major thiol of animal tissues. Intracellular glutathione concentrations are relatively high (0.5-10mM), particularly in the liver (Meister, 1982). This tripeptide has a number of functions, one of which is the detoxification of xenobiotic molecules. Depletion of hepatic glutathione by administration of diethyl maleate to mice was found to increase their susceptibility to paracetamol-induced hepatic necrosis (Mitchell et al., 1973).

7.2 Biliary metabolites of [^{14}C]benzo(a)pyrene

Administration of an intravenous dose of BP resulted in 33% of the dose being eliminated in the bile of guinea pigs fed normal diet within 4h, with less than 1% in the urine (see Table 3.1). Biliary elimination of [^{14}C]benzo(a)pyrene metabolites in rabbit is comparable with that in the guinea pig (approximately 30% of the dose in 4-6h), whereas elimination is much greater in the rat (60% in 6h) (Chipman et al., 1982; Chipman et al., 1981). In all 3 species, biliary metabolites of [^{14}C]benzo(a)pyrene consisted mainly of polar conjugates. Only 15% of the metabolites in guinea pig bile were extractable into ethyl acetate, whilst in rabbit and rat bile, the proportion of extractable metabolites was slightly lower ($<$ 12% and 8% respectively). These percentages increased to 30-40% after incubation of bile with hydrolytic enzymes.

The major non-polar metabolites in rat bile, after hydrolysis, were BP 4,5-dihydrodiol, 3,6-quinone, 9-hydroxybenzo(a)pyrene and 3-

hydroxybenzo(a)pyrene, whereas these comprised less than 10% of rabbit bile. The major conjugated metabolite in rabbit bile was BP 9,10-dihydrodiol (18% of the ^{14}C in bile; Chipman et al., 1982). In contrast to these findings, the bile from guinea pigs fed normal diet contained predominantly dihydrodiol metabolites of benzo(a)pyrene after hydrolysis with β-glucuronidase. In rat and rabbit bile, the proximate carcinogen BP 7,8-dihydrodiol was not detected, but this metabolite was present in guinea pig bile (see Table 3.11). Therefore the guinea pig would appear to be the most appropriate of these three species as a model for colon carcinogenesis. The species differences in biliary metabolites of BP could be explained by differences in hepatic isozymes of cytochrome P450 present in these animals showing selectivity for different areas of the BP molecule. The differences in biliary metabolites are summarised in Table 7.1.

Rabbit bile was found to contain substantial quantities of sulphate conjugates of BP metabolites, but these were only very minor constituents of rat bile. In the guinea pig, sulphate conjugates also appeared to be very minor components. Incubation of bile with β-glucuronidase/aryl sulphatase did not produce a noticeable increase in ethyl acetate-extractable metabolites when compared with β-glucuronidase incubates. An increase would have indicated that the difference was due to the presence of sulphate conjugates. However, definite conclusions cannot be drawn from these experiments, as β-glucuronidase/aryl sulphatase frequently resulted in less hydrolysis than β-glucuronidase alone. This may have been due to decreased hydrolysis of conjugates per se, or increased decomposition of the aglycone after release from the conjugate.

Approximately 30% of the radioactivity in bile was extractable with ethyl acetate after incubation of bile with caecal contents, and

40-50% after incubation with β-glucuronidase, or β-glucuronidase/aryl sulphatase (Table 3.6). Therefore 50-70% of the dose remains in the aqueous layer after extraction of metabolites hydrolysed by these enzymes, and may be in the form of glutathione or other thio-ether conjugates. Activated PAH such as naphthalene-1,2-oxide have been shown to be conjugated with glutathione in vitro (Booth et al., 1960 and 1960a) and in vivo (Pue et al., 1982), and glutathione conjugates of BP 4,5-oxide have been detected in rat bile (Plummer et al., 1980).

7.3 Effects of high fat and high cholesterol diets on biliary metabolites of [^{14}C]benzo(a)pyrene

The administration of high fat and high fat/high cholesterol diets resulted in a significantly greater proportion of the radioactivity in bile being eliminated as glucuronide conjugates of benzo(a)pyrene dihydrodiols when compared with animals fed normal diet. In normal animals, the 4,5- and 9,10-dihydrodiol glucuronide conjugates were present to a similar extent (about 3% of the radioactivity in the bile). In animals fed high fat and high cholesterol diet, the BP 4,5-dihydrodiol glucuronide predominated, accounting for 13% of the radioactivity in the bile. The quantity of 7,8-dihydrodiol eliminated in the bile was higher in animals fed high fat and high cholesterol diets when compared with normal animals (see Table 3.11), but the increases were not statistically significant due to large inter-individual variations.

7.4 Bacterial metabolites

Production of polar conjugates by the liver, and elimination of these compounds in the bile brings metabolites of BP into contact with the bacteria present in the gut (see Figure 7.2). Under the influence

of a high fat diet, quantitative changes in the strains of intestinal bacteria occur and also the activities of various enzymes of the flora (see Chapter 1, section 1.3(d), and Chapter 4, section 4.1). A number of pure strains of intestinal bacteria have been shown to be capable of hydrolysing BP conjugates in vitro. Such activity in vivo will result in the release of the aglycone within the colon. These aglycones were stable to further degradation by the strains of bacteria tested.

Incubation of bile containing [^{14}C]benzo(a)pyrene metabolites collected from animals fed normal diet with caecal contents removed from the same animals, resulted in the hydrolysis of conjugates of BP dihydrodiols and the phenolic metabolite (peak 8 in hplc). A greater proportion of dihydrodiols was released after incubation of bile from animals fed high fat or high cholesterol diets with their caecal contents, although in most cases the differences were not significant. This is probably a reflection of the increased quantity of BP dihydrodiol glucuronides in the bile of these animals when compared with controls, rather than increased activity of the bacteria in the caecal contents since the incubations were carried out overnight at 37°C.

Kinoshita and Gelboin (1978) have reported that the hydrolysis of benzo(a)pyrene-3-glucuronide by β-glucuronidase to 3-hydroxybenzo-(a)pyrene yields a BP derivative which binds to DNA to a far greater extent than either the 3-hydroxybenzo(a)pyrene or the glucuronide. Therefore a simple measurement of metabolites before and after hydrolysis by the gut flora may not provide a complete indication of the potential for the release of active metabolites.

7.5 Enzyme activity of the gut mucosa

Cytochrome P450 was detectable only in the mucosa of the small intestine of guinea pigs fed normal diet, and not in colonic or rectal mucosa. Levels in small intestinal mucosa were unaffected by a high level of fat in the diet, or fat plus cholesterol, pretreatment with BP, or a combination of high fat diet and BP pretreatment.

Aryl hydrocarbon hydroxylase was also undetectable in the colon and rectum. Activity was found in small intestinal mucosa, however, which was inducible in normal animals by BP administration. Lack of induction in high fat and high cholesterol groups suggests that these diets are counteracting the inducing effects of orally administered BP. An increased lipid content in the diet increases bile acid production by the liver, and thus aids solubility and absorption of the lipids. A high fat diet would therefore be expected to increase indirectly the solubility of a lipid-soluble compound such as BP. The inability of BP to induce AHH in small intestinal mucosa of animals fed high fat and high cholesterol diet may reflect alterations in endoplasmic reticulum structure brought about by the fat or cholesterol in the diet. An alternative explanation is that the induction of AHH activity in normal animals by BP is assisted by the vehicle, DMSO. The administration in coconut oil is possibly more representative of dietary intake.

Failure to detect measurable levels of AHH in colonic mucosa in the guinea pig, or to demonstrate inducibility by elevated quantities of fat or cholesterol in the diet was not altered by BP administration. The presence of AHH in this tissue would have allowed the possibility of reactivation of benzo(a)pyrene metabolites released from conjugates by the intestinal microflora, after absorption by the cells of the colonic mucosa. Its absence indicates that the proposed model using the guinea pig may be unsuccessful.

Although guinea pig colon was unable to produce detectable quantities of BP metabolites, Autrup et al. (1978) have shown that cultured human colon cells can metabolise primary metabolites of BP, including the activation of the 7,8-dihydrodiol. In addition, the cytochrome P450 system appears not to be the only pathway for BP oxidation in rat and human colonic microsomes. Arachidonate, linoleate or their hydroperoxides were found to increase BP metabolism by these microsomes by up to 5 times whilst palmitate and NADPH had no effect (Craven and DeRubertis, 1980). Arachidonate-stimulated BP metabolism was inhibited by indomethacin, but this inhibitor was ineffective against linoleate-stimulated increases in xenobiotic oxidation. This indicates that the fatty acid-induced increases in metabolism were not solely mediated by cyclooxygenase. Craven et al. (1983) have also reported a significant increase in protein-bound [^{3}H]BP metabolites in rat colonic microsomes in the presence of arachidonate or linoleate. Both increases were lowered by the addition of an inhibitor of fatty acid oxidation, indicating the involvement of fatty acid hydroperoxides in the production of active, macromolecular-binding BP metabolites.

7.6 DNA Binding

Following an oral dose of [^{3}H]benzo(a)pyrene, the levels of tritium covalently bound to DNA of liver and gut mucosa were low, and showed no difference between the dietary groups. A large proportion of this "binding" could be attributed to tritium exchange during metabolism, as at least 40% of this "binding" was found in animals which had received a single oral dose of tritiated water. Therefore, although arachidonate-dependent oxidation was not measured, it appears unlikely that this is of significance in vivo since the absence of cytochrome P450 was reflected in very low covalent binding.

7.7 Enterohepatic circulation

Guinea pigs fed normal diet excreted 33% of an intravenous dose of [^{14}C]benzo(a)pyrene in the bile within 4h, with less than 1% in the urine (Table 3.1). Animals fed high fat and high cholesterol diets excreted a similar proportion (27% and 37% respectively) in the bile within 4h. Thus, the epoxidation rate of BP remains unchanged by diet, which is consistent with the observation that overall cytochrome P450 content of guinea pig liver is unaltered by high fat or high cholesterol diet, and similarly AHH is unaffected in this tissue.

Measurement of tritium excretion after [^{3}H]benzo(a)pyrene administration showed that, in normal guinea pigs, 34.9% of an oral dose was eliminated in the faeces within 72h, with 7.9% in the urine (Table 6.2). In animals fed high fat and high fat/high cholesterol diets, a greater proportion of the excreted tritium was recovered in the urine, and less in the faeces. These differences were statistically significant for the animals fed high fat diet (see Table 6.2). This suggests that an increased percentage of fat in the diet increases enterohepatic circulation of BP metabolites, possibly due to increased solubility of these lipid soluble compounds. Enterohepatic circulation of [^{14}C]benzo(a)pyrene metabolites has been shown to occur in rat (Chipman et al., 1981) and rabbit (Chipman et al., 1982).

7.8 Conclusions

Guinea pigs fed high fat and high cholesterol diets excrete the same precentage of an intravenous dose of [^{14}C]benzo(a)pyrene in the bile (approximately 30%) within 4h when compared with normal animals. However, the metabolic profile of these biliary metabolites is altered, such that a greater proportion of the radioactivity is attributable to glucuronide conjugates of BP dihydrodiols in the animals fed high fat

and high cholesterol diets. The source of the extra dihydrodiols was not an increase in epoxide hydrase, since the activity in the liver was unaltered by the administration of diets containing high levels of fat or cholesterol. The conversion of epoxides to phenols (see Figure 7.1) is believed to be spontaneous. Therefore reduction of glutathione S-transferase activity in guinea pigs fed high fat and high cholesterol diets seems to be the most likely explanation (see Figure 7.1). Vessey and Zakim (1981) have shown that bile acids, at physiological concentration, inhibit the total soluble fraction glutathione S-transferase activity from rat liver by about 50%. This may also true for guinea pigs. An inhibition was found for microsomal glutathione S-transferase from guinea pig liver, but may have been in part due to a detergent effect of the bile acids on the membrane-bound enzyme. The reduced activity of glutathione S-transferase under the influence of the greater quantity of bile acids secreted in the presence of high fat and high cholesterol diet will leave a greater proportion of BP metabolites available for the other pathways of metabolism (see Figure 7.1).

In summary, the proximate carcinogenic form of benzo(a)pyrene, the 7,8-dihydrodiol, was detected in guinea pig bile. The animals showed an altered profile of biliary metabolites when fed diets containing a high proportion of fat, or fat and cholesterol, with an increased formation of dihydrodiols. However, colonic microsomal drug-metabolising enzymes were undetectable in normal animals, and were apparently not induced by administration of these diets, by pretreatment of the animals with benzo(a)pyrene, or a combination of BP administration and high fat or high cholesterol diets. This was reflected in a very low level of covalent binding to DNA in vivo and the feeding of high fat and high cholesterol diets did not increase the

binding. This indicates that there is negligible interaction between DNA and active metabolites of benzo(a)pyrene within the colonic mucosa. Therefore the administration of benzo(a)pyrene to guinea pigs fed high fat or high cholesterol diets appears unlikely to provide a suitable animal model for studies on mechanisms of colon carcinogenesis.

Table 7.1   Species differences in biliary elimination of
[^{14}C]benzo(a)pyrene metabolites

Species	% dose in bile in 4-6h	Major metabolites	Reference
Rat	60%	Glucuronide conjugates of 4,5-diol, 3,6-quinone, 3OHBP and 9OHBP.	a
Rabbit	30%	9,10-Diol glucuronide. Also sulphate conjugates.	b
Guinea pig	30%	Glucuronide conjugates of 9,10- and 4,5-diols.	c

References

a. Chipman et al. (1981)
b. Chipman et al. (1982)
c. This thesis

Figure 7.1   METABOLIC ACTIVATION

Figure 7.2

The Fate of Benzo(a)pyrene in the Body
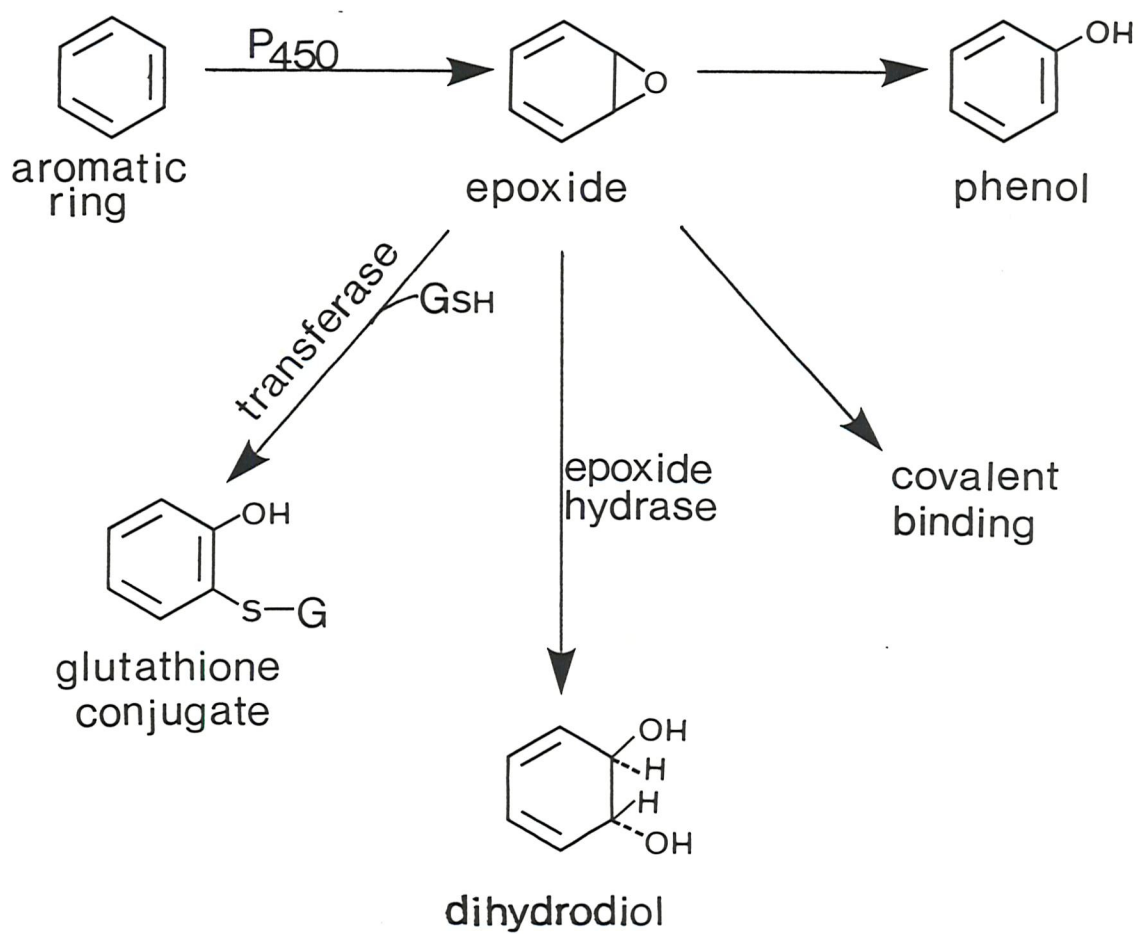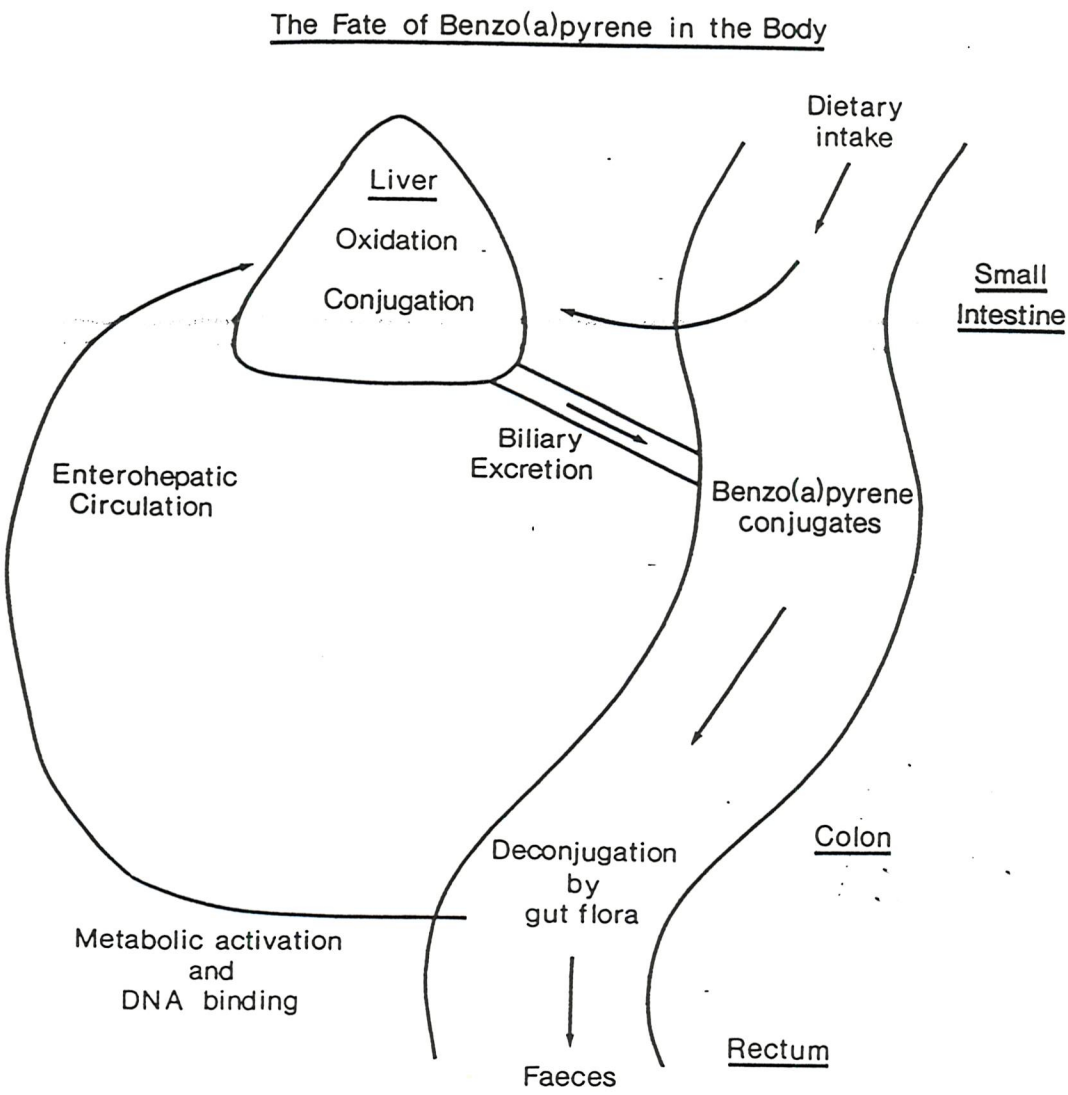
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