INFLUENCE OF ENDOGENOUS FEMALE SEX-STEROIDS ON MUTAGEN METABOLISM

by

RICHARD DAVID GOOLD

A Thesis Submitted to
Rhodes University
in Partial Fulfillment of the
Requirements for the Degree of

MASTER OF SCIENCE

January 1985

School of Pharmaceutical Sciences
Rhodes University
Grahamstown

Acknowledgements

I would like to express my sincere gratitude to the following people :

My supervisor, Dr BJ Wilson, for her invaluable guidance, constructive criticism, and encouragement throughout this study.

Prof B Potgieter, for his interest and advice.

Prof BN Ames, for the kind donation of the *Salmonella* strains, and Dr DM Maron, of the Ames laboratory, for her valuable suggestions.

Prof E van der Merwe and Prof DJ van Schalkwyk, for their assistance with the statistical analysis of the data.

Dr B McCulloch, for the work done by the State Veterinary Laboratory, Grahamstown, on my behalf.

Mr CL Beuthin and Mr HC Murrell, for help with the SPSS programme.

Dr RS Stafford, of the EMIC, Union Carbide Corporation, Oak Ridge, Tenneessee, for supplying mutagenicity data on the test compounds.

Mr S Goott, of MPS laboratories, for the donation of mitomycin C.

Prof I Kanfer, for access to departmental facilities.

Mr LH Purdon, for his technical assistance and encouragement.

The contributions of others to this study are also appreciated :

Prof EE Baart, Dr PG Berwick, Miss BR Davies, Mrs V Hodgson, Mr P January, Prof JFE Newman, Dr AE Russell, Mr AW Sonemann, Prof PD Terry, and the staff of the School of Pharmaceutical Sciences.

Finally, I am grateful for the financial support afforded by a South African Medical Research Council grant, and by a Glaxo bursary.

ABBREVIATIONS USED

A : adenine
AFB_1 : aflatoxin B_1
AHH : aryl hydrocarbon hydroxylase
ATP : adenosine triphosphate
bio : biotin gene
BP : benzo(a)pyrene
BSP : sulphobromphthalein
C : cytosine
CcR : NADPH-dependent cytochrome c reductase
chl : nitrate reductase gene
cyt : cytochrome
°C : degrees Celsius
DMN : dimethylnitrosamine
DMSO : dimethylsulphoxide
DNA : deoxyribonucleic acid
E_1 : oestrone
E_2 : oestradiol
E_3 : oestriol
ECOD : ethoxycoumarin-*O*-deethylase
EROD : ethoxyresorufin-*O*-deethylase
EMIC : Environmental Mutagen Information Centre
FF : undefined hypothalamic feminizing factors
G : guanine
g x 1000 : gravity
gal : 'rough' deletion
GH : growth hormone
his : histidine deletion
HSDH : hydroxysteroid dehydrogenase
IARC : International Agency for Research on Cancer
ip : intraperitoneal
K_m : Michaelis-Menten constant
kg : kilogram
l : litre
LPS : lipopolysaccharide
LSI : liver somatic index
M : molar
3MC : 3-methylcholanthrene
MGA : minimal glucose agar
MMC : mitomycin C
mRNA : messenger RNA
NADP : nicotinamide adenine dinucleotide phosphate
NADPH : NADP (reduced form)
OD_{650} : optical density at wavelength 650 nm
+ : wild type gene
pAQ1 : multicopy plasmid
PB : sodium phenobarbital
PBG : progesterone binding globulin
PCB : polychlorinated biphenyl
PCH : polycyclic hydrocarbon
PCN : pregnanolone-16α-carbonitrile
pKM101 : R-factor
PRG : progesterone
PRL : prolactin
psi : pounds per square inch
RCF : radial centrifugal force
rfa : 'deep rough' deletion
RNA : ribonucleic acid
rpm : revolutions per minute
S : Svedberg units (sedimentation)
S9 : 9000 *g* supernatant
SKF-525-A : 2-diethylaminoethyl 2,2-diphenylvalerate
SNK : student-newman-keuls procedure
SPSS : statistical package for the social sciences
SRF : spontaneous reversion frequency
T : thymine
TEBG : testosterone-oestradiol binding globulin
$t_{½}$: biological half-life
U : uracil
UDP : uridine diphosphate
UDPGA : uridine diphosphoglucuronic acid
uv : ultraviolet
uvrB : uv repair B gene deletion
V_{max} : maximal velocity (enzyme kinetics)
W : watt

CONTENTS

List of Figures

List of Tables

ABSTRACT

Cytochrome P-450, the terminal oxidase of the metabolic mono-oxygenase system, is thought to exist in multiple forms, which have differing substrate specificities, and are variably inducible by different enzyme inducers. Many mutagens, themselves unreactive, require metabolic activation by one or more of these cytochrome P-450-dependent microsomal enzymes for mutagenic activity. Such mutagens may be detected in the *Salmonella* mutagenicity test only by the incorporation of an hepatic microsomal (S9) fraction into the assay (as a first approximation to *in vivo* metabolism). Induction of the microsomal enzymes by different agents enhances the metabolic activation of mutagens; in fact, many mutagens are only detected when the S9 fraction has been induced by appropriate agents. Inducers of the phenobarbital-type are known to enhance microsomal steroid hydroxylation and, in turn, the steroids, when administered at supraphysiological levels, have been reported to be inducers of several mono-oxygenase activities.

The inductive effects of the female sex-steroids and the combined effects of steroid and phenobarbital (PB) pretreatment on the metabolic activation of four mutagens have been investigated using the *Salmonella* assay. Female Sprague-Dawley rats were pretreated with 17β-oestradiol (E_2) or progesterone (PRG), at a level of either 1 mg/kg or 20 mg/kg daily for 14 days. A duplicate set of similarly pretreated groups were also induced with PB. Hepatic microsomal fractions were prepared from each group and incubated with each of the test mutagens in the presence of a tester strain known to detect each particular type of mutagen. Induction of the hepatic metabolizing system by PB increased the activation of the mutagens significantly (as reflected by an increased number of revertant prototrophic *S.typhimurium* colonies). The administration of PRG also caused significant, and dose-dependent, induction of the activation of aflatoxin B_1, benzo(a)pyrene, and dimethylnitrosamine. In general, E_2 exhibited no inductive effect, but it did produce an increase in the activation of aflatoxin B_1 (a reaction which is known to be catalysed by a mono-oxygenase preferentially inducible by PB). When use was made of a microsomal fraction that was prepared from animals which were both steroid-pretreated and induced by PB, mutagenic activation was of the same order of magnitude as that observed when induction was brought about by PB alone. The absence of additive effect, taken together with the observations already mentioned, indicate that steroids induce the same cytochrome isozymes that are induced by PB.

The implications of sex-hormonal regulation of the metabolic activation of mutagens are briefly discussed.

CHAPTER 1 : INTRODUCTION : CYTOCHROME P-450, MUTAGEN METABOLISM AND THE AMES TEST

1.1 THE EXISTENCE OF MULTIPLE FORMS OF CYTOCHROME P-450

Cytochrome (cyt) P-450 is the terminal oxidase of the microsomal electron transport system, responsible for the oxidative metabolism of endogenous steroids and fatty acids, as well as of many drugs, and other xenobiotics. The chemical nature and reactivity of cytochrome(s) P-450-linked oxidations have been the subject of several reviews (86,114,232,258).

A growing body of evidence suggests the existence of multiple forms of cyt(s) P-450 which have distinct (but sometimes overlapping) specificities with regard to substrate interaction and mono-oxygenation (114,165,232,288). Two (or more) mono-oxygenase systems may exist in the liver microsomes, one employing cyt P-450 (also called cyt $P\text{-}450_{LM2}$) as its terminal oxidase, the other cyt P-448 (also called cyt P_1-450 or cyt $P\text{-}450_{LM4}$) (114,117,232). Antibodies prepared against purified cyt P-450 do not cross-react with cyt P-448, and vice versa, a finding which shows that the two haemoproteins are dissimilar (165,232). However, it is now known that the terms cyt P-450 and cyt P-448 also define classes of P-450 haemoproteins, rather than specific cytochromes (118).

In this study, cyt P-450 is considered to be the labile P-450 haemoprotein found predominantly in the liver of the untreated or phenobarbital-treated rat; cyt P-448 is considered to be the stable P-450 haemoprotein found predominantly in livers of rats treated with certain polycyclic hydrocarbons (the prototype of which is 3-methylcholanthrene (3MC)) (118). The term cyt(s) P-450 designates any cytochrome which has a spectral maximum of about 450 nm when it is reduced and complexed with carbon monoxide; this includes both cyt P-450 and cyt P-448 (117).

1.2 DIFFERENTIAL INDUCTION OF CYTOCHROME(S) P-450

Many structurally-diverse compounds are capable of inducing the microsomal mono-oxygenases (66). These inducing agents are often generally classified as being either phenobarbital-like (preferentially inducing the cyt P-450-dependent systems), or 3MC-like (preferentially inducing the cyt P-448-dependent systems) (117,232). Recently, the existence of a third type of inducing agent has been propounded, the administration of which induces enzymes in a pattern similar to that observed after the coadministration of phenobarbital (PB) and 3MC. In this class of compounds belong the polychlorinated biphenyls (for example, Arochlor) and polybrominated biphenyls. Certain of the polychlorinated biphenyls have however been resolved into isomers which, when administered separately, are either PB-type or 3MC-type inducers (232). Therefore, it is possible that this class may not constitute a new type of inducer, but that their induction characteristics may be due to the fact that these agents are mixtures of PB-type and 3MC-type inducers. The mechanism of enzyme induction by these different compounds, and the physiological and pharmacological implications of their action, have been reviewed by several authors (66-68,103,112,232,248).

1.3 CYTOCHROME(S) P-450-DEPENDENT ACTIVATION OF MUTAGENS

The cyt(s) P-450 isozymes are known to be responsible, more so than other enzymes, for the *in vivo* metabolism of certain toxic compounds to mutagenically active intermediates (119,144,172,195,197, 212,220,222,223,279,289,299). In addition, antibodies against cyt P-450 and cyt P-448 inhibit the *in vitro* mutagenicity of several mutagens almost completely (165).

Mutagenic metabolites may be electrophiles (epoxides, carbonium ions or nitrenium ions) capable of random covalent binding to numerous cellular nucleophiles, including the oxygen and nitrogen atoms of DNA bases (119,157). Alternatively, reactive intermediates may be of the free radical variety, thought to induce lipid peroxi-

dation and the destruction of several cellular components, including DNA (12,144). If the mutagenic metabolite is a planar aromatic molecule, it may also intercalate with the DNA structure, at a sequence of repeating nucleotides, resulting in frameshift mutations (80,211,212). The complex subject of mutagenesis is outside the immediate scope of this work, except insofar as it is mediated by the four test mutagens studied (see Chapter 2). A number of comprehensive reviews of the biochemistry of mutagenesis have been published (80,81,95,113,130,195,220,221,223,264,285,287).

The contribution of each of the molecular species of cyt(s) P-450, towards the metabolic activation of a mutagen, is specific for the particular compound (144,165). In addition, the content of individual cyt P-450 isozymes in the microsomes is variable according to the induced state of the liver (165). Therefore, the contribution of each of the individual forms of cyt P-450, to the total activation of a promutagen to mutagenic metabolites, is dependent, not only on the specific catalytic activity, but also on the content (level of induction) of the isozyme in the hepatic microsomes (144,165). If the specific activity is assumed to be constant, then a quantitative estimation of the contribution of each cyt P-450 isozyme is possible (144,165).

Also present in the liver are a number of metabolic pathways by which mutagenically reactive intermediates may be detoxified; these include binding to 'scavenger nucleophiles' such as glutathione, as well as various enzyme-catalysed conjugation reactions (119,144, 172,195,267).

The net toxicity of mutagens in any *in vivo* (or *in vitro*) system is thought to depend on the balance between the pathways of activation and those of detoxification. This 'balance' is influenced by the levels of induction of both the particular cyt P-450 isozyme (or other enzyme) responsible for the activation of the mutagen, and of the enzymes of deactivation, and also by the competition for any of these enzymes by other endogenous or exogenous substrates (39,119, 144,157,172,267).

1.4 HORMONAL REGULATION OF MUTAGEN METABOLISM

Present studies on species- and sex-differences in metabolism have indicated that the hormonal status of an animal may be an important factor in the regulation of mutagen metabolism (24,31,173,209,291).

The sex-steroids are known to modify, directly or indirectly, macromolecular function in the cells of many tissues, binding to specific protein receptors which promote their preferential retention in hormonally sensitive cells (31). Although steroids, *per se*, are not metabolized to mutagens, they appear to have a highly specific regulatory action on hepatic metabolism (31,209). At physiological levels, the endogenous female sex-steroids are competitive inhibitors of the same cyt P-450-dependent mono-oxygenases that are responsible for mutagen activation, and are, therefore, probably alternative substrates of these enzymes (Section 3.3) (233,282).

These steroids have been reported to enhance the metabolic activation of mutagens, when administered at high doses, possibly predisposing the organism to increased risk (4,31,180). The interaction of the steroids with cyt(s) P-450 and their effects on the metabolism of xenobiotics are discussed in Chapters 3 and 4.

The aim of this study was the investigation of the influence of supraphysiological levels of the endogenous female sex-steroids, 17β-oestradiol and progesterone, on the metabolism of four mutagens. The metabolic biotransformation of the four test compounds to mutagenically reactive intermediates was quantitatively determined in this investigation using the Ames test as a bioassay (see Section 1.5.2).

1.5 THE AMES TEST

1.5.1 Introduction

Short-term *in vitro* and submammalian mutagenicity tests have evolved in response to the disadvantages of longer-term animal carcinogenicity tests. The time and expense required for animal bioassays, as well as their low sensitivity, limit their utility in screening the vast number of synthetic chemicals to which humans are exposed (11,12,208). The short-term mutagenicity assays in current use have been described in the literature (75,79,127,169,208,212,240,277), and have been most comprehensively reviewed by Hollstein *et al.* (133;678 references).

Of the microbial mutagenicity assays, the Ames test is regarded as being the only test which presently meets the criteria of an established predictive test in genetic toxicology (56,237,239). The relative rapidity, sensitivity, simplicity, and low-cost of the assay, have contributed to its current use in more than two thousand government, industrial, and academic laboratories (11,16,39, 125,237). Test results on over 2600 chemicals have been reported in the Environmental Mutagen Information Centre (EMIC) bibliography (202). A fully computerized system for the performance and analysis of the Ames test became available in 1984 (Mutascreen^{R}, Labsytems Oy, Helsinki).

The assay method has been recently updated in a detailed publication by Maron and Ames (202), and is described in Chapter 5.

The Ames test is performed by the combined incubation, in a histidine-deficient medium, of a small amount of test compound, about a billion histidine-auxotrophic bacteria, and an hepatic microsomal homogenate, usually prepared from rats (39).

The strain of bacteria used may be one of a set of specially-constructed mutant *Salmonella typhimurium* strains, selected for their sensitivity and specificity in being reverted from a require-

ment for histidine to prototrophy (11,20,202). The *Salmonella* tester strains are described in some detail in Section 1.5.4. Because these bacteria have few of the enzymes required for the metabolic activation of mutagens (214,265), a microsomal (S9) fraction is incorporated, as an *in vitro* first approximation to *in vivo* mammalian metabolism (10,11,13,50,184,197,215,216,237). This facilitates the detection, by the *Salmonella* strains, of most promutagens which require metabolic activation for their mutagenicity (16,37,47,100, 125,212).

After incubation on a petri-plate at 37°C for 48-72 hours, the number of revertant prototrophic *Salmonella* colonies are counted (22,33). Each colony is descended from a bacterium that has mutated from a defective histidine gene (his^-) to a functional one (his^+) (11,212). The number of revertant colonies per plate provides a quantitative estimate of the mutagenic potency of the test compound (11,40,75,79,212,214). Normally, test results are either clearly negative or clearly positive, and a linear dose-response curve is usually observed with positive responses (76,212,214).

1.5.2 Applications of the Ames Test

The primary applications of the Ames test are the screening of new synthetic chemicals for mutagenicity, and the testing of established products for mutagenic impurities (214).

It is estimated that at least one thousand new chemicals are introduced into the human environment each year, and that over sixty-three thousand are in everyday use in households and industry (11,17,144,208). These products include various drugs, food additives, pesticides and cosmetics (144). Although mutagenicity testing *per se* is not yet a premarketing requirement for new consumer products, it may become so in the near future (55). For the purposes of general mutagenicity screening, efforts have been made towards the standardization of the test protocol (2,77,105,111,129, 260,261), and a recent international collaborative study has indicated that such standardization significantly increases the reproducibility of the assay (3,23,62,83,128,200).

Several modifications of the Ames assay protocol have broadened the utility of the test (19,97,196,202,207,224,301).

Environmental mutagenic pollution of air and water supplies is routinely tested in modified Ames tests, as are complex mixtures such as cigarette smoke condensates, food, and the protein pyrolysis products of foods (11,12,202,211,214). The urine and faeces of animals may also be tested for the presence of the mutagenic metabolites of ingested products (9,64,84,156,166,214,254,308).

The Ames test finds further application as an enzyme assay, with the number of *his*$^+$ revertant *Salmonella* colonies being a measure of the enzymatic activity of the S9 preparation (20,196). Differences among S9 fractions prepared from various tissues and species may thus be determined, and the effect of various pretreatments on the metabolic activity of S9 can be investigated (39,125,267).

The rate of mutagenic activation, as measured by mutagenesis in the *Salmonella* strains, is assumed to quantitatively reflect the metabolic capacity of the liver in the intact animal (31). The *Salmonella*/microsomal test, used in this way, is not a simple enzyme assay, specific for a particular activating enzyme, since the S9 homogenate contains the full complement of enzymes involved in the metabolism of mutagens (13,281). Therefore, the resulting mutagenicity reflects the effects of pretreatment on the overall balance of the pathways of both activation and detoxification of the test mutagens (281).

1.5.3 Correlations Between *In Vitro* Mutagenicity and *In Vivo* Carcinogenicity

The use of short-term *in vitro* and submammalian tests as a predictive indicator of animal carcinogenicity is the subject of some controversy. While it is clear that the liver homogenate is able to metabolize most promutagens to their active mutagenic forms, it cannot faithfully duplicate the absorption, distribution, metabolism, and excretion of a compound in the intact animal (16,27,42, 125,175,212,237).

Again, although it may well be that mammalian and bacterial DNA are basically similar, and that it is possible to expose considerably more DNA to mutagens using bacterial populations (211,237), the DNA repair capability of mammalian cells appears to be different to that of bacteria (175,212).

Classes of carcinogens which appear to cause cancer by nonmutagenic mechanisms (for example, the steroid hormones), are not detected by microbial mutagenicity assays (15-17,19,249). It is possible that these carcinogens affect cell growth and thus may act as 'promoters' of tumours rather than 'initiators', which are thought to interact with DNA (11,16,175,212).

Nevertheless, over 80% of known animal carcinogens are mutagenic in *Salmonella*, and in the majority of reports, there is an excellent correlation between *in vitro* mutagenicity and *in vivo* carcinogenicity (11,56,74,207,211,212,219).

The value of *in vitro* mutagenicity tests, including the Ames test, as predictors of animal carcinogenicity has been reviewed extensively (11,15-17,28,35,39,50,55,56,65,74,79,99,125,127,143,207,208, 211,212,214,215,219,236,240,241,243,250,277,284). A 'battery' of short-term tests is favoured by many investigators, so that the strengths of one test can compensate for the inadequacies of another in detecting specific classes of mutagens (9,11,15-17,50,74, 182,202). *In vitro* mutagenicity tests are regarded as an excellent method of prescreening environmental chemicals to be submitted to a more rigorous set of animal bioassays (39,50,212,214).

1.5.4 The *Salmonella* Tester Strains

The parent of the strains used in the Ames test is *Salmonella typhimurium LT-2*, the causative organism of infective diarrhoea (20,202). Each of the numerous tester strains contains one of several different known mutations of the histidine operon, resulting in a growth requirement for histidine (146). Reversion requires a compensating mutation, almost invariably of the same

general type as the original (146). In addition to the basic histidine mutation, the strains possess other mutations which greatly enhance their sensitivity to mutagens (202).

Table 1.1 shows the genotypes of the most useful strains. A set of four standard strains (TA97, TA98, TA100 and TA102) is currently recommended for general mutagenicity screening. Used in concert, they detect a wide range of mutagen classes with greater efficiency than other strains (7,202). The different histidine mutations are discussed in the sections dealing with the the individual strains, and the additional mutations are described below.

Table 1.1 : Genotypes of *Salmonella typhimurium* TA Strains Used in Mutagenicity Testing

Histidine Mutation						Additional Mutations		
hisD6610 hisO1242	*hisD3052*	*hisG46*	*hisC207*	*hisG428*	*hisC3076*	LPS	Repair	R-factor *(pKM101)*
TA90	TA1538	TA1535	TA1536	-	TA1537	*rfa*	*uvrB*	*-R*
(TA97)	(TA98)	(TA100)	-	TA104	TA2637	*rfa*	*uvrB*	*+R*
-	TA1978	TA1975	TA1976	-	TA1977	*rfa*	+	*-R*
TA110	TA94 TA2420	TA92	-	TA103	TA2425	+	+	*+R*
-	TA1534	TA1950	TA1951	-	TA1952	+	*uvrB*	*-R*
-	-	TA2410	-	-	-	+	*uvrB*	*+R*
TA89	TA1964	TA1530	TA1531	-	TA1532	*gal*	*uvrB*	*-R*
-	TA2641	TA2631	-	-	-	*gal*	*uvrB*	*+R*
-	-	-	-	(TA102)	-	*rfa*	+	*+R*
TA88	*hisD3052*	*hisG46*	*hisC207*	-	*hisC3076*	+	+	*-R*

All strains were originally derived from *S.typhimurium LT-2*.
Strains in brackets are the current standard tester set (recommended for general mutagenicity screening).
Abbreviations : LPS : Lipopolysaccharide of bacterial cell wall
+ : wild type gene
gal : "rough" deletion (defective LPS)
rfa : "deep rough" deletion (defective LPS)
uvrB : deletion of the ultra-violet repair B gene
R-factor : the plasmid *pKM101*, which increases error-prone DNA repair
In TA104, the *hisG428* mutation is on the chromosome, whereas in TA102 it is on the multicopy plasmid *pAQ1*.
See text for details of mutations.

Table adapted from 7, 18, 20, 185, 186, 201, and 202.

The lipopolysaccharide (LPS) molecules of the normal bacterial cell wall act as a barrier to the passage of large molecules, including many mutagens, into the cell. It is possible to increase cell wall permeability to these molecules by introducing mutations that remove part of the polysaccharide side-chain of the LPS molecule (18). The *gal* ('rough') deletion prevents the synthesis of galactose, causing the loss of that part of the LPS side-chain distal to the first galactose unit (18).

The *rfa* ('deep rough') deletion prevents the synthesis of a polysaccharide which is distal to the ketodeoxyoctanoate-lipid core of the cell wall LPS. This results in the maximum 'stripping' of the LPS molecule that is possible without cell lethality, and greatly increases the permeability to large molecules (18,202).

The *uvrB* mutation inactivates the gene coding for the DNA excision repair system, resulting in greatly increased sensitivity to mutagens that form repairable DNA lesions, without quantitatively altering the mutagenic response (18,21,22,202,214). Intercalating agents are equally effective on $uvrB^-$ and $uvrB^+$ strains, but compounds that alkylate as well as intercalate are detected with much increased sensitivity with $uvrB^-$ strains (8). The *uvrB* deletion also extends through the *gal*, nitrate reductase (*chl*) and biotin (*bio*) genes (the latter resulting in a requirement for biotin) (186,202).

The four standard tester strains all contain the R-factor plasmid, *pKM101*, which increases chemical and spontaneous mutagenesis by enhancing the rate of error-prone DNA recombinational repair (202, 216,279). Strains containing the plasmid show a marked increase in sensitivity to mutagens, especially those that cause DNA damage that is not directly mutagenic, but becomes so when repaired by error-prone repair.

The details of these additional mutations are presented by Ames *et al.* (18), and the development of the current tester strain set, and the characteristics of the less frequently used strains are well documented (8,13,14,18,21,22,76,146,185,186,202,215,216).

1.5.4.1 TA 97 : Frameshift Mutagens

Runs of repetitive base sequences in DNA are often 'hot-spots' for frameshift mutagenesis. Shifted base pairings may occur more easily in these repetitive sequences (212), and frameshift mutagens can, by intercalation into the DNA structure, stabilize the mispairings by restoring the correct reading frame for histidine biosynthesis (202). The critical site for reversion in TA97 is such a run, consisting of six cytosines (*hisD6610*). The development and characterization of TA97 (outlined briefly below) is detailed by Levin *et al.* (186), and the lineage of *hisD6610* shown in Figure 1.1.

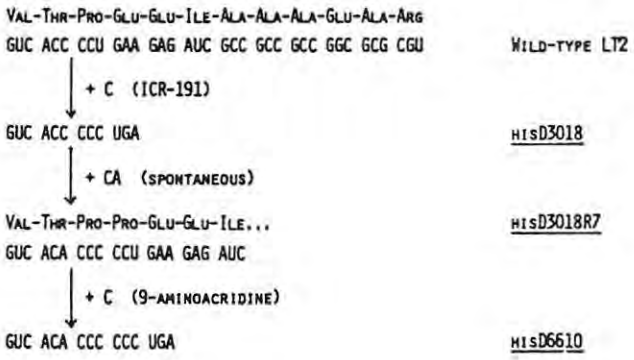


Figure 1.1 : Lineage of the *hisD6610* mutation of TA97 (185)

The wild-type strain, *S.typhimurium LT-2*, has four cytosines in the *hisD* gene (which encodes for histidinol dehydrogenase) of the mRNA. Another cytosine is added to this by mutagenesis with ICR-191 (an acridine half-mustard derivative) to form the *hisD3018* mutation. A spontaneous revertant of *hisD3018*, designated *hisD3018R7*, has an extra proline triplet, due to the insertion of two additional bases in the run of cytosines at the *hisD3018* site. Another cytosine is introduced to *hisD3018R7* by the frameshift mutagen 9-aminoacridine, resulting in a run of six cytosines, the *hisD6610* mutation. There is also a run of alternating -G:C- base pairs several codons to the 3' side of the *hisD6610* mutation (see Figure 1.1), constituting another 'hot-spot' (186).

Note on TA97 : Subsequent to the completion of this research, TA97 has been replaced by a new strain TA97a. This new strain was constructed from TA97 in response to difficulties encountered with the strain, namely, poor viability and growth, pin-point colonies, and hypersensitivity to mutagen toxicity. TA97a has a smaller *uvrB* deletion than TA97, and is claimed to lack the disadvantages of TA97, while still retaining a mutagenic response identical to its parent (7,201).

1.5.4.2 TA 98 : Frameshift Mutagens

The *hisD3052* mutation is introduced into the wild strain by mutagenesis with ICR 364-OH, detailed by Isono and Yourno (146), and shown in Figure 1.2. It arises from the deletion of a single -G:C- pair from the *hisD* gene, and results in a nonfunctional gene product (146). Close to this original -1 frameshift mutation is a rare mispairing-prone sequence of eight repeating -G:C- pairs, another 'hot-spot' for frameshift mutagenesis. Restoration of the original base sequence (replacing the deleted cytosine) is extremely rare with *hisD3052* (146). However, deletion of a -G:C- base-pair doublet early in the repeating sequence restores the normal reading frame in the mRNA, except for the sequence between the two frameshifts (146,212). Hence a gene-product is synthesized with only a short region of wrong codes (212).

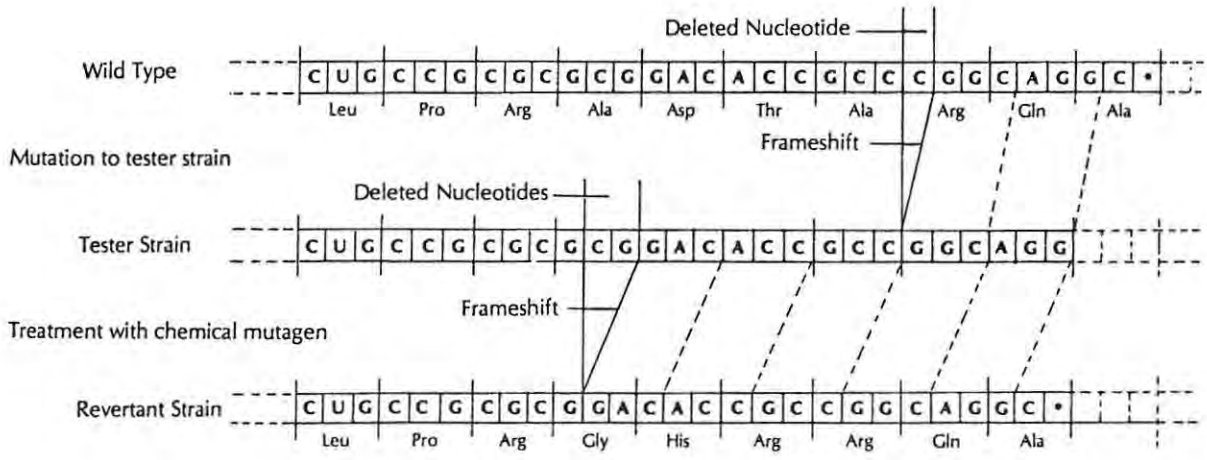


Figure 1.2 : Formation and reversion of the *hisD3052* frameshift detecting mutation of TA98 (212)

1.5.4.3 TA 100 : Base-Pair Substituting Mutagens

TA100 contains the *hisG46* mutation in the gene which encodes for the first enzyme of histidine biosynthesis (22,104). The substitution of a cytosine nucleotide for a thymine changes the RNA triplet code to encode for proline instead of leucine (Figure 1.3) (21,202, 212). The incorporation of proline, which disrupts the secondary structure of proteins, results in a nonfunctional enzyme. Reversion to a functional gene can occur either by restoration of the original wild-type code, or by a variety of extragenic suppressor mutations (22,197,212). The lack of specificity of the revertant codes may explain why strains containing *hisG46* are so efficient for detecting a wide variety of base-pair substituting mutagens acting at one of the -G:C- pairs, since almost any base change that results in an amino acid other than proline will allow the enzyme to function (202,212).

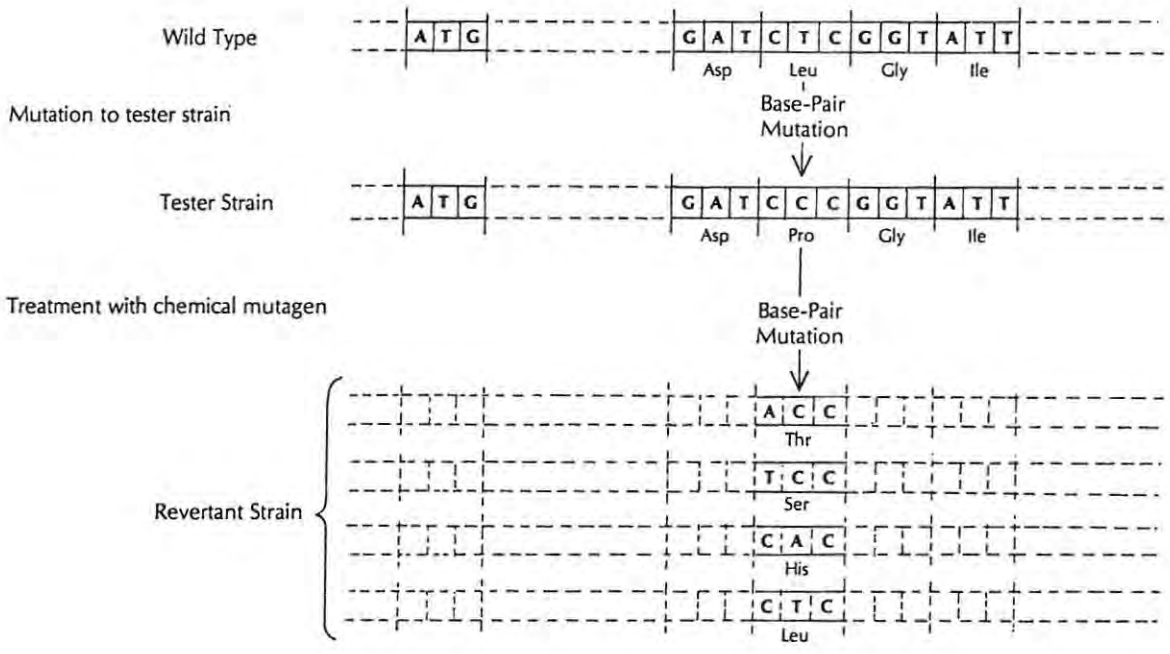


Figure 1.3 : Formation and reversion of the *hisG46* base-pair substitution detecting mutation of TA100 (212)

1.5.4.4 TA 102 : Oxidizing Mutagens

TA102 detects the oxidizing mutagens, which may be the most important class of mutagens contributing to cancer and , more controversially, to aging (12,74,185,202). These mutagens, which are not detected by the other strains, include X-rays, mitomycin C, bleomycin, hydroperoxides and quinones; a variety of aldehydes and psoralens, and UV light (185,202). The unique sensitivity of TA102 is due to its ochre histidine mutation, *hisG428*, selected as the histidine mutation best reverted by oxidative DNA damage (185).

HisG428 has -A:T- base pairs at the critical site for reversion, not the -G:C- pairs of the other histidine mutations (202). The sensitivity of TA102 to reversion was increased markedly by increasing the number of copies of the histidine mutation in each cell (185,202).

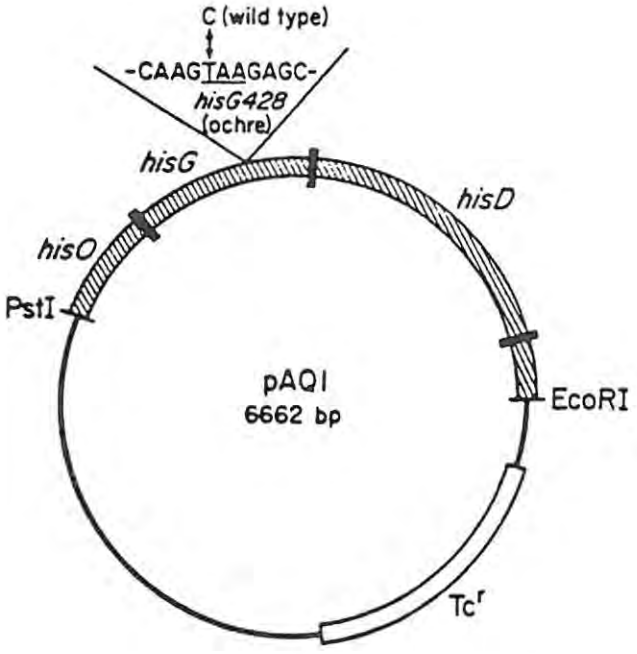


Figure 1.4 : Structure of the multicopy plasmid, *pAQ1*, of TA102, showing the ochre mutation, *hisG428*. Tc^r : tetracycline resistance marker (185)

This was achieved by the cloning of the *hisOGDC* region (which includes the *hisG428* mutation) onto a multicopy plasmid, *pAQ1* (Figure 1.4), which also carries a marker for tetracycline resistance (the basis of the genotype confirmation test for TA102). This plasmid was then introduced into a *Salmonella* strain which contained both the *rfa* marker and the *pKM101* plasmid, forming the tester strain TA102. Because the detection of cross-linking mutagens requires an intact DNA excision repair system, TA102 was constructed with a $uvrB^+$ background (185,202).

It has been determined that there are about 30 copies of *pAQ1* and 7-8 copies of *pKM101* per cell (185). The sensitivity of TA102 to mutagens is increased five- to twelve-fold (and SRF increased by six-fold) over strains with the *hisG428* on the chromosome (e.g. TA104), rather than on a multicopy plasmid (185,202).

CHAPTER 2 : THE METABOLISM AND MECHANISM OF MUTAGENICITY OF THE TEST MUTAGENS

2.1 CHOICE OF MUTAGENS

Each of the four standard tester strains detects a different class of mutagenic compound; for this reason, mutagens were selected, for each tester strain, which are representative of the class detected by the strain. It is known, however, that some of the tester compounds may cause more than one kind of mutation and, therefore, may also be detected by others of the tester strain set.

Wherever possible, mutagens were selected from those to which human environmental exposure may be significant. However, the choice of mitomycin C was largely governed by the sensitivity of TA102, which detects oxidizing mutagens.

Compounds which require metabolic activation for mutagenicity were chosen for this study, in order to investigate the efficiency of different pretreatments of the liver on this activation. Although the mutagenicity of mitomycin C may be detected without the incorporation of a microsomal fraction, metabolic activation has been reported to enhance the mutagenic response several-fold (184,185).

2.2 DIMETHYLNITROSAMINE

Dimethylnitrosamine (DMN) is representative of a large class of compounds, the nitrosamines, which are metabolically activated to potent carcinogens.

Exposure to nitrosamines is primarily brought about through the intragastric nitrosation of dietary nitrate and nitrite (as well as other nitrogen-containing compounds) (11,141). This nitrosation is catalysed by the nitrate reductases of the bacterial flora normally resident in the gastrointestinal tract and is optimal under weakly acidic conditions (11,141,256). Although the use of nitrates and

nitrites as food preservatives is much less prevalent today than in the past, DMN can be detected in human blood after the ingestion of a normal meal, and it has also been found in stored meat, fish, cheese, alcoholic beverages, vegetables and vegetable oils, as well as in pesticides and drinking water (141).

Although DMN is carcinogenic in several species (reviewed in the IARC monograph (141)), and mutagenic in *Drosophila*, it cannot be detected in bacterial mutagenicity assays, nor in *Neurospora*, without the incorporation of an hepatic microsomal (S9) fraction. This phenomenon is thought to be due to the inability of the microorganisms to perform the metabolic dealkylation of DMN, which yields mutagenically reactive metabolites (95,127,197,237).

As well as inducing tumours in rat liver, kidney, and lung, DMN has acute toxicity, causing haemorrhagic centrilobular necrosis of the liver, damage to the hepatic central veins, and less severe injury to other organs (primarily the kidney) (195).

DMN undergoes oxidative demethylation in the liver, a process catalysed by the microsomal enzyme, DMN demethylase, and the reaction exhibits a requirement for NADPH and molecular oxygen, indicating the participation of cytochrome(s) P-450, the terminal oxidase of the microsomal MFO system (Figure 2.1) (8,13,71). The products of dealkylation are formaldehyde (which is rapidly converted to CO_2 and released in expired air), and the unstable monomethylnitrosamine. This monomethyl derivative apparently decomposes spontaneously, both *in vitro* and *in vivo*, to yield two alkylating species, the methyl carbonium and diazomethane ions (38,71,95,195,198,220, 221,223,238).

The alkylating metabolites of DMN react with cellular nucleophiles, including DNA, RNA and proteins (195,220). The active DNA alkylating agent is not the diazoalkane, but the carbonium ion, which alkylates the N-7 position of guanine, as demonstrated with deuterated DMN in rats (95,190,195,197,220). This position (N-7 of guanine) is the preferred position for alkylation of double-stranded DNA (95,220).

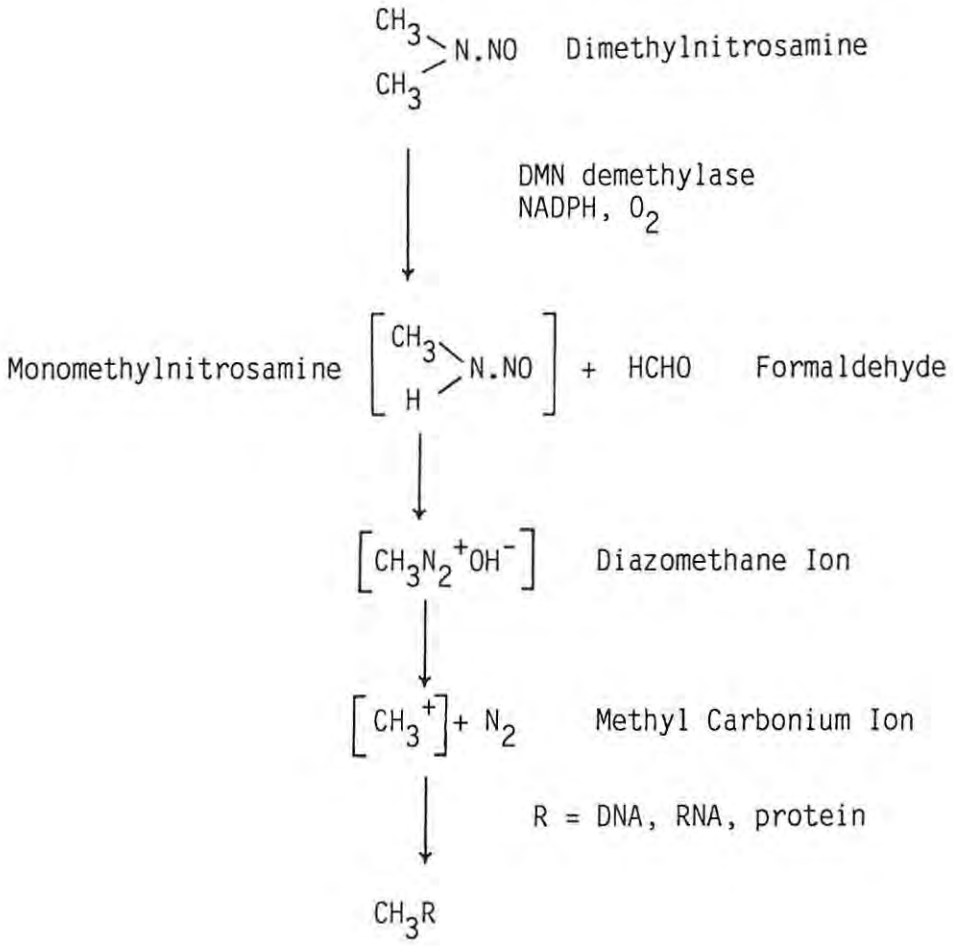


Figure 2.1 : Metabolic biotransformations of dimethylnitrosamine (adapted from 195,197,222)

N^7-methylguanine readily ionizes to a form of the base which can pair, through hydrogen bonding, with the normally noncomplementary base, thymine (220). This constitutes a G→A base-pair transition (base-pair substitution mutations involving transitions always retain pyrimidine:purine pairings, whereas transversions can produce purine:purine or pyrimidine:pyrimidine pairings) (13,80,95).

As well as causing transitions initially, N^7-alkylation of guanine may, much more slowly, cause the hydrolysis of guanine from DNA (depurination), and consequently, cleavage of the DNA backbone (80, 95,220).

False negative results are obtained with DMN in the standard plate-incorporation Ames assay, with both uninduced and induced rat hepatic S9 fractions. However, DMN can be detected as mutagenic when the preincubation modification of the Ames test is utilized (16,38,214,237,238,278,307). The failure of the standard test was thought to be the result of the short-lived, but highly reactive, alkylating metabolites of DMN becoming trapped on the nucleophilic hydroxyl groups of the soft agar component, and so being unable to reach and methylate the nucleophilic groups of the bacterial DNA (36,38,50). Now, however, it is attributed to insufficient enzymatic activation in the standard assay (the mutagen, S9 fraction, and bacteria are generally incubated at higher concentrations in the preincubation assay) (16,207,214,237,238).

In support of this latter hypothesis, it has been found that, if the concentrations of S9 or DMN in the preincubation assay are reduced to those of the standard test, little or no mutagenicity results. However, when the concentrations of the standard assay are increased to those encountered in preincubation, the mutagenic response is equal to that found in the preincubation procedure (238).

Kinetic data have demonstrated that DMN demethylase exists in two forms, with different substrate affinities (26). In the rat (and the mouse), the apparent K_m values of these forms are 0,2 mM and 51 mM, and the two isozymes appear to be controlled independently by enzyme inducers (26,238). These considerations suggest that more than one cyt(s) P-450 species (and possibly, other types of enzyme) may be involved in DMN activation (26).

Induction of the rat, hamster, or mouse hepatic microsomal MFO system with PB or 3MC causes a two-fold increase in the activation of DMN to mutagenic metabolites, although, *in vivo*, such induction appears to protect these species from the acute toxicity and carcinogenicity of DMN (38,71,195).

2.3 AFLATOXIN B_1

Aflatoxin B_1 (AFB_1) is a mycotoxin produced by *Aspergillus flavus* and *A.parasiticus*, as well as, under some conditions, by *A.oryzae* (136,195,278,304). These widespread fungi thrive on grain and grain products under moist storage conditions, though virtually all foodstuffs have potential for aflatoxin contamination (140,278).

AFB_1 is the most potent hepatocarcinogen known in rats, and it is also carcinogenic in several other species, including the primates (136,140,195,223,273,278,304). Positive correlations between the intake of aflatoxin-contaminated dietary staple and the incidence of hepatic carcinoma are reported from Central and Southern Africa, and also from Thailand, providing circumstantial evidence for a causal relationship in man (140,222,223). AFB_1 is also mutagenic in most submammalian test systems incorporating an hepatic microsomal system (136,140,195,273).

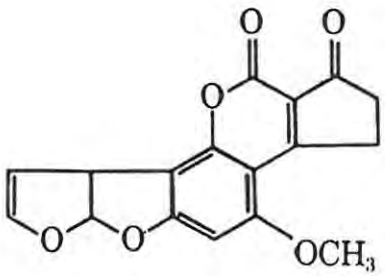


Figure 2.2 : Aflatoxin B_1 (304)

The molecule consists of a bisulfuran ring system, fused to a substituted cyclopentanone moiety, with a methoxy group attached to carbon-9 of the benzene ring (see Figure 2.2) (100,136,195,223, 304). The 2,3-vinyl ether double bond, methoxy group, and cyclopentanone ring are each crucial to toxicity, and any alteration of these structural features decreases the mutagenic activity of AFB_1 (136,304).

Although AFB_1 is, itself, unreactive towards nucleophiles, nucleic acid- and protein-bound radioactivity, after administration of ^3H-labelled AFB_1, suggests the metabolic conversion *in vivo* to mutagenically active intermediates (100,195). In addition, AFB_1 requires a microsomal enzyme fraction and an NADPH-generating system for mutagenicity in *Salmonella* (136,140,278,304). The hepatic biotransformations of AFB_1, both various oxidative conversions catalysed by the microsomal mixed-function oxidases, as well as reduction by the cytoplasmic reductase system, are shown in Figure 2.3 (136,140,165, 223,267,304).

Of the AFB_1 metabolites isolated and tested in the *Salmonella*/ microsome assay, aflatoxicol is the most mutagenic, followed by aflatoxin Q_1 and aflatoxin M_1 with low activity, and then aflatoxin P_1 and aflatoxin B_{2a}, which are both nonmutagenic (100,213,267,304). All these metabolites are, however, less mutagenic than AFB_1, a finding which implies that none is the ultimately toxic AFB_1 metabolite, and that they may be detoxification products (304).

Although its high reactivity precludes its isolation, it is thought that the AFB_1 metabolite, which is the ultimate mutagen, may be the AFB_1-2,3-epoxide (136,140,195,267,273,304). Evidence in support of epoxidation has come from the isolation of 2,3-dihydro-2,3-dihydroxy-AFB_1 from mild-acid hydrolysates of covalent adducts of AFB_1 with nucleic acids and proteins (dihydrodiols are detoxification products of epoxide hydrase activity) (40,100,136,195,223,304).

An electrophilic carbonium ion, formed at C-2 of the AFB_1 epoxide, is stabilized by lone electron pairs on the oxygen atom of the reduced furan, and it is proposed that covalent adduct formation may occur between this ion and a nucleophilic oxygen or nitrogen of either a nucleic acid or a protein (195,273). The major DNA adduct is between the AFB_1 epoxide and the N-7 atom of guanine (2,3-dihydro-2-(N^7-guanyl)-3-hydroxy AFB_1), this being the preferred site of alkylator action, as it resides in a sterically accessible position in the major groove of DNA (223,273). This alkylation can,

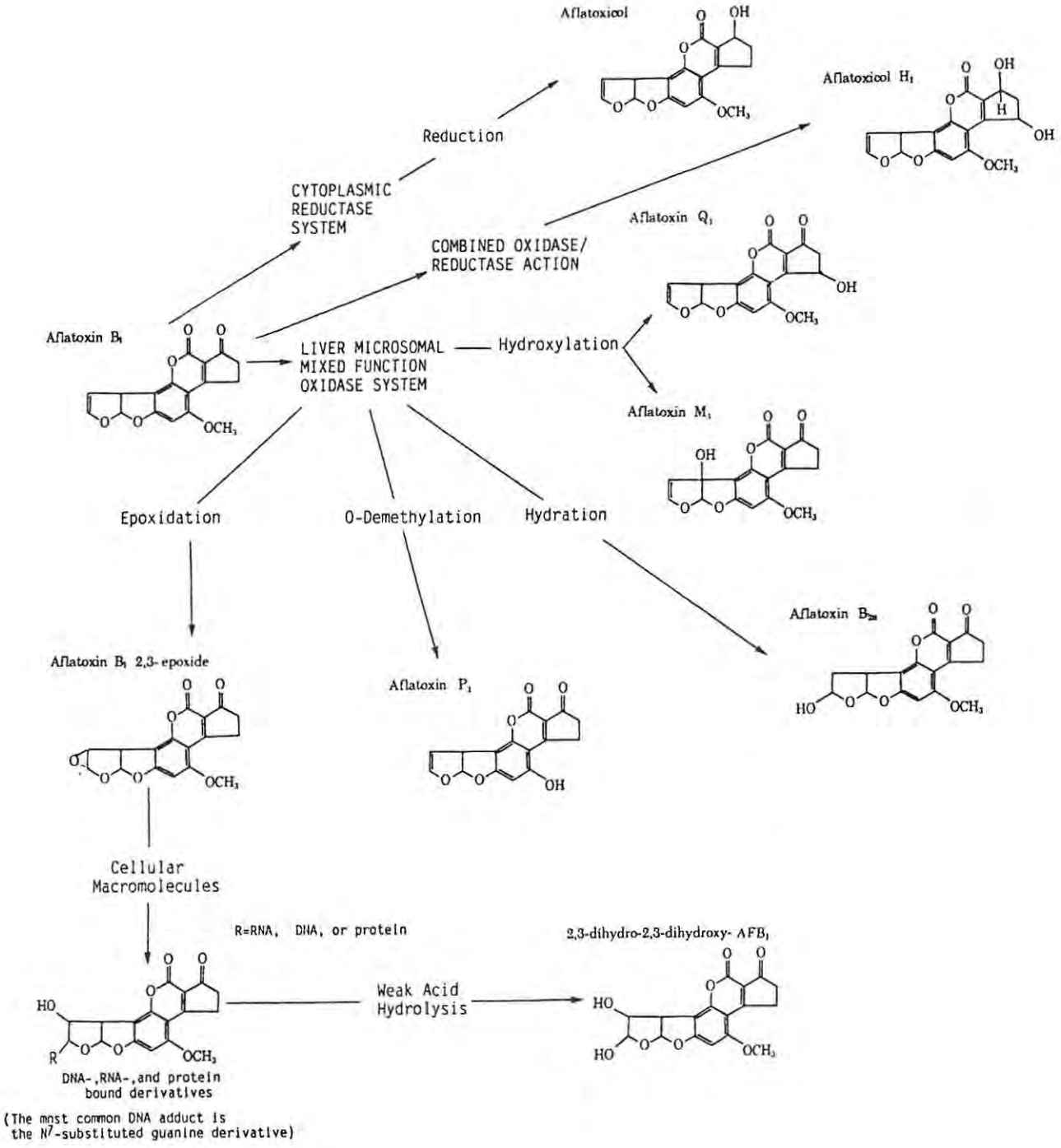


Figure 2.3 : Metabolic biotransformations of aflatoxin B_1 (adapted from 136, 223, 304)

in a similar manner to that described in Section 2.2, result in a G→A transition (a base-pair substitution mutation) (273). Although simple methylation or ethylation of guanine can induce such base-pair substitution, the large polycyclic AFB_1 molecule is also capable of frameshift mutations through intercalation with DNA (although the relative frequencies of the two mutation types is unknown) (216,223).

Unlike the metabolic activation of other polycyclic hydrocarbons, the epoxidation of AFB_1 is catalysed by a cytochrome P-450 isozyme which is preferentially inducible by PB (antibodies against this isozyme inhibit AFB_1 mutagenicity by 60-75%) (165,267). The cytochrome P-448 isozyme supports the hydroxylation of AFB_1 to the weakly mutagenic AFM_1 (267). The induction of rat liver microsomes with PB increases the mutagenic response to AFB_1 in the Ames test (121,267), although , *in vivo*, induction appears to protect animals from the carcinogenic effects of the mycotoxin (120,121,195,217).

AFB_1 toxicity has been found to depend, not only on the catalytic activity of the oxidase isozyme, but also on the level of induction of the isozyme in the microsomal fraction (165) as well as on the balance between activating phase I metabolic reactions (epoxidation), and detoxifying, phase II reactions (primarily conjugation with glutathione) (195,267). Therefore, the susceptibility of a species to AFB_1 carcinogenicity may reflect the net bioactivation resulting from the simultaneous activities of various metabolic pathways in the liver (136).

2.4 BENZO(a)PYRENE

Benzo(a)pyrene (BP), a product of the incomplete combustion of organic matter, is representative of the polycyclic hydrocarbons (PCH). It is a component of automobile and industrial exhaust fumes, as well as of cigarette smoke, and it is also formed in the preparation of many cooked and smoked foods (262,264). The environmental occurrence of BP, as well as its carcinogenicity, are reviewed in the IARC monograph (138).

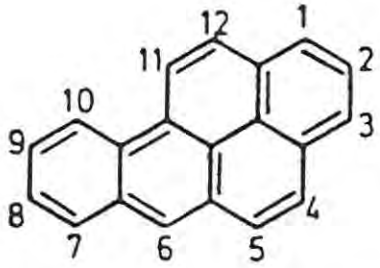


Figure 2.4 : Benzo(a)pyrene (264)

The BP molecule itself has no sites of high chemical reactivity (Figure 2.4). Sufficient reactivity for covalent interaction with cellular nucleophiles (including DNA) is introduced however by enzymatic epoxidation at various positions (119,195,223,262,302). The further metabolism of BP is complex, involving some transformations which serve to deactivate and eliminate these active intermediates, and others which potentiate their toxicity (119,157). Figure 2.5 summarizes the proposed metabolic transformations of BP, while some of the possible fates of the active electrophilic intermediates are illustrated by the three pathways (A, B, and C) shown in Figure 2.6.

The first metabolic transformation of BP to electrophilic species consists of the formation of either the K-region epoxide, BP-4,5-oxide (Figure 2.6, C), or the non-K-region epoxides, primarily BP-7,8-oxide and BP-9,10-oxide (Figure 2.6, A and B). The formation of the K-region epoxide is supported by a cytochrome P-450-dependent mono-oxygenase, which is inducible by PB, whereas the non-K-region epoxidations are catalysed by a mono-oxygenase which is inducible by 3MC (151,157,172,267). It has been suggested that these enzymes may be two forms of the same mono-oxygenase, aryl hydrocarbon hydroxylase (AHH), one form being inducible by PB, and the other inducible by 3MC (195,233,290).

The epoxides (or arene oxides) may spontaneously isomerize to form phenols (Figure 2.6, B), undergo conjugation with glutathione (catalysed by glutathione-*S*-transferases) (Figure 2.6, C), or may

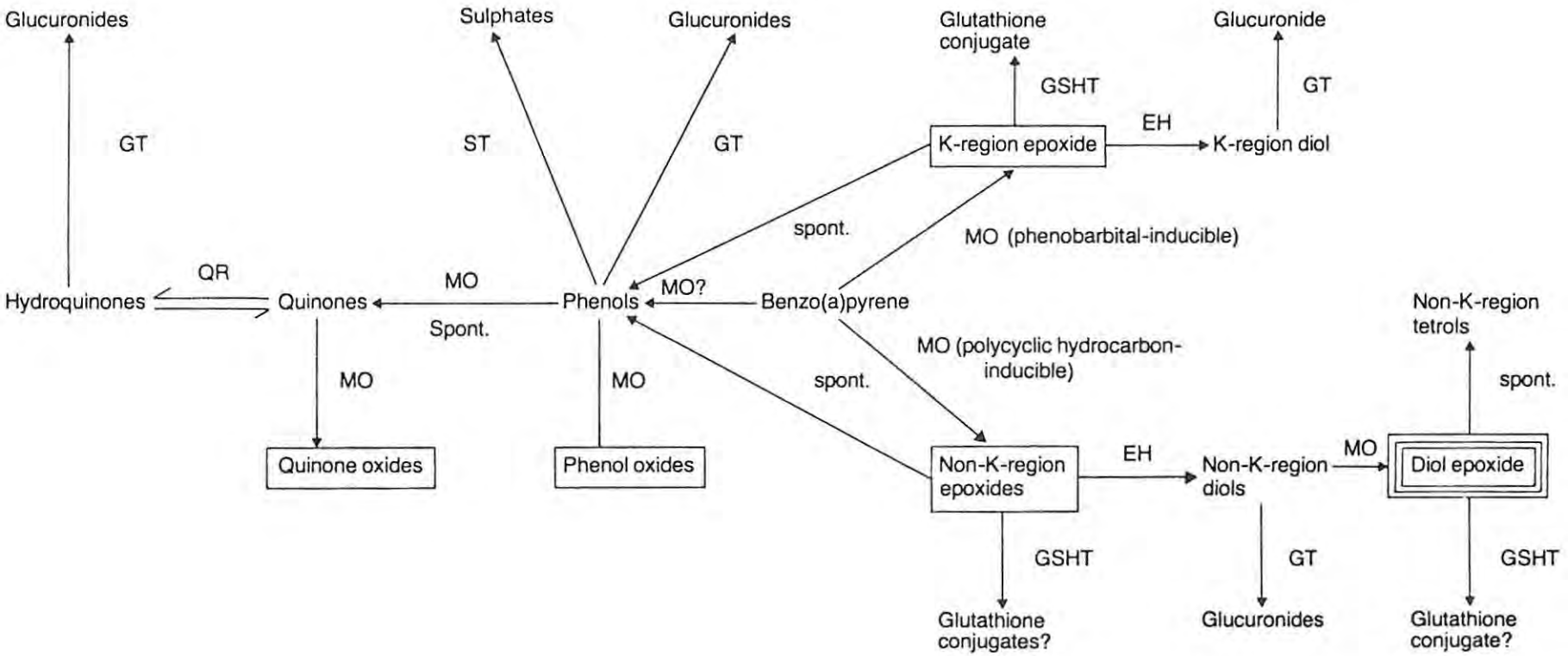


Figure 2.5 : Metabolic Biotransformations of BP. Enzymes : GT = glucuronyltransferase; ST = Sulphotransferase; GSHT = Glutathione transferase; MO = Mono-oxygenase; EH = Epoxide Hydrase; QR= Quinone reductase; spont. = Nonenzymatic process; ▭ = DNA binding metabolite; ▣ = Ultimate carcinogen.(Adapted from 157)

be converted to dihydrodiols by the microsomal enzyme epoxide hydrase (Figure 2.6, A) (58,119,159,188,195,262,305). The phenols may be oxidized, either spontaneously, or by a mono-oxygenase, to form BP-quinones (Figure 2.5) (58,59,262). These quinones are redox-active agents, which can accept electrons enzymatically from NADPH to produce hydroquinones, or, when reduced, may donate electrons to molecular oxygen to form quinone oxides (59,157). The phenols may also undergo a second oxidation (mediated by a mono-oxygenase which is inducible by 3MC) to form the phenol oxides (Figure 2.5) (58,116,287). Both the phenols and quinones of BP have little, or no, inherent mutagenic activity, but their respective oxides have the potential for covalent interaction with DNA. However, they are not thought to contribute significantly to the mutagenicity of BP (58,157,159,287,303,306). The phenols, dihydrodiols, and the hydroquinones are all substrates for UDP glucuronyl- and sulpho-transferases, and the polar conjugates so formed (which are also mutagenically inactive) are readily excreted (58,115,116, 119,150,264).

The conjugations with glucuronide, sulphate, or glutathione are true phase II detoxification reactions. While hydration by epoxide hydrase (EH) is also considered to be a detoxification pathway, this enzyme also plays a role in the further activation of BP (119, 172). In the special case when the dihydrodiol formed by EH is on an angular benzene ring (a so-called 'bay region'), as is the case with BP-7,8-dihydrodiol, a secondary oxygenation by the microsomal mono-oxygenases produces a 'vicinal diol-epoxide', BP-7,8-dihydrodiol-9,10-oxide (Figure 2.5 and Figure 2.6, A) (58,116,172,267,306).

Early observations suggested that the K-region epoxide was the ultimate carcinogenic BP metabolite. Certainly, BP-4,5-oxide has been found to be a potent alkylating mutagen in bacteria and isolated mammalian cells, whereas the non-K-region epoxides are only weakly mutagenic in these systems (137,167,188,251,303,305). However, BP-4,5-oxide (and BP-9,10-oxide) induced few, if any, tumours when applied to the skin of mice, whereas the BP-7,8-oxide was found to be highly carcinogenic in the mouse-skin test (119,157, 251,303,305,306).

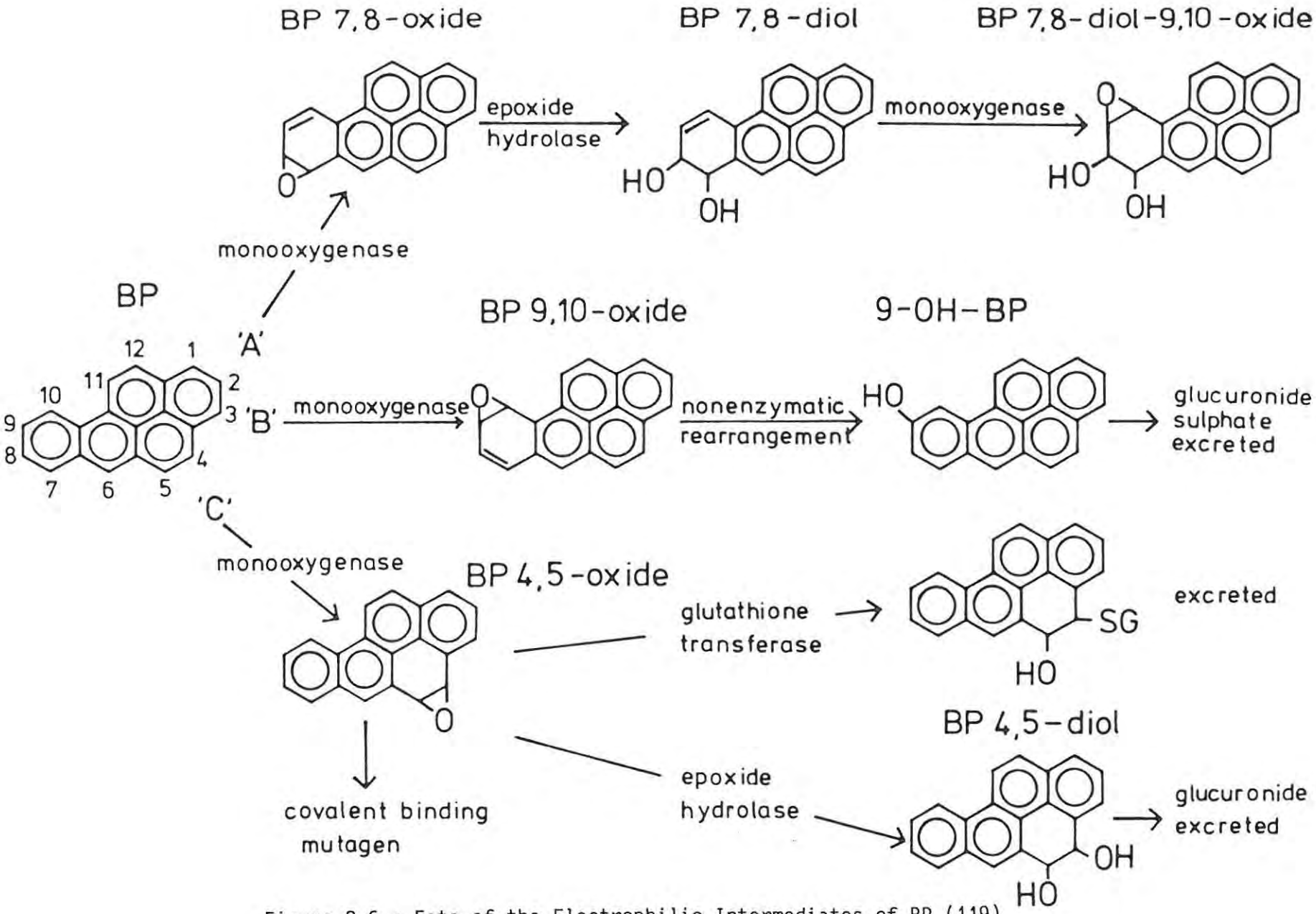


Figure 2.6 : Fate of the Electrophilic Intermediates of BP (119)

In addition, the incorporation of an hepatic microsomal fraction, or of purified EH, into *in vitro* mutagenicity test systems (including *Salmonella*), completely eliminated the mutagenicity of BP-4,5-oxide, while the number of mutations induced by BP-7,8-diol-epoxide was unchanged, or considerably enhanced (306). Hence, while BP-4,5-oxide may account for a significant amount of DNA-binding in the absence of EH, it is rapidly deactivated by EH, and it is excreted either unchanged, or as the glucuronide (119,157,306). The diol-epoxides on the other hand are poor substrates for EH because of steric hindrance by the 7,8-diol group, and, therefore, their deactivation by way of hydrolysis, to form tetrols, is primarily nonenzymatic (119,157,167,306).

It is now thought that it is not BP-4,5-oxide but the vicinal epoxide, BP-7,8-diol-9,10-oxide, produced from BP-7,8-oxide through the secondary mono-oxygenation of BP-7,8-diol, that is the ultimate carcinogenic BP metabolite (116,119,137,157,262,305,306). In particular, the (+)anti-BP-7,8-diol-9,10-oxide enantiomer has been identified as the major DNA-binding metabolite in several biological systems, including mouse skin, rodent embryo cells, and human bronchial transplants (131,150,151,306). This epoxide is also more mutagenic than the primary epoxides both in bacterial and mammalian cells (151).

DNA-binding by BP metabolites was enhanced about three-fold in the presence of TCPO (1,2-epoxy-3,3,3-trichloropropane), an inhibitor of EH activity, although BP-dihydrodiol formation was completely prevented. This indicates that an electrophilic metabolite(s), other than those derived from the BP-7,8-dihydrodiol, may contribute to DNA-binding (58,150). There is evidence that an active electrophilic product of 9-hydroxy BP is an important DNA-binding species, and it has been tentatively identified as the 4,5-epoxide of 9-hydroxy-BP (116,150,151,167).

Susceptible sites for the binding of the reactive intermediates formed from BP-7,8-diol have been shown to involve the exocyclic and N-7 nitrogen atoms of guanine, the exocyclic nitrogen of adenine, and phosphate; the sites involved in the binding of 9-OH-BP intermediates are largely unknown (116,149,150).

The profile of the primary metabolites of BP, as well as the proportions of the major DNA-binding intermediates (BP-7,8-dihydrodiol-9,10-oxide and 9-hydroxy-BP-4,5-oxide) are altered significantly by enzyme inducers such as 3MC and PB (150,151). Induction with 3MC resulted in DNA-binding by product(s) of 9-OH-BP, almost exclusively, while induction with PB was associated with the binding of BP-4,5-oxide and product(s) of BP-7,8-dihydrodiol to DNA (151,245).

EH is inducible by PB and also, to a much lesser extent, by by 3MC (149,245). Following pretreatment with PB, the principle dihydrodiol formed was the 4,5-dihydrodiol, with the production of other diols little increased over controls; after pretreatment with 3MC, the production of all three dihydrodiols was enhanced, with that of the 9,10-dihydrodiol increased to the greatest extent (245).

The overall activation of BP in rat liver microsomes, to mutagenically active intermediates, appears to be preferentially mediated by a cyt P-448-dependent mono-oxygenase; induction by 3MC enhanced the activation of BP four- to thirty-fold (20,132,267). In addition, monospecific antibodies against cyt P-448 purified from the livers of rats pretreated with 3MC were found to inhibit the mutagenicity of BP completely (165,267).

In the metabolism of BP then, several pathways exist which activate intermediates to DNA-binding or mutagenic species, and these are variously affected by different types of enzyme inducers. There are also a number of pathways by means of which the active metabolites can be detoxified. These include various conjugation reactions as well as hydrolysis by EH, which is similarly affected by different inducers and also plays a part in reactivation of certain dihydrodiols. The enzyme-catalysed addition of glutathione, a polar tri-

peptide and general scavenger of electrophiles within the cell, may be another pathway of deactivation for the primary epoxides (58,119,131,150). The formation of glutathione conjugates of the diol-epoxides has been postulated but not directly demonstrated (119). Nonspecific binding of the active intermediates of BP to macromolecular nucleophilic sites in the cell may also constitute a pathway of deactivation (116,150).

It appears then that the balance between the pathways of activation and those of detoxification is critical with regard to the net activation and resulting mutagenicity of BP. This 'balance' affects not only the total amount of DNA-bound metabolites but their relative proportions as well (58,116,150,157,267).

In the Ames test, BP is detected, after metabolic activation by an hepatic microsomal fraction, as a reactive mutagen capable of both frameshifts and base-pair substitutions (281). BP is detected with greatest efficiency in strains containing the R-factor (186,216).

2.5 MITOMYCIN-C

Mitomycin C (MMC), an antitumour antibiotic isolated from *Streptomyces caespitosus* (70,140,192), is active against a variety of human malignancies (70,192). Although human data are not available, MMC has been found to be carcinogenic in mice and rats (140), and has been detected as an oxidizing mutagen in several short-term mutagenicity assays (140,221). MMC has limited mutagenic activity in the the Ames test without the incorporation of an hepatic microsomal fraction, but the inclusion of S9 increases the mutagenic response several-fold (140,184,185,202,216).

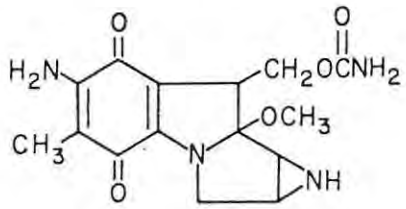


Figure 2.7 : Mitomycin C (193)

The MMC molecule (Figure 2.7) possesses three potentially reactive groups: a quinone nucleus, an aziridine ring and a carbamate group. After chemical or enzymatic reduction, MMC becomes a reactive bifunctional alkylator, although it is also capable of causing other DNA lesions (147). In its natural oxidized form however MMC shows hardly any alkylating function; it is thought that this lack of reactivity may be related to the partial withdrawal of electrons from nitrogen-4 into the quinone ring (Figure 2.8, A) (147).

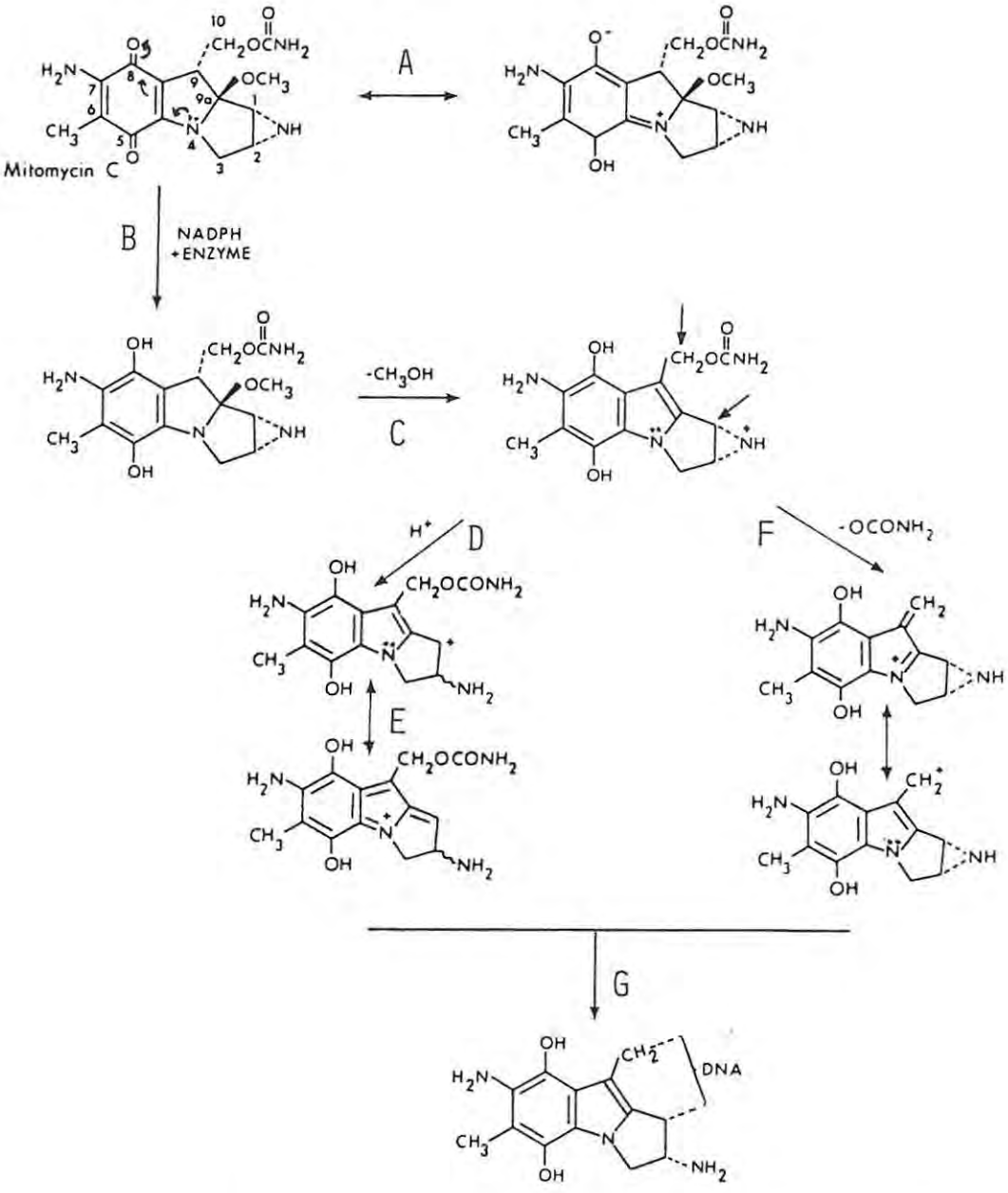


Figure 2.8 : Proposed mechanism for the metabolic activation of mitomycin C to alkylating species (adapted from 147, 192)

NADPH-dependent enzymatic reduction of the quinone ring (Figure 2.8, B), by two consecutive 1-electron steps to form the hydroquinone, is a prerequisite for MMC activation *in vivo* (147,192,246). The subsequent spontaneous loss of the tertiary-9a-methoxy group (Figure 2.8, C) is probably caused by the regaining of the electrons by nitrogen-4, coupled with the high driving force for the formation of the fully aromatic indole system (12,70,147,185,247).

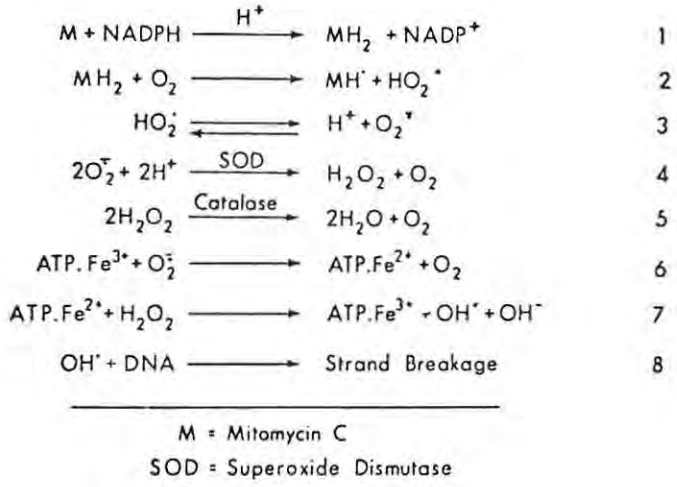
The loss of the methoxy group facilitates the opening of the aziridine ring (Figure 2.8, D) because of the stabilization of the positive charge by the indole ring which occurs in the transition state of the reaction before ring fission (Figure 2.8, E) (147). This event results in an alkylating carbonium ion being exposed at carbon-1 (147). A second carbonium ion arises at carbon-10 of the substituted indole (Figure 2.8, F), once again due to the stabilization of the positive charge by the indole nitrogen, but also because the carbamate ion is a good leaving group (70,147,192). These two resonance-stabilized electrophilic centres account for the bifunctional alkylating activity of MMC and, therefore, for its ability to cause DNA cross-linking (Figure 2.8, G) (95,192,247).

The presence of interstrand cross-links in DNA has been confirmed with the ethidium fluorescence assay and by S_1-endonuclease sensitivity (192,193). It is thought that the carbonium ion at carbon-1 is the first to bind covalently to DNA (as the aziridine ring opens), followed by that at carbon-10 (192,247). The most likely sites for interstrand linkages by MMC seem to be those between the amino groups of adenines or cytosines and the oxygen groups of guanine (especially oxygen-6) (147,192). The intercalation of the MMC ring system into the DNA double helix may be a preliminary step to the covalent cross-linking of DNA (94).

Monoalkylation of DNA is a potentially mutagenic event (base pair substitution), and it may also lead to depurination, which often results in single DNA strand breaks. DNA crosslinking on the other hand prevents replication; further DNA synthesis ceases whenever the replicating fork reaches a crosslink (80). In addition, mono-

alkylation presents a more easily-repairable DNA lesion than cross-linking. Therefore, although DNA cross-linking by MMC is less common (by a factor of ten-twenty) than monoalkylation, the former is more likely to constitute a lethal cytotoxic event rather than a mutagenic one (192,193,247). Both monoalkylation and the concomitant (interstrand and intrastrand) DNA cross-linkage increase proportionally with the guanine and cytosine content of the DNA (70, 147,192,193).

In addition to both alkylation and DNA cross-linkage, MMC has also been shown to degrade DNA by means of successive cyclic redox processes with oxygen, resulting in the production of reactive hydroxyl free radicals (12,185,192,247). The presence of free radical intermediates has been confirmed by electron paramagnetic resonance spectroscopy (246).



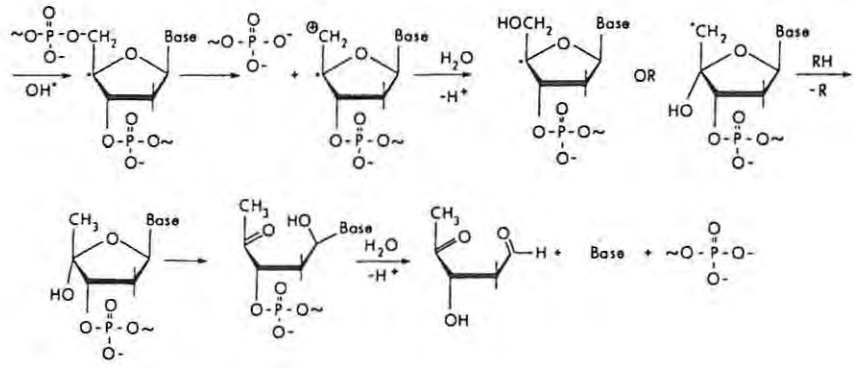


Figure 2.9 : Proposed mechanism of DNA degradation by reduced mitomycin C, showing DNA strand breakage by the hydroxyl radical (adapted from 192,193)

The proposed mechanism for cleavage of DNA by free radicals is outlined in Figure 2.9.

The antibiotic, after reduction by NADPH to the hydroquinone (MH_2) (step 1), is reoxidised to produce the semiquinone ($MH^{\cdot}$) and the hydroperoxy radical ($HO_2^{\cdot}$) (step 2). The latter species exists in equilibrium with the superoxide anion ($O_2^{\cdot-}$) (step 3). The cell is normally protected from the latter by the enzyme superoxide dismutase (step 4) and, similarly, the enzyme catalase removes H_2O_2 (step 5). Hydroxyl radicals ($OH^{\cdot}$) are thought to be generated by the reaction of H_2O_2 with traces of iron complexed with either protein or with ATP in a Fenton-type reaction (steps 6 and 7).

Hydroxyl radicals are very short-lived, but they may give rise to membrane lipid peroxidation and/or DNA damage if formed in close proximity to these cellular macromolecules (95,193). The degradation of DNA by the $OH^{\cdot}$ radical is thought to occur in a series of reactions involving initial hydrogen abstraction from position-4 of the ribose (192,193) (Figure 2.9).

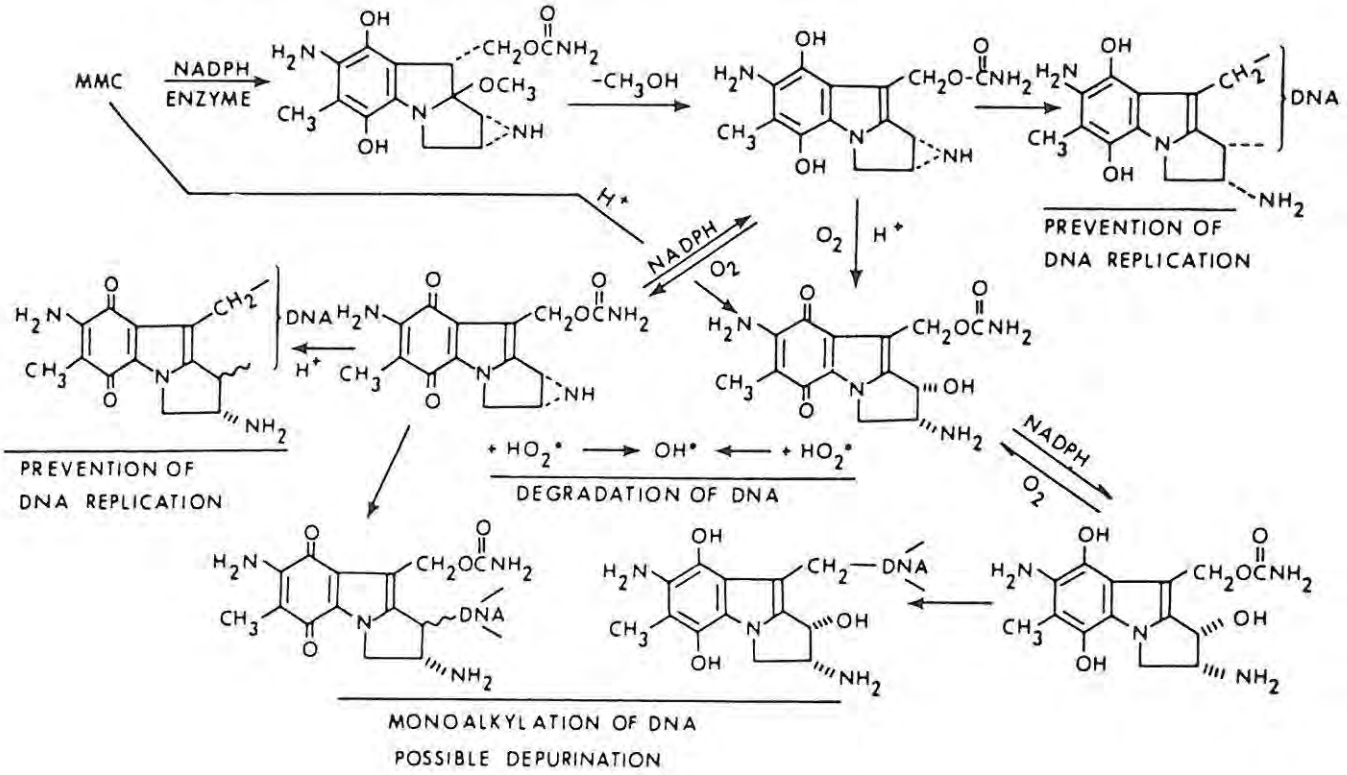


Figure 2.10 : Summary of transformations involved in mitomycin C activation (192)

Figure 2.10 summarizes how species are formed from MMC, which may either alkylate, cross-link, depurinate, or cleave DNA (in cyclic redox processes).

TA102 is the only strain of the standard set that can detect MMC since this strain has an intact (*uvrB*$^{+}$) DNA excision repair system (185,186,202,214,216,277). DNA cross-linking is lethal to those *Salmonella* strains which lack a functional repair system (*uvrB*$^{-}$) (185,186,201,202). Detection of MMC also requires a strain which contains the R-factor (185,186). Previously, mutational reversion of TA102 by MMC was attributed to -A:T- base pair substitution at the *hisG428* ochre mutation (see Section 1.5.4.4), but it has recently been shown that the antibiotic reverts by causing extragenic suppressor mutations (7).

CHAPTER 3 : THE ENDOGENOUS FEMALE SEX STEROIDS

3.1 INTRODUCTION

The two steroids investigated, 17β-oestradiol (E_2) and progesterone (PRG), are naturally occurring female sex hormones in all vertebrate species.

E_2, shown in Figure 3.1, is the most active of the natural oestrogens, which, together with other hormones, are responsible for the maintenance of the functions of female sexual organs as well as for the regulation of the menstrual cycle in primates, and for the oestrus cycle in other mammals (44,139).

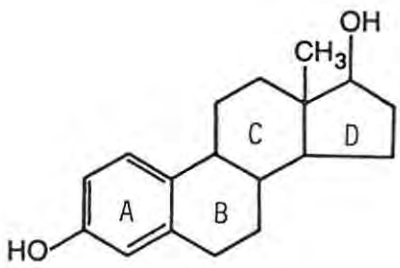


Figure 3.1 : 17β-Oestradiol (229)

Progesterone (PRG), shown in Figure 3.2, is the only important natural progestin, but many synthetic ones have similar biological properties. The normal role of PRG is to prepare the uterus for implantation of the fertilized ovum and to maintain pregnancy (139).

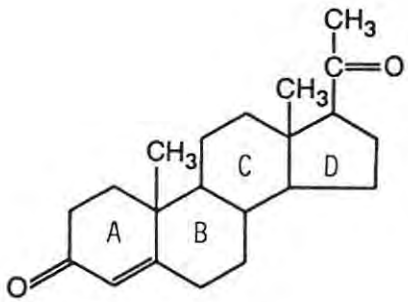


Figure 3.2 : Progesterone (229)

E_2 is secreted by the ovaries and circulates in the plasma non-covalently bound to a specific β-globulin, Testosterone-Oestradiol Binding Globulin (TEBG) (191). PRG, secreted by the corpus luteum in large quantities during pregnancy, also binds to a specific plasma glycoprotein (Progesterone Binding Globulin - PBG) (30,142).

In this protein-bound form the steroids are transported in the bloodstream and become available to both target tissues (primarily the uterus) and to certain nontarget tissues, which include liver, kidney, lung, pituitary, intestine, adrenal, and testes (4,53,276). Unbound steroid molecules can pass freely through the cell membranes of these tissues, by passive diffusion, due to their lipophilic nature and (relatively) small size (228).

E_2 and PRG are similar in several respects, including their biosynthesis from a common precursor (cholesterol), their molecular modes of action, and their metabolism and excretion (51,139). However, considerable species- and sex-dependent variation is noticed in steroid metabolism, particularly in the proportions of metabolites produced and in the types of conjugations they undergo (30,49,142).

3.2 METABOLISM OF THE FEMALE SEX-STEROIDS

3.2.1 Introduction

Both E_2 and PRG undergo rapid metabolic conversions to more polar metabolites in the cells of certain nontarget tissues. Although some peripheral conversions do occur, the liver is the primary organ of metabolism (30,106,139,142). PRG has a reported $t_{1/2}$ of between 3 and 90 minutes (30), and the $t_{1/2}$ of E_2 is estimated to be about 20 minutes (139).

The principal reactions in steroid metabolism are the oxidative-reductive interconversions of hydroxyl and keto groups at certain positions, catalysed by the steroid dehydrogenases, and the introduction of molecular oxygen, through cytochrome P-450-dependent hydroxylations, at various other sites (30,51,53,86,139).

Metabolites of the steroids are conjugated to form water-soluble compounds, which are eliminated mainly in the urine, often after cleavage, reabsorption, and reconjugation in the enterohepatic circulation (51,53,106,139). The metabolism of both E_2 and PRG will be described, together with the common features of their metabolic fate.

3.2.2 Metabolism of 17β-Oestradiol

3.2.2.1 Primary Biotransformations

The major metabolic transformations undergone by E_2 are shown in Figure 3.3.

Although E_2 and E_1 can be interconverted by a 17β-hydroxy steroid dehydrogenase, the oxidation of E_2 to E_1 predominates quantitatively (53,106,142,155), so that E_1 is the main substrate for the subsequent metabolic hydroxylations (44). E_1 is converted to E_3 via the reduction of the 16α-hydroxylated intermediate (44,106).

The metabolism of the three oestrogens is very similar in character; in rats, nonhuman primates and in humans, transformation occurs principally by 2-hydroxylation and, to a lesser extent, by the competitive 16α-hydroxylation (44,46,51,57,106,121,142,191).

High levels of thyroid hormone cause an increase in 2-hydroxylation and a decrease in 16α-hydroxylation (91), while in liver cirrhosis, 2-hydroxylation is diminished and 16α-hydroxylation is enhanced (44). A decrease in the oxidation of E_2 to E_1 is also observed in human liver cirrhosis (44).

Other possible hydroxylation sites are at the aromatic C-4 atom, and the cycloaliphatic positions 6, 7, 11, 14, 15 and 18 (44,108). About 1% of injected E_2 is recovered as 4-hydroxyoestrone from the urine of the human (44). Hydroxylations at C-6 or C-7 of ring B of either E_1 or E_2 are minor pathways in rats (142).

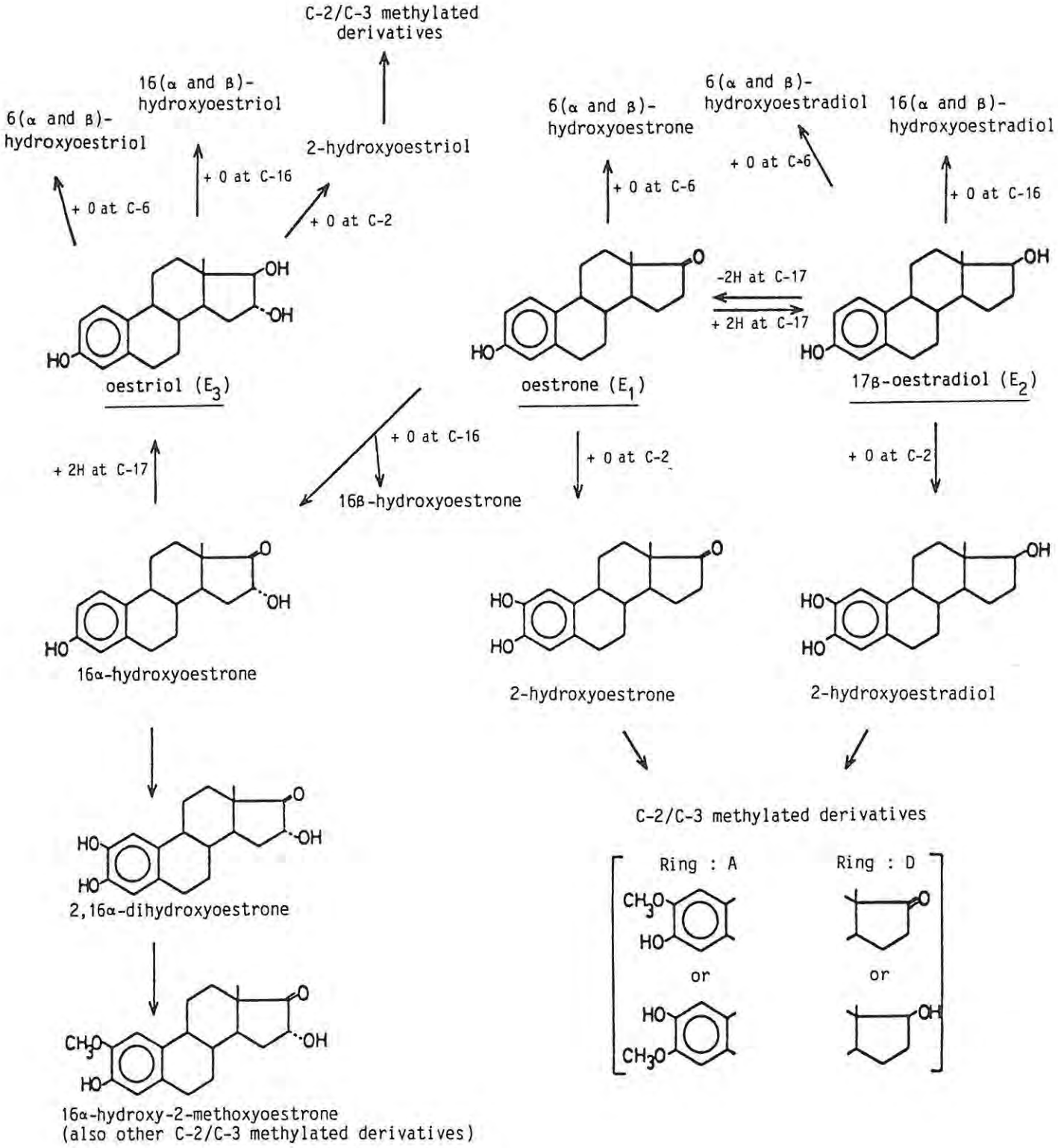


Figure 3.3 : Metabolism of 17β-oestradiol - structures represent intermediates of the major *in vivo* metabolic pathways in rats (adapted from 44, 51, 142)

Polyhydroxylated (that is, with more than three hydroxyl groups) and other polyoxygenated oestrogen metabolites are also reported (not shown in Figure 3.3) (44). In rat bile, 2-hydroxyoestriol, 2-methoxyoestriol, 16α-hydroxy-2-methoxyoestrone, 2,6α-dihydroxy-oestradiol, 2-hydroxy-16-keto-oestradiol and its 2-methylether, have all been identified (44). From human bile, 15α-hydroxyoestrone and 15α-hydroxyoestriol (oestetrol) have been isolated, and 2-hydroxyoestriol has been identified in the urine of human late pregnancy (44).

3.2.2.2 Secondary Biotransformations

The 2-hydroxyoestrogens ('catechol oestrogens'), and the 4-hydroxy-oestrogens, may undergo other biotransformations, besides conjugation (44).

The most important of these biotransformations is methylation at C-2 or C-3 (shown in Figure 3.3), catalysed by catechol-*O*-methyl-transferase (44,142). The main methylated metabolite is 2-methoxy-oestrone, though all the catechol oestrogens are probably methylated, as catechol-*O*-methyltransferase has a relatively low substrate specificity (44).

In both the rat and the human (*in vitro*), the catechol oestrogens may also undergo further oxidation to the semiquinone, followed by conjugation with glutathione at C-6 or C-4 (44).

It is thought possible that further metabolism of the oxidized catechol structure may eventually result in the opening of ring A, although no conclusive evidence has yet been presented in support of this hypothesis (44).

3.2.2.3 Conjugation of the Oestrogens

The polyhydroxylated oestrogens are sufficiently water-soluble to be excreted without further modification, but conjugation is a prerequisite for the urinary or biliary excretion of all other steroids (44). Conjugation occurs mainly in the liver, but also takes place in the kidney, and in the intestinal mucosa (44,102). E_3 is conjugated in the liver as rapidly as it is formed from E_1 and E_2, and little or none appears free in the blood (142).

The main conjugating reactions are glucuronidation and, to a lesser degree, sulphation of the 2- and 3- methyl ethers of the catechol oestrogens (44). Glucuronidation has been reported at ring D, as has 'double conjugation' (44).

Sulphation is catalysed by the cytoplasmic sulphotransferases, which appear to utilize 3'-phosphoadenosine-5'-phosphosulphate as the sulphate donor, and also require ATP and Mg^{2+} (44,102,108).

Glucuronides are formed from the glucuronide donor, uridine diphosphoglucuronic acid (UDPGA), and are catalysed by specific UDP-glucuronyltransferases, present in the microsomal fraction of the liver and other tissues (51,102,108,294). Glucuronyltransferases seem to be specific both for the steroid as a substrate, and for the position of the steroid to be glucuronidated (44,126).

Although conjugation is generally regarded only as a prerequisite for excretion, it has been reported that oestrone-3-sulphate may play a special role (44). It is formed rapidly from E_2 in the liver and its plasma level exceeds that of E_2 by about ten-fold. In addition, it may be rapidly reconverted to E_1 by arylsulphatases and, then, to E_2 by 17β-hydroxysteroid dehydrogenase in both the liver and the target tissues (44). On the basis of these observations, it has been proposed that oestrone-3-sulphate, in equilibrium with E_2 (or E_1), may represent a 'storage form' of the free and physiologically active oestrogens (44).

3.2.2.4 Excretion

In the human, conjugated oestrogen metabolites are preferentially excreted *via* the kidney, though some may undergo biliary excretion. Steroid glucuronides excreted in the bile may be deconjugated by the enzymes of the intestinal microflora and reabsorbed into the enterohepatic circulation, where reconjugation can occur (53,142). In man, most conjugated steroid metabolites are ultimately excreted in the urine, with small amounts in the faeces; in the rat, however, faecal excretion predominates (44,106,139,142).

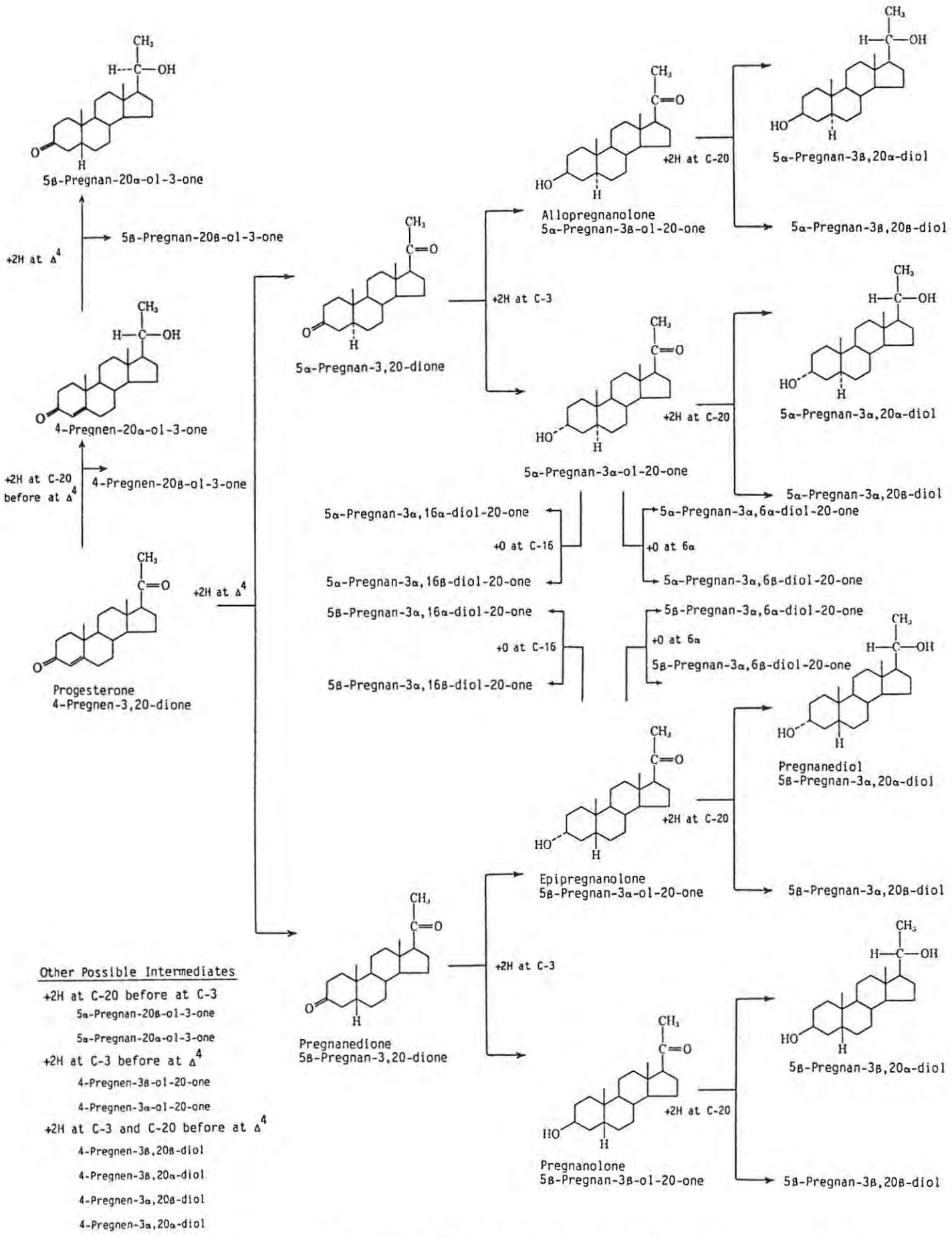
In man, the main urinary metabolites of E_2 and E_1 are the conjugates of E_3, 2-hydroxyoestrone, E_1, 16-hydroxyoestrone, and E_2, given in order of decreasing quantitative importance; approximately twenty other minor metabolites are also eliminated *via* this route (106). Ball *et al.* (32) have reported, however, that urinary excretion of 2-hydroxyoestrone during the menstrual cycle reaches, or even exceeds, that of E_3. During pregnancy, however, the excretion of the 'classical' oestrogens (E_1, E_2, and E_3) predominates, as a result of direct oestrogen production by the 'foeto-placental unit'(44).

3.2.3 Metabolism of Progesterone

3.2.3.1 Primary Biotransformations

The first transformation in the hepatic metabolism of PRG (shown in Figure 3.4), is the reduction of the Δ^4-3 ketone group (catalysed by 4-ene-reductase, a microsomal cytochrome P-450-dependent enzyme), to 5α- and, predominantly, 5β-pregnanediones (51,53).

The hepatic hydroxysteroid dehydrogenases reversibly reduce the keto groups at positions C-3 and C-20. At both positions, α-hydroxy dehydrogenases are more active in humans, so that the α-hydroxy steroid form predominates (53). In rats, 6α- and 6β-hydroxy PRG formation is significant (280).



Other Possible Intermediates

+2H at C-20 before at C-3
- 5α-Pregnan-20β-ol-3-one
- 5α-Pregnan-20α-ol-3-one

+2H at C-3 before at Δ^4
- 4-Pregnen-3β-ol-20-one
- 4-Pregnen-3α-ol-20-one

+2H at C-3 and C-20 before at Δ^4
- 4-Pregnen-3β,20β-diol
- 4-Pregnen-3β,20α-diol
- 4-Pregnen-3α,20β-diol
- 4-Pregnen-3α,20α-diol

Figure 3.4 : Metabolism of progesterone - structures represent intermediates of the major *in vivo* human metabolic pathways (adapted from 30, 51, 142)

The metabolism of PRG in extrahepatic, nonendocrine tissues, for example the uterus, appears to differ somewhat; the main metabolites here have been found to be 5α-pregnenes, with 3α- and 20α-hydroxyl groups (51).

3.2.3.2 Conjugation and Excretion

As in the case of the oestrogens, the progestins are conjugated, with either glucuronic acid or with sulphuric acid, and then excreted, primarily in the urine (about 50-60% of a labelled dose). Although about 30% of a labelled dose of PRG is originally excreted in the bile, only about 10% is eliminated in the faeces, a finding which indicates that considerable reabsorption into the enterohepatic circulation occurs (30,106,139,142).

In humans, the main urinary metabolite has been found to be the glucuronide of 5β pregnan-3α,20α-diol (pregnanediol) (30,106,108). About 5% of an administered dose of PRG is excreted in the form of pregnanolones, and about 2% as pregnanediones (51). PRG itself does not appear in the urine, except for the small amounts which have been detected in late pregnancy (30).

In the rhesus monkey, and in some other nonhuman primates, pregnanediol is only excreted in trace amounts, yet PRG metabolism in the chimpanzee and baboon resembles the human pattern much more closely (30).

3.2.4 Metabolism of the Synthetic Steroids

The synthetic oestrogens and progestins (which are frequently used components of oral contraceptive formulations) are metabolized by mechanisms similar to those by which the natural steroids are transformed, and both natural and synthetic steroids have similar modes of action. The metabolism of the synthetic oestrogens has been reviewed by Bolt (44).

3.3 SIMILARITIES BETWEEN STEROID- AND DRUG-METABOLIZING ENZYMES

Certain similarities have been found to exist between the enzymes of drug metabolism and those which metabolize the steroids. These similarities suggest that the steroids are the natural substrates for the same microsomal enzymes as are responsible for the metabolism of many exogenous compounds, including drugs (44,66,67,109,160, 270,282).

The similarities between the drug- and steroid-metabolizing enzymes are briefly listed below :

1. The enzymes are localized in the hepatic microsomes, and both exhibit a requirement for NADPH and molecular oxygen for activity (which is a characteristic of the mixed-function oxidases) (51,57,66,154,171,270).

2. Higher enzyme activity is noticed in the adult male rat than in the adult female (66). See Section 4.1.

3. Higher enzyme activity is noticed in adult male rats than in immature male rats (234). See Section 4.1.

4. The enzymes are inhibited *in vitro* by the addition of SKF-525-A and *in vivo* by administration of CCl_4 to rats (66-68,300).

5. The activity of the microsomal enzymes is inhibited by carbon monoxide, and this inhibition is reversed by monochromatic light at 450 nm. This behaviour indicates the dependence of the enzymes on cytochrome P-450 (44,66,67).

6. The steroid hormones appear to be alternative substrates for, and therefore competitive inhibitors of, the enzymes of drug metabolism (66,67,96,282).

Both E_2 and PRG, as well as the other steroids, have been found to inhibit competitively numerous cyt P-450-mediated drug oxidations by rat liver microsomes *in vitro* (46,109,154,170,227,233, 282), resulting in the metabolism of both substrates (steroid and drug) being slower than if either were the only substrate (231).

Pasleau *et al.* (233) have reported that increasing the concentration of PRG or testosterone rapidly inhibited the *in vitro* hepatic activity of AHH in the rat. Maximal inhibition was attained at equimolar substrate (BP) / inhibitor (steroid) concentrations, and greater amounts of steroids did not further inhibit AHH activity.

AHH has been found to be supported by two cyt(s) P-450 forms ; a specific form, which is inducible by 3MC, and a less specific form, which is inducible by PB, which is capable of accepting a wider range of substrates (including the steroids) (233). The less-specific form was inhibited *in vitro* by the steroids in the livers of both male and female rats (233). However, the 3MC-inducible form of AHH was not inhibited, even at steroid concentrations of up to ten times the substrate concentration (233).

In the adult female rat, the administration of BP inhibited the 16α-hydroxylation of progesterone slightly, but did not inhibit any of the other steroid 16α-hydroxylases. These steroid-16α-hydroxylases have been found to be completely independent of cyt P-448, and they are not inducible by 3MC (233).

These observations suggest therefore that while the 16α-hydroxylation of steroids is not affected by BP, the steroids are, nevertheless, able to inhibit part of the activity of AHH (233).

7. The enzyme activity of the hepatic enzymes is enhanced after protracted pretreatment of rats with various drugs or halogenated hydrocarbons.

The pretreatment of rats with PB for several days has been found to increase the activity of the hepatic microsomal enzymes that hydroxylate the steroids (67,68,231). This increased steroid hydroxylase activity was reflected, *in vivo*, by accelerated steroid metabolism and by alterations in the physiological action of the steroids (66-68,187,191,300).

Many inducers of drug metabolizing enzymes, such as diphenylhydantoin, chlorcyclizine, orphenadrine, phenylbutazone, and halogenated hydrocarbon insecticides (such as chlordane, DDT and dieldrin) also appear to be capable of inducing the steroid hydroxylases (30,66-68). The metabolism of the synthetic steroids is similarly enhanced by inducers of the microsomal enzymes (67,187).

The 16α-hydroxylation of PRG, and the 2-hydroxylation of E_2 can be induced by pretreatment with PB but not by inducers of the 3MC-type (57,232). This fact may indicate that the steroid hydroxylases are independent of the cytochrome P-448 isozyme (57,66,107,109,112,232,233).

The rate of the 4-hydroxylation of E_2 in the human placenta has been found to be elevated three- to five-fold in cigarette smokers (34,66,155).

8. The *in vivo* metabolism of both steroids and drugs is similarly affected by alterations in the intake of dietary macronutrients (25,160).

3.4 THE STEROID-RECEPTOR MODEL

3.4.1 The Steroid-Receptor-Response Sequence

In the cells of target tissues, both E_2 and PRG bind to specific high-affinity saturable receptor proteins in the cytosol. Subsequent to this noncovalent binding to the cytosolic receptor, the steroid-receptor complex is translocated to the nucleus, where it interacts with specific 'acceptor' sites (chromatin DNA and associated histones) on the genome (4,30,53,106).

This interaction results in the unmasking of a number of cistrons from which specific mRNA is transcribed. These mRNAs code for either initiation, or acceleration, of the biosynthesis of certain structural and functional proteins (see Figure 3.5) (48,189,228, 229).

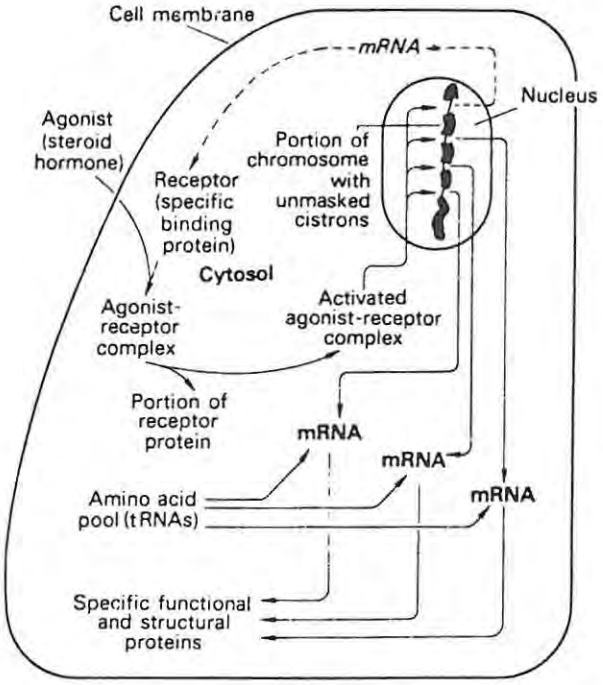


Figure 3.5 : Diagram of steroid-receptor-response sequence (48)

3.4.2 The Oestrogen-Receptor

The longest, and therefore, best known of the steroid receptors is the oestrogen-receptor, which is thought to be a globular protein with a molecular mass of around 200 000 dalton. The oestrogen-receptor, originally with a sedimentation coefficient of 8 S, dissociates into a 4 S form when it binds to oestrogen. Following an additional modification, the complex is converted to a 5 S species, which is transported to the nucleus (106). Oestradiol has a greater affinity for the oestrogen-receptor binding site than any of the other natural steroids (44). For example, the affinity of E_2 for the oestrogen-receptor is about ten times that of oestrone (E_1) (48).

3.4.3 The Progesterone-Receptor

The progesterone-receptor is estimated as having a molecular mass of between 60 000 and 110 000 dalton and a sedimentation coefficient of around 4 S (30). The interaction of the PRG-receptor complex and the nuclear chromatin fractions is analogous to that of the oestrogen-receptor complex, although there is no apparent need for receptor alteration, as is the case with the oestrogen-receptor (106).

3.4.4 Physiological Manifestations of the Receptor-Response Sequence

Considerable evidence supports a unitary hypothesis of steroid action; *viz.*, that the physiological manifestations of the steroid hormones result from their initial interaction with specific receptors in target cells (30).

A steroid hormone may induce the synthesis of its own specific receptor protein, as well as that of another steroid hormone (4,48, 229,276).

For example, the PRG-receptor concentration appears to be dependent on the action of both oestrogen and PRG, as E_2 administration results in an eight-fold increase in the basal concentration of the PRG-receptor after twenty hours (30,48,189). The observation that, under most circumstances, PRG can only exert its physiological effects after 'priming' by oestrogen is now understood in terms of the stimulation of PRG-receptor synthesis by oestrogen, thereby mediating the progestational response (30).

The receptor-response sequence, which results in the increased synthesis of specific proteins, may also possibly account for the reported ability of the steroids to induce enzyme synthesis (see also Section 4.2.) (4,183).

3.5 STEROID NONMUTAGENICITY

Despite evidence of their carcinogenicity, neither E_2 nor PRG (at low concentrations) can be detected as mutagenic by the use of *Salmonella* strains, even with the addition of a metabolic activation system. Such nonmutagenicity is found even when the metabolic system (usually a microsomal fraction of hepatic tissue) is induced by any one of a variety of inducing agents (125,174,208,237,259).

It has been reported that as the steroid concentration in the assay mix was increased to concentrations of greater than 500 µg/plate, there was a tendency for the number of visible colonies to fall below the count range of spontaneous reversion and for 'thinning' of the background lawn as a result of cytotoxicity (145,174,202, 259).

IARC monographs (139,142), and a computer-generated review of mutagenicity data on the two hormones (requested from the EMIC) confirm the nonmutagenicity of E_2 and PRG both in *Salmonella* and in other short-term test systems.

Microbial mutagenicity assays are not capable of detecting carcinogens which act by nonmutagenic mechanisms, and there is, in these test systems, little evidence of a direct genotoxic interaction between the steroids and DNA (240,279).

The present concept of sex-steroid carcinogenicity strongly implicates the involvement of the cytoplasmic receptor protein, and it may be that the steroid hormones act by promoting the growth of tumours previously initiated, either spontaneously or by true mutagens (129,139,145,174,183,214).

CHAPTER 4 : THE INFLUENCE OF THE ENDOGENOUS FEMALE SEX-STEROIDS ON METABOLISM

4.1 SEX-RELATED DIFFERENCES IN HEPATIC METABOLISM IN THE RAT

4.1.1 Introduction

In the rat, marked sex-related differences have been observed in hepatic cytochrome(s) P-450-dependent microsomal enzyme activities. In general, hepatic microsomes from male rats have been found to metabolize various drugs more rapidly than those from female rats, with a correspondingly shorter duration and intensity of action often observed in the intact male (4,66,103,290).

The magnitude of these sex-related differences has been found to depend on the substrate and the nature of the reaction; for example, the hydroxylation of hexobarbital and the *N*-demethylation of aminopyrine appear to be sex-dependent, whereas the hydroxylations of aniline and zoxazolamine do not (109). Differences in enzyme activities are reportedly reduced, to varying extents (again, depending on the substrate), by starvation, adrenalectomy, hypoxia, and various drugs (66,103,109,210,232).

The observed sex-related differences in the metabolism of the rat have been reviewed by Kato (162) and Colby (63), and several of these are shown in Table 4.1.

4.1.2 The Role of Discrete Species of Cytochrome(s) P-450

Sex-related variations in metabolism are sometimes accompanied by differences in the activity (V_{max}) and apparent affinity constant (K_m) of the particular cytochrome(s) P-450-supported enzyme involved (109). However, sex-differences in the microsomal content of cytochrome (cyt) P-450 and in the activities of NADPH-dependent cyt c reductase (CcR) and cyt P-450 reductase, reportedly, are slight, and it is thought that these, alone, cannot be responsible for the

Table 4.1 : Sex-Related Differences in Hepatic Metabolism in the Rat

Enzyme Activity or Metabolic Parameter Measured	Sex in which Metabolic Activity/Level is Greater	Literature Reference
Cyt P-450 content	male (minor diffs.)	(5,103,158,290)
Cyt P-450 reductase	male (minor diffs.)	(290)
NADPH-dependent CcR	male (minor diffs.)	(63,103,124,162,290)
ECOD	male	(162,290)
EROD	female	(290)
E_2 hydroxylation	male	(4,66)
Testosterone hydroxylation	male	(4,66)
PRG-16-α-hydroxylase	male	(4,66,101)
Mestranol-*O*-demethylation	male	(181)
Cholesterol-7-α-hydroxylase	male	(101)
17-Hydroxysteroid oxidoreductase	male	(122)
6-β- & 16-α-HSDH	male	(122)
11-β-HSDH	male	(177)
3-α- & 3-β-HSDH	male	(180)
5α-Reductase	female	(180)
Hexobarbital hydroxylation	male	(4,45,103,164,210)
Pentobarbital demethylation	male	(109)
Ethylmorphine-*N*-demethylation	male	(103,109,210)
Aminopyrine-*N*-demethylation	male	(101,109,164,210)
Aryl Hydrocarbon (BP) Hydroxylase (AHH)	male	(4,101,158,210)
Aniline-*p*-hydroxylation	no sex diffs.	(109,210)
Zoxazolamine hydroxylation	no sex diffs.	(109,210)
Glutathione reductase	no sex diffs.	(310)
p-Nitroanisole-*O*-demethylation	male	(101,109)
Dimethylaniline mono-oxygenase	female	(310)
Steroid glucuronyltransferases	female	(244)
PB metabolism	male	(210)
Carisoprodol metabolism	male	(210)
Ethanol dehydrogenase	female	(283)
Ethanol oxidation	male	(283)
3-hydroxy-3-methyl glutaryl Coenzyme A	female	(60)
Morphine & Methadone-*N*-dealkylation	male	(45,102,210)
Chlorpromazine demethylation	male	(45)
Nicotine dealkylation	male	(45)
Guthion metabolism	male	(102,210)
Schradan metabolism	male	(102,210)
Sulphanilamide metabolism	male	(45,102)
Picrotoxin metabolism	male	(45,102)
Strychnine metabolism	male	(45,102)

observed sex-differences in metabolism (109,290). It has been suggested that sex-dependent differences in drug and steroid metabolism may be related to the existence of varying amounts of discrete species of cyt(s) P-450 in the liver (103,117,158,290).

Male rats have been found to have greater hepatic cyt P-450-dependent mono-oxygenase activity than females, but females possess inherently greater cyt P-448-mediated mono-oxygenase activity (290). These conclusions have been drawn partly on the basis of the activities of two enzymes; ethoxyresorufin-*o*-deethylase (EROD), and ethoxycoumarin-*o*-deethylase (ECOD) (290).

EROD has been found to be supported almost exclusively by cyt P-448 and, therefore, its activity levels reflect cyt P-448-mediated mono-oxygenase activities (290). Similarly, ECOD activity is an indicator of cyt P-450-mediated mono-oxygenation since it has been found to be supported by this isozyme (290). ECOD activity was significantly higher in hepatic microsomes from the intact male rat than in those from the female, whereas EROD activity was found to be higher in microsomes from female rat liver (290).

There is some evidence to support the hypothesis that sex-differences in mono-oxygenase activities in rats may be due to a difference in substrate affinity for cyt(s) P-450 (158,164,290). The 'feminizing' effect of oestrogen administration on the metabolism of male rats (Section 4.1.7) may also be explained by this hypothesis (an increase in substrate binding to cyt P-448 mediated by oestrogens) (290).

Alternatively, oestrogens may stimulate the production of a haemoprotein P-450 that is highly specific for cyt P-448-type substrates (290).

4.1.3 The Effect of Gonadectomy and Antagonistic Hormone Treatment

Orchidectomy of the adult rat reduced the activities of several hepatic microsomal enzymes (103,109), including aminopyrine-*N*-demethylase, hexobarbital hydroxylase, ECOD, AHH, and the 3α- and 11β-hydroxysteroid dehydrogenases (HSDH) (5,164,242,290). A small (15%) decrease in hepatic cyt P-450 content after castration of the male has been reported (5,93,290). The effects induced by orchidectomy can be reversed by the prolonged exogenous administration of testosterone (5,162,164,180,234,288,290).

The administration of testosterone to the intact male rat had no effect on CcR activity, cyt P-450 content, or on either ECOD or EROD activity (290). E_2 administration to intact male rats caused slight decreases in hepatic ECOD activity and in CcR and cyt P-450 content, while it increased EROD activity (290). The drug-metabolizing activities of immature male rats were increased significantly after testosterone pretreatment (232).

Ovarectomy of the adult rat had no effect on the activities of AHH, ECOD, or several other androgen-dependent microsomal enzymes (4,5, 109,180,242) ; in addition, no effect was observed on hepatic cyt P-450 content (5).

The pretreatment of intact females with E_2 had no effect on cyt P-450 content or ECOD activity, although it decreased CcR and increased EROD activities (290).

The administration of testosterone (and other androgens) to intact or ovarectomized female rats, or to orchidectomized males (177), resulted in an increase in the rate of metabolism of several drugs (102,164,180): aniline (234), hexobarbital (43,45,232), and guthion (45,102), among others. Pretreatment of the intact female rat with testosterone had no effect on hepatic cyt P-450 levels or EROD activity, but increases in the activities of ECOD and CcR were observed (290).

4.1.4 The Development of Sex-Differences in Metabolism

4.1.4.1 Foetal Metabolism

Cytochrome P-450-dependent mono-oxygenase activities are barely detectable in the foetal liver of many species, including that of the rat (117,118,288).

The absence of activity of these enzymes in the foetus may be due to their competitive inhibition by high levels of maternally-derived substances, such as reduced progesterone metabolites (69,88,89,117,118). Alternatively, their absence may be due to either a lack of stimulation by endogenous inducing agents, of the enzymes responsible for the synthesis of hepatic microsomal mono-oxygenase systems, or to the inability of the mechanisms responsible for the synthesis of these systems to respond to endogenous inducing agents (117).

It has been reported in support of the latter hypothesis that hepatic cyt P-450 content (118) and various cyt P-450-dependent mono-oxygenase activities, which are normally inducible by PB in the adult and neonatal rat liver, were not inducible by PB in the foetus (although PB freely crosses the placental barrier) (117). Although both AHH activity and cyt P-448 levels are not inducible by PB in the foetal liver, they may be induced by 3MC (117,118). These findings suggest that, in foetal liver, cyt P-448-dependent mono-oxygenases are responsive to appropriate inducing agents, whereas cyt P-450-dependent mono-oxygenases are not (117,118).

Thus, a mechanism which suppresses the induction of cyt P-450-mediated mono-oxygenases, without preventing the induction of cyt P-448-dependent enzymes, may exist in the foetus (117,118).

4.1.4.2 Neonatal Metabolism and the Onset of Sexual Maturity

No sex-related difference in hepatic metabolism has been observed in the neonate or the sexually-immature rat (69,102).

The *in utero* suppression of foetal P-450 haemoprotein systems seems to be lost when the foetus is removed from the maternal environment, and at this time also, both mono-oxygenase systems appear to become responsive to inducing agents (117). It has been reported that a gradual increase in mono-oxygenase levels and cyt P-450 content, in both sexes, begins to occur immediately post parturition, reaching adult levels on about days 60-75 (117,288). Up until puberty, no difference between the two sexes was observed in mono-oxygenase activities, but, at the onset of sexual maturation, an abrupt and lasting increase in both these activities and in cyt P-450 content (117), was observed in the male; activity in the female remained at the levels attained at puberty (66,180,242,288).

The delayed development of hepatic metabolism in the neonate is possibly due to the gradual removal of the *in utero* suppression of the induction of foetal metabolism (117). Alternatively, it may be mediated by the stimulation of the enzymes responsible for the synthesis of the drug-metabolizing system, by increasing amounts of either endogenous or exogenous inducing agents (or both) (117).

4.1.4.3 Metabolism During Pregnancy

In the pregnant rat, during the course of pregnancy, a progressive decrease, has been observed in the *in vitro* hepatic metabolism of aniline, *p*-nitroanisole, aminopyrine and *p*-nitrobenzoic acid (73). Other *in vitro* studies have also shown decreases in the specific activity of the glucuronyltransferases (nmol/min/mg protein) *vis-a-vis* several substrates (bilirubin, morphine, *p*-nitrophenol, 4-methylumbelliferone, E_1 and E_2) during pregnancy (69,89,226,270,294). Conjugations with sulphate were also reduced in the pregnant rat (226), as was the biliary excretion of the glucuronide-conjugated metabolites of phenytoin and sulphobromphthalein (BSP) (52).

An inverse correlation was found to exist between these *in vitro* findings and the *in vivo* duration of pentobarbital- or hexobarbital-induced anaesthesia during pregnancy (73,89,226).

Pregnant women, or women using oral contraceptives, have also been reported to metabolize several drugs more slowly than nonpregnant adult women (52,69,230).

These diminished enzyme activities have been attributed to competitive inhibition of the enzymes by the elevated levels of female gonadal hormones (particularly PRG and its reduced metabolites) which are present in pregnancy (73,87,89,226,271). However, the period of maximal depression of enzymatic activities, in late pregnancy (days 19-20), did not correlate well with the period of maximal elevation of plasma PRG levels (days 14-16) (73). In addition, no difference in the level of PRG was observed between microsomal enzyme preparations from pregnant and nonpregnant female rats (73). These findings tend to rule out direct competitive inhibition as the mechanism for reduced *in vitro* metabolism during pregnancy in the rat (73).

The administration of PRG to nonpregnant rats, in doses designed to mimic the plasma levels which occur in pregnancy, failed to depress metabolism, a finding which possibly indicates that PRG also has no indirect role in controlling levels of microsomal enzyme metabolism (73).

The depression of drug metabolism in pregnancy has been found to be linked most closely to reduced levels of hepatic cyt P-450 (226), although correlations were not consistent for all pathways (73). A differential depression of cyt(s) P-450-dependent enzymes, however, might explain these results (73).

In support of the latter hypothesis, a selective depression of induction by PB of the hepatic cyt(s) P-450 mono-oxygenase system, similar to that which occurs in the foetus, was found to develop in the adult female liver in pregnancy (118,226). In the liver of the

pregnant rat, as in the foetal liver, the induction of AHH and cyt P-448 by 3MC was not impaired (52). Therefore, the suppressive mechanism that is present in the foetal liver of rats (see Section 4.1.4.1) appears to exist in the maternal liver as well (118).

This suppression (of the induction of the cyt P-450-dependent mono-oxygenases) may be a reflection of the need to maintain elevated levels of the female sex-hormones in pregnancy (the endogenous steroids are preferentially metabolized by these enzymes) (73,117). The elevated levels of PRG observed in pregnancy may therefore be the consequence of, and not the reason for, the observed decrease in enzyme activity (73).

It has been reported that the liver mass, and the total protein content of the hepatic microsomes, increased during pregnancy (226). Therefore, the decrease in enzyme activities appeared less when measurements were based on the metabolic activity of the whole liver than when calculated as activity per unit mass of liver tissue, or per unit mass of hepatic microsomal protein (73,226). An increase in the total body mass, and in the liver mass/body mass ratio has also been observed in pregnancy (73). These findings may indicate a differential synthesis of hepatic proteins during pregnancy, with little or no synthesis of the cyt P-450 mono-oxygenases (73).

It may also be possible that pituitary growth-promoting factors are responsible for the depression of hepatic cyt P-450-mediated drug metabolism in pregnancy (see also Section 4.1.8) (52,63,73,226,252).

It has been established that cyt P-450 content and microsomal drug-metabolizing enzyme activities are lower than normal in many conditions characterized by the rapid growth of liver cells, for example in the foetus and neonate, after partial hepatectomy, and in hepatic tumours (226). In all of these circumstances, and in pregnancy, the presence of increased levels of growth-promoting factors has been reported (226).

4.1.5 Androgen-Dependent and Androgen-Independent Enzyme Activities

Sexual variations in metabolism appear to be under the control of the sex hormones, particularly the androgens; for gonadectomy, or the administration of an antagonistic gonadal hormone, reverses sex-related differences (102). In addition, no difference in metabolism has been observed among sexually immature rats of either sex (109,242).

It has been suggested that drugs may be classified into two groups; those which are metabolized by androgen-dependent mechanisms, and those which are metabolized by androgen-independent ones (4,158,180). The effects of inducers on the enzymes that catalyse the metabolism of these two groups of drugs have been shown to differ; androgen-dependent activities were induced by pretreatment with PB but not by pretreatment with 3MC, whereas androgen-independent activities were induced by pretreatment with either enzyme inducer (158). 3MC (a cyt P-448-type inducer) has been shown to decrease androgen-dependent drug-metabolizing activities of the rat liver but to increase markedly those which are androgen-independent (290).

AHH, which has higher activity in the male, is unusual in that it has been found to be supported by two cyt(s) P-450 forms; the type predominating in the male is inducible by PB, while the type pre-eminent in females is inducible by 3MC (233,290).

Reportedly, androgen-independent enzymes are present at similar activity levels in both male and female rats, but the androgen-dependent enzymes are present at significantly higher levels in the male. It has been suggested that this phenomenon may be due to the continuous presence of androgens (acting as enzyme inducers) (45,180,288). The androgens may inactivate an active endogenous repressor which represses the action of a regulator gene responsible for the synthesis of the activities of the microsomal drug-metabolizing system (see also Section 4.2 and Figure 4.2) (167).

It appears then that the enzyme inducing ability of the androgens may be more closely related to their anabolic activity rather than to their androgenic activity (45,102,109,164,232).

4.1.6 Oestrogen-Dependent Enzyme Activities

Only a very limited number of enzymes seem to be oestrogen-dependent (180), and they appear to require the presence of an intact pituitary gland for activity (63,290).

Female rats were found to have a two- to three-fold higher level of hepatic 3-hydroxy-3-methylglutaryl coenzyme A reductase activity than do males; gonadectomy reduced this activity level to that of the male, and the administration of E_2 reversed the latter effect (60). Therefore, physiological levels of oestrogen appear to play a role as a positive effector of reductase activity (60).

The activities of the specific UDP-glucuronyltransferases that catalyse the glucuronidation of E_1, E_2, and testosterone, were found to be higher in female rats than in males (244). Ovarectomy reduced E_1 glucuronyltransferase activity by 50%, and E_2 administration for a period of twelve days induced the enzyme to normal levels (244). The activity of E_1 glucuronyltransferase was not induced by prolonged pretreatment with PB, whereas a single dose of 3MC led to moderate induction (244). Gonadectomy of the male rat had no effect on testosterone glucuronyltransferase, and the subsequent administration of testosterone did not influence the activity levels of the enzyme either (244).

Hepatic EROD activity is also higher in the female rat than in the male, and the administration of E_2 to intact, or gonadectomized, male rats increased hepatic EROD activity (290). It is not clear whether the effect of E_2 on EROD activity is due to the direct action of the hormone on the liver or whether it is dependent on the pituitary (290).

5α-Reductase activity has been found to be higher in the liver of the female rat than in that of the male, and the administration of E_2 restored the loss of activity which resulted from ovarectomy (180). Lax *et al.* (180) have suggested that 5α-reductase may not be oestrogen-dependent but may be androgen-dependent; only, androgens may act as repressors, not as inducers (as with other androgen-dependent enzymes), of enzyme synthesis.

4.1.7 The Androgen-Antagonizing Action of Oestrogens

The administration of E_2, at relatively low doses (5 µg-100 µg/kg), exerts a powerful antagonizing effect on the androgen-dependent enzyme activities of hepatic metabolism in the intact male rat (43, 122,170,180,189,234,252,290). In addition, E_2 also causes nearly complete reversal of androgen-mediated metabolic effects in the intact female and in castrates of either sex (176,179,180).

The 'feminizing' effects of E_2 on metabolism were still demonstrable in intact male rats receiving exogenous 5α-dihydroxy-testosterone (180). In fact, the E_2-mediated decrease of androgen-dependent 3β-HSDH activity in the male rat liver was not reversed by a 100-fold higher dose of androgen, a finding which shows the potent anti-androgenic nature of E_2 on androgen-dependent enzyme activities (176,179).

These observations suggest that E_2 acts on the mono-oxygenase system independently of its inhibition of androgen biosynthesis (mediated by suppression of gonadotropin release) (180). The androgen-antagonizing nature of oestrogens may be mediated by their interference with the binding of androgens to the repressor of the gene responsible for the synthesis of the microsomal enzyme system. Therefore, oestrogens may prevent the inactivation of this repressor by androgens and, thus may block the subsequent induction of enzyme synthesis (164).

However, it has been suggested that the androgen-antagonizing effects of low doses of oestrogens are not likely to be mediated by hepatic oestrogen receptors since the lowest E_2 doses which can still influence enzyme activities (1-10 μg) are insufficient to cause translocation of the steroid receptors (180). In fact, at physiological levels it may be that steroid receptors do not play a role in mediating sex-steroid action on the liver (4,180) since hepatic steroid metabolism is so rapid that endogenous steroids (including the androgens) are unlikely to reach the nucleus of hepatocytes (204,297).

4.1.8 The Role of the Hypothalamo-Pituitary Axis

A more conceivable target for the androgen-antagonizing action of oestrogen is the hypothalamus, and experiments on hypophysectomized rats support this theory.

Sex-differences in xenobiotic and steroid metabolism in rats only become apparent after the maturation of the hypothalamo-pituitary system (63,92,275). Hypophysectomy of the male rat had little effect on hepatic androstenedione metabolism (122), or on 11β-HSDH activity (177). However, masculinization of hepatic microsomal mono-oxygenase activities followed hypophysectomy of the female rat (122,123,177), and once the pituitary was removed, the activities of these enzymes were no longer influenced by the administration of either androgen or oestrogen (177).

These observations indicate an obligatory role for the hypophysis in modulating sex-steroid action on the liver; specifically, a repressive influence on the metabolism of the female rat (92,170,177,275). Several reports have also demonstrated that the 'feminizing' effects of oestrogens in the intact female rat can be mimicked by the administration of growth hormone (GH) to the hypophysectomized rat (180,252). However, it is not known at present which hypophyseal hormone(s) might be physiologically responsible for *in vivo* sex differences in the rat. GH and prolactin (PRL) are the most likely candidates, but an as yet undefined 'feminizing factor' (FF) may be involved (180).

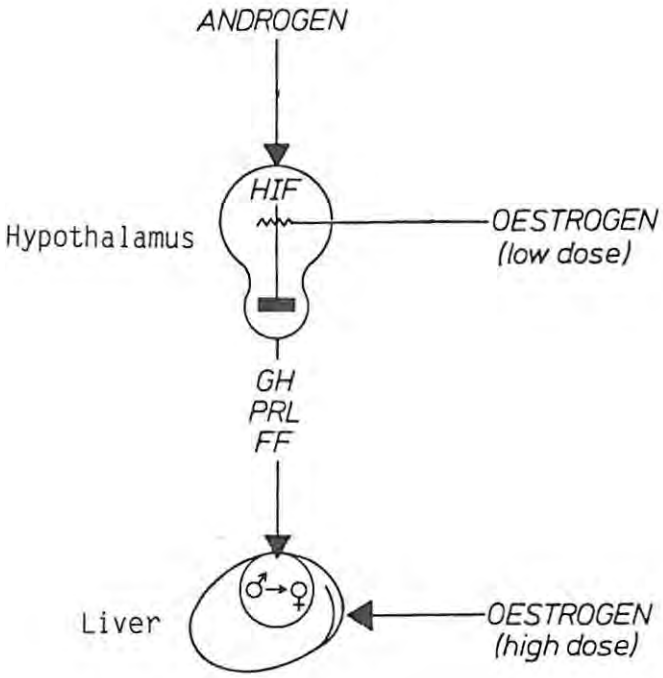


Figure 4.1 : Schematic representation of the possible modes of action of androgens and oestrogens on hepatic androgen-dependent enzyme activity in rats. HIF; undefined hypothalamic inhibiting factors: ⟶ ; inductive influence : ⟶■ ; repressive influence: ⟶〰 ; antagonizing action (adapted from 180)

According to this model of hormonal regulation of the activity of androgen-dependent hepatic enzymes (represented in Figure 4.1), pituitary feminizing factors (GH, PRL, or FF) are the prime physiological factors responsible for changes in hepatic enzyme activity. In the presence of androgens, hypothalamic FF release is inhibited, with a resultant masculinization of metabolism. Low doses of oestrogens override the androgenic suppression of FF release, and high doses of oestrogens saturate hepatic steroid metabolism and, by way of the oestrogen receptor system, cause induction of enzyme activity (see also Section 4.2) (180).

The masculinization of hepatic metabolism in the female rat, which occurs following hypophysectomy, may therefore be due to the removal of the source of the 'feminizing factor', thought to maintain female-type metabolism in the liver (122).

4.1.9 Effect of Adrenalectomy

Adrenalectomy is reported to decrease microsomal enzyme activity in male rats (66). Normal levels were restored, not by the administration of testosterone (levels of which should be normal in adrenalectomized rats), but by cortisone (66,232). No difference in cytochrome P-450 levels have been observed in adrenalectomized rats (205,232). Therefore, although the adrenal may have a regulatory role in the increased enzyme activity of males, the role of cortisone is unclear, as adrenalectomy has no effect on the enzyme activities of the female rat (232).

4.1.10 Sex-Related Metabolic Differences in Other Species

Although it is generally reported that no sex-related differences in drug-metabolizing activities exist in the rabbit, cat, dog, guinea-pig, or hamster, differences are thought to exist in certain strains of mice and fish, but reported findings often conflict (66, 164,232,288,290).

Adult female Swiss-Webster mice were found to metabolize dimethylaniline, aminopyrine, pentobarbital, and ethylmorphine faster, than the male mouse, and also to have a higher hepatic cyt P-450 content (232,288,310). The sex-related difference in dimethylaniline-*N*-oxidase activity was nullified by the administration of testosterone to the female mouse (288) and decreased by ovarectomy (although the differences were partially restored by PRG administration) (82,310).

Castration of the male mouse generally resulted in an increase in mono-oxygenase activity (abolishing the sex-difference) (310), although the activity of dimethylaniline-*N*-oxidase was reduced (82). These effects were reversed by testosterone administration (82,310). Testosterone administration to intact male mice reduced the metabolism of nortestosterone, but increased that of hexobarbital (66). Testosterone administration to gonadectomized mice of both sexes caused a decrease in AHH activity (4,295). Pretreatment

with E_2 caused an increase in the activity of hepatic AHH in the gonadectomized female but no similar change in the male (189,295). Testosterone or E_2 administration to the neonate increased AHH activity in the male mouse but not in the female (295).

These findings infer an inhibitory action of androgens in mice, contrary to their stimulatory effect in rats (288).

Hepatic cyt P-450 content and mono-oxygenase activities are reportedly higher in the male rainbow trout than in the female fish (92). The administration of E_2 to juvenile rainbow trout resulted in a statistically significant decrease in the activity of mono-oxygenase activity (92)

4.2 ENZYME INDUCTION BY THE STEROIDS

Several studies have supported the suggestion that the sex-steroids exert their physiological effects, in both target and nontarget tissues, by influencing the synthesis of protein at the level of gene transcription (228).

Although short-term treatments of rats with sex-steroids have shown competitive inhibition of drug metabolism, inductive effects on the enzymes of drug metabolism have been reported after protracted administration of high doses of steroids (66,152,154,230,248).

According to the Jacob-Monod model (Figure 4.2), an enzyme inducer alters the configuration of a repressor protein, which is responsible for inhibiting the synthesis of a specific enzyme. The inactivated repressor can no longer block DNA transcription, and synthesis of the specific mRNA molecules, which encode for the enzyme, is enhanced (108,148).

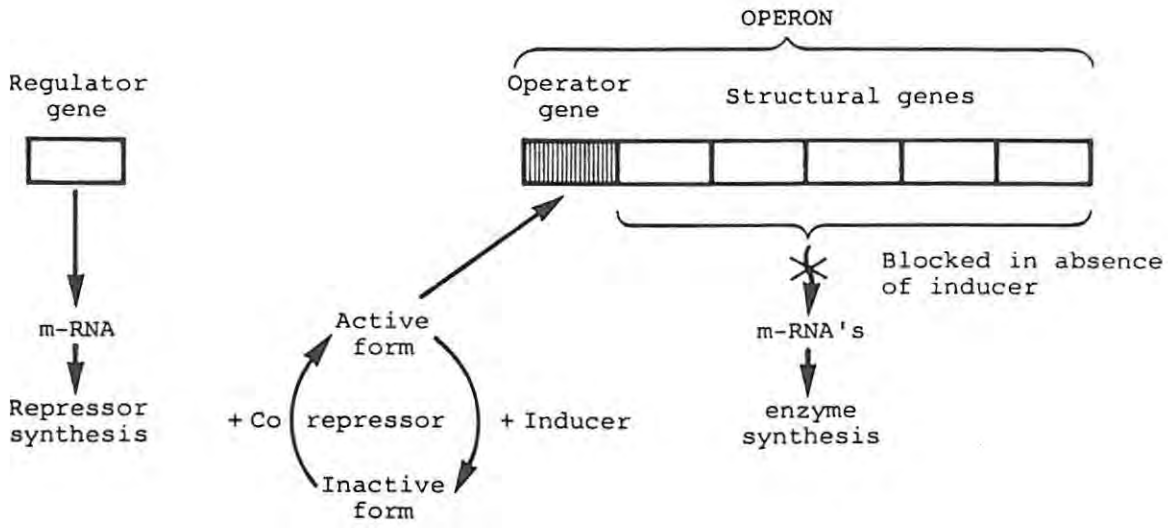


Figure 4.2 : Suggested Scheme for the Mechanism of Enzyme Induction (108)

The 'steroid-receptor' model of steroid action in target tissues (Section 3.4) illustrates a mechanism whereby a steroid may enter a nontarget cell, translocate, and interact with nuclear DNA to exert an inductive effect on metabolic enzymes (31,108). In addition, the presence of oestrogen-receptors in the (nontarget) hepatic tissue of rats has been demonstrated (4).

Kato *et al.* (163) observed that pretreatment with E_2 (200 µg/kg/day for 15 days) had no effect on the hepatic drug-metabolizing activities of ovarectomized rats. Similarly, Bulger and Kupfer (57) found that E_2 administration (100 µg/kg/day, ip for 4 days) did not alter the *in vitro* hepatic microsomal activities of E_2-2-hydroxylase, E_2-3-methoxy-*O*-demethylase, benzphetamine-*N*-demethylase, or ethylmorphine-*N*-demethylase, in the intact or gonadectomized female rat.

However, Al-Turk *et al.* (4) observed that protracted pretreatment with E_2 or E_1 at doses which were ten-fold higher than these (1 mg/kg/day, for 7 days) induced a two-fold increase in the *in vitro* hepatic and intestinal AHH and ECOD activity in the female rat (52,57,189,276). At these supraphysiological doses, significant increases in cyt P-450, parallelling those of AHH activity, were also observed (4,276).

The protracted pretreatment of nonpregnant female rats with E_2 (1 mg/kg/day, for 14 days) has been reported to induce E_1 and morphine glucuronyltransferase activity by 53% and 50%, respectively, in the isolated perfused liver (52). Rao *et al.* (244) have also reported that E_2 administration for 15 days to ovarectomized rats increased the *in vitro* hepatic microsomal activity of E_1 glucuronyltransferase, to 140% of the normal values of the untreated, nonovarectomized rat (244).

Vodicnik *et al.* (290) have reported that treatment with E_2 (2,5 mg/kg, twice daily for 4 days) induced the hepatic microsomal activity of EROD in male and female rats *in vitro*.

Therefore, although the reported action of E_2 on androgen-dependent (and therefore normally sexually differentiated) enzyme activities in male rats is of a feminizing nature, higher doses can have the reverse effect, masculinizing the activity of female rats (85,178).

The administration of PRG to virgin female rats (10 mg/kg/day, i.p. for 4 days) has also been found to result in enhanced hepatic drug-metabolizing activities *in vitro* (73,98).

Several synthetic oestrogens and progestins of the type commonly used in oral contraceptive formulations have been found to induce microsomal drug-metabolizing enzymes in rats, although at concentrations greater than those required for contraception (98,230).

Juchau and Fouts (154) have reported that norethynodrel, administered to male rats, exerted a biphasic effect on the hepatic hydroxylations of hexobarbital and zoxazolamine *in vitro*; following an initial inhibition, induction of these enzyme activities was observed. Jori *et al.* (152) have noticed that protracted administration of medroxyprogesterone acetate, alone or in combination with ethinyloestradiol, and norethynodrel combined with mestranol, increased the metabolism of pentobarbital *in vivo*.

The norethynodrel + mestranol combination has also been found, by Carter *et al.* (61) to stimulate the hepatic metabolism of ethylmorphine *in vitro*, and to increase cyt P-450 levels, after the protracted pretreatment of female rats. Pretreatment with lynoestranol enhanced the metabolism of phenobarbital, hexobarbital, and phenytoin (253,286).

The combinations of lynoestranol + mestranol, norethynodrel + mestranol, and norethindrone + mestranol, administered to female rats for 4 days or more, at doses adequate for contraception, induced aniline hydroxylase, *p*-nitroanisole-*O*-demethylase and aminopyrine-*N*-demethylase activities (153). Two other contraceptive combinations, norethisterone + mestranol, and quingestranol acetate + ethinyl oestradiol, have also been reported to be inducers of hepatic microsomal enzymes (88,90).

Daily administration of oestradiol benzoate to female rats (25 µg and 100 µg/kg for 21 days), resulted in a 1,7- and 2,5-fold increase in β-hydroxy-β-methylglutaryl coenzyme A reductase activity in normal and ovarectomized rats respectively (1). Increases in AHH activity have also been observed in foetal rat liver cultures which were exposed to diethylstilboestrol (DES) in the medium (189).

Some steroids with anabolic (testosterone propionate, 19-nortestosterone (45), and methyltestosterone (124)), anti-mineralocorticoid, or glucocorticoid (spironolactone (124,268,269)) properties have been shown to enhance hepatic microsomal *N*-demethylation and aliphatic hydroxylation (124,268). Pregnanolone-16α-carbonitrile (PCN), as well as being a potent inducer of hepatic drug-metabolizing activities, increased microsomal protein, CcR, and cyt P-450 content (194,269,270).

As described in Section 4.1.7, it may be that the oestrogens, at physiological levels, are metabolized too rapidly for them to regulate metabolism by acting on the hepatic steroid-receptor system (180). However, high doses of physiological oestrogens, or moderate doses of less easily-metabolized synthetic oestrogens, may

saturate or bypass hepatic steroid metabolism respectively (180). These steroids may then activate the cytosolic receptor system, translocate, and activate or modify the genome, causing induction of androgen-dependent enzymes (or, not impossibly, repression of some enzymes) (4,180,276). There is some evidence that these general observations about induction by oestrogens may also be true of progestins as well as of other steroids. However, the levels of endogenous steroid which are required to induce the hepatic mixed-function oxidases have been supraphysiological; lower doses have either no effect or an inhibitory effect on these enzymes (4,170).

4.3 MULTIHORMONAL REGULATION OF HEPATIC ENZYME ACTIVITIES

There is no substantial evidence supporting a unifying theory of hormonal regulation of hepatic drug- and steroid-metabolism. The manner in which different enzyme activities react to changes in the endocrine system suggests that the opposite may be true (180). The balance between male and female sex-hormones does however seem to be important in determining the activities of the metabolizing systems. Evidence to date points to the presence of distinct multi-hormonal regulatory modes for different types of enzyme activity (180).

In general, the observed effects of the steroids on the mixed-function mono-oxygenases are dependent on the organ studied, the steroid administered, and the dose and duration of steroid administration (4,52,88,180,230,276).

CHAPTER 5 : METHODS

5.1 RAT PRETREATMENT

5.1.1 Establishment and Maintenance of the Animal Colony

Only virgin female Sprague-Dawley rats, bred from original stock acquired from the S.A. Institute for Medical Research, were used to eliminate possible strain and stock differences in metabolism (66). Twelve groups (A to L) of two rats each were established in separate plastic cages, and all groups were subject to a 12 hour dark/light cycle, in a room with stable temperature and humidity (210). The animals were allowed food and drinking water *ad libitum* as starvation has been shown to alter the microsomal enzyme system to varying degrees (110,210).

All drinking water was freshly distilled to eliminate the possibility of any impurities in tap water altering the microsomal enzymes (64).

The use of insecticides in the animal room was prohibited as several of these are known to be inducers of the mixed-function oxidases (MFO) (93), and no bedding was provided for the same reason (67,210,289). As it has been reported (289) that the frequency of cage cleaning may also alter the activity of the microsomal enzymes, all cages were cleaned every day to standardize any such effects. The animals were allowed one month to equilibrate under these conditions before treatment commenced.

The test animals weighed 210 ± 25 g on their first treatment day. They were weighed daily thereafter, which provided an opportunity both to examine the animals for any observable changes in condition and to accustom them to handling.

5.1.2 Pretreatment - Rationale and Protocol

The rat pretreatments are summarized in Table 5.1, and the methods of preparation of all the reagents used may be found in Appendix 1.

5.1.2.1 Steroid Pretreatment

The test rat groups received daily ip injections of E_2 or PRG, at either the 1 mg/kg or 20 mg/kg level, in an ethyl oleate vehicle. One control group was injected with vehicle only while another was untreated, making a total of six groups.

The steroid dose levels were chosen after a literature survey (4,52, 96,135,152,154,186,276). Doses of E_2 greater than 0,5 mg/kg/day have been found to be necessary to elicit inductive changes in the activities of ECOD and AHH and to increase the hepatic cyt P-450 content (4). Brock and Vore (52) have reported that the administration of E_2, at 1 mg/kg/day for 14 days, increased hepatic RNA and DNA synthesis, and liver size to those observed in the pregnant rat.

Table 5.1 : Summary of the Rat Pretreatments

TREATMENT	RAT GROUP	
	UNINDUCED	PB-INDUCED
No Treatment	A	G
Ethyl Oleate Only	B	H
17β-Oestradiol 1 mg/kg	C	I
17β-Oestradiol 20 mg/kg	D	J
Progesterone 1 mg/kg	E	K
Progesterone 20 mg/kg	F	L

Treatment was administered daily at a fixed time (08h00-09h00), for 14 days, and animals were sacrificed 24 hours after the last treatment (4,96,158,276).

Animals were regularly monitored for abdominal pain and, on dissection, were examined for inflamed intestinal membranes, both symptomatic of peritonitis, which, along with other pathological states, may affect the microsomal enzymes (218). Any animal showing signs of morbidity was excluded from the study.

5.1.2.2 Enzyme Induction

For the purposes of routine environmental screening with the Ames assay, the hepatic microsomes are normally maximally induced to increase the efficiency of the test in mutagen detection (20,202). It has, in fact, been reported that many mutagens are undetected without the incorporation of induced S9 (202). The usual method of maximal induction is by a single ip injection of Arochlor (see Section 1.2), 5 days before sacrifice (6,125,166,202).

This investigation did not require the sensitivity essential for mutagenicity screening, since well-established mutagens were used. In addition, deliberate maximal induction may mask any steroidal inductive effects, especially if these are slight. Arochlor is also a stable, hazardous carcinogen with a very viscous nature, and it is thus difficult to handle (202). The presence of Arochlor in the S9, albeit in small amounts, may influence the mutagenic reversion of the tester strains (202).

Consequently, each treatment group was duplicated, with one set of groups (A to F) uninduced, and another set (G to L) induced by PB, a generally effective inducer, although less efficient than Arochlor (20,202). Induction was achieved by substituting a 1 mg/mℓ PB solution for the drinking water, starting 7 days prior to sacrifice (13).

The rats were sacrificed by cervical dislocation 24 hours after the last steroid administration. Sacrifice was at a fixed daily time (between 09h00 and 09h30) to avoid circadian variation in the activity of the microsomal enzymes (210).

5.2 PREPARATION OF THE S9 FRACTION

The S9 fractions were prepared according to the method of Garner *et al.* (100), as adapted by Ames (13,20,202). All procedures were performed at 0-4°C to prevent degradation of the microsomal enzymes.

All glassware was soaked in chromic acid solution for 24 hours before being rinsed three times with double-distilled water and then autoclaved.

The livers were excised immediately after sacrifice, under aseptic conditions, to avoid microbial contamination of the S9 fraction. The skin was swabbed with 95% ethanol before incision, and care was taken to exclude fur from the abdomen and not to rupture the oesophagus or the gastrointestinal tract.

The freshly excised liver was weighed in a beaker containing approximately 1 mℓ 0,15 M KCl solution per gram of wet liver. The liver was then washed several times with 0,15 M KCl, to help ensure a sterile preparation, and to remove haemoglobin, which may inhibit cytochrome P-450 (202,210).

The liver was not perfused before homogenization, as this has been reported to reduce the hepatic cyt P-450 content (210). Furthermore, the increase in liver size and mass (by as much as 25%) which occurs on perfusion must then be taken into account when preparing subcellular fractions to study induction (210).

The washed liver was transferred to a beaker containing 3 mℓ 0,15 M KCl per gram of wet liver and minced as finely as possible (to shorten homogenization time), using sterile scissors. At this point, the livers of both rats in each group were pooled.

The minced liver was homogenized using a Braunsonic 1510 ultrasonicator, with the homogenate flask held in crushed ice. Four 15 sec homogenization cycles were interspersed with cooling cycles of 2 min. Only small quantities of tissue were homogenized at one time, since the heat generated by prolonged homogenization may result in the destruction of the microsomal enzymes (210).

The homogenate was centrifuged (in teflon centrifuge tubes) at 9 *g* x 1000 for 10 min in an MSE 18 refrigerated (4°C) centrifuge. With the centrifuge head used (MSE :69181, R = 10,7 cm), centrifugation was at 8,7 x 10^{-3} rpm, calculated from :

$$RCF = 0{,}0000111 \times R \times N^2$$

Where RCF : Radial Centrifugal Force (*g* x 1000)
R : Radius (cm) from shaft to tip of centrifuge tube.
N : revolutions per minute

The supernatant (the S9 fraction) was decanted and distributed into sterile plastic cryotubes. These were frozen and stored in liquid air (at -180°C) in a 5 ℓ high-vacuum storage flask.

5.2.1 Stability of the S9 Preparations

The period for which frozen S9 preparations retain their activity on storage is still an unresolved issue. The storage periods used by different investigators range from several weeks to a few years (78,100,202,245).

Stanton *et al.* (272) have found that the microsomal enzymes were stable for several days in soft agar, at the temperatures employed in the Ames test.

5.3 RECEIPT AND STORAGE OF THE *SALMONELLA* STRAINS

The *Salmonella* strains were received from the Ames laboratory as fresh cultures on paper discs and surrounded by soft agar. On the arrival of the strains, the culture discs were immediately placed into 10 mℓ sterile nutrient broth and incubated for 10 hours in a shaking waterbath at 37°C. This results in a culture density of approximately 1-2 x 10^9 cells/mℓ (7,20).

After incubation, sterile DMSO was added to the cultures as a cryoprotective agent (0,09 mℓ DMSO/mℓ culture). The cultures were then distributed into sterile cryotubes; each tube was filled nearly to the top to protect against oxidative damage, but allowing for expansion on freezing. The cultures were frozen and stored in liquid air (-180°C) until required. Tester strains stored at these low temperatures have been found to retain their viability and genetic characterisitics for up to three years (7,29,185,202).

In this investigation, tester strain cultures were always grown directly from these 'frozen permanents' (Section 5.5.2.3). Enough permanents were prepared to avoid the problems associated with frequent inoculation from one frozen culture (202). Tester strains were not subcultured since this may result in the loss of the R-factor (202).

5.4 TESTER STRAIN GENOTYPE CONFIRMATIONS

Initial genotype tests were performed immediately upon arrival of the tester strains to ensure that they had retained their genetic markers in transit. Strains are expected to arrive in good condition if received within a week of dispatch, though they are affected by prolonged delivery time and/or hot weather (7). Genotype confirmations were also routinely incorporated into each mutagenicity assay, as were sterility tests on all reagents. Test methods were those of Maron and Ames (202), and the reagent formulae may be found in Appendix 1.

5.4.1 The Histidine Requirement

All four tester strains have a *his*$^-$ character, that is, require histidine for growth. Since the *uvrB* deletion also extends through the *bio* gene, TA97, TA98, and TA100 also require biotin (but the *bio* gene is not revertable, and therefore it is not necessary to test for biotin requirement) (20,202).

By means of a glass spreader, 0,1 mℓ of a 0,1 M histidine solution and 0,1 mℓ of a 0,5 M biotin solution were distributed over a minimal glucose agar plate. Control plates, as well as those for TA102, contained biotin but no histidine. An inoculum of each strain was streaked across the control plate and then the histidine/biotin plate. After overnight incubation at 37°C, growth on the histidine/biotin plate, and none on the control, is indicative of the presence of the *his*$^-$ deletion. The wild strain should grow on both plates.

5.4.2 The *rfa* Mutation

The *rfa* mutation permits large molecules such as crystal violet to enter and kill the bacterium and, therefore, strains with this character were tested for crystal violet sensitivity.

Fresh overnight *Salmonella* culture (0,1 mℓ) was mixed with 2 mℓ of molten soft agar (at 50°C), overlayed onto a nutrient agar plate, and allowed to set. A disc, impregnated with crystal violet, was placed onto the surface of the seeded plate, which was then incubated overnight at 37°C. A clear zone of inhibition surrounding the disc indicates the presence of the *rfa* mutation. Wild-type strains, or strains with the *gal* deletion, are not inhibited since crystal violet cannot penetrate the cells.

5.4.3 The *uvrB* Deletion

This mutation is quite stable and is not easily lost; however, its presence can be confirmed by demonstrating uv-sensitivity in bacterial strains which contain the deletion.

The four tester strains were streaked across the same nutrient agar plate in parallel stripes and a piece of foil so placed over the plate that it covered half of each streak. The plate was irradiated with a 15 W germicidal lamp at a distance of 33 cm for 8 sec (non R-factor strains were streaked on a separate plate and irradiated for 6 sec). After incubation at 37°C for 12 hr , the strains with the *uvrB* deletion (TA97, TA98, and TA100) grow only on the nonirradiated section of the plate. TA102 and the wild-type strains are not inhibited by irradiation.

5.4.4 The R-Factor

The four standard strains all contain the R-factor (*pKM101*), which also contains an ampicillin marker, the basis of the genotype test for this plasmid. Nutrient agar plates were seeded with the tester strain (as for the *rfa* mutation) and commercially available ampicillin discs were embedded in the overlay (76,309). In this case, a zone of inhibition indicates the wild-type genome, and uninhibited growth the presence of the R-factor.

5.4.5 The *pAQ1* Plasmid

TA102 is the only standard strain containing this plasmid, which contains a tetracycline resistance marker. This strain was therefore tested with both ampicillin and tetracycline discs (on the same plate), and an R-factor strain was used as a control for tetracycline sensitivity.

5.4.6 Spontaneous Reversion

Spontaneous reversion of *his*$^-$ mutants to histidine prototrophy, in the absence of a mutagen, occurs at a characteristic frequency for each strain (the SRF), and this phenomenon may be used as a diagnostic indicator for genotype (20,201).

Reportedly, the SRF of any strain may differ from one laboratory to another, but should be consistent within each (202), and each should establish its own acceptable SRF ranges (76). Historically encountered SRF ranges for the standard tester strains are shown in Table 5.2.

Table 5.2 : Acceptable SRF Ranges for Standard Strains

Data are for plates without S9, and the SRF may be very slightly higher if S9 is included. The SRF with the preincubation procedure is no different to that using the plate incorporation assay (307). Adapted from 202.

Salmonella Strain	SRF Range
TA97	90 - 180
TA98	20 - 50
TA100	100 - 200
TA102	240 - 360

Although the SRF may vary over an extended period of time, extreme fluctuations between experiments, or deviations from the acceptable ranges, mean the genotype of the strain should be retested and, if necessary, the strain should be reisolated (202).

5.5 THE MUTAGENICITY ASSAY

5.5.1 Introduction

The method followed was that of the plate-incorporation test (13, 18,20), modified by the use of a preincubation step (207,307) and by certain of the recommendations of Belser (40), which reportedly increase the precision of the assay.

The mutagen, bacterial strain and the differently pretreated S9 preparations were incubated together for a period at 37°C (pre-incubation), then soft agar was added, and the mix poured onto a minimal glucose agar plate. After incubation at 37°C for 48 hour, the revertant colonies visible on the plates were counted.

The preincubation modification, recommended by several workers (78, 125,184,202,277), is as sensitive, and with some mutagens (such as DMN, AFB_1, and BP) more so than the standard plate-incorporation test (20,36,202,207).

All glassware, instruments, and reagents were sterile. Since ethylene oxide, used to sterilize petri-plates, is a potent mutagen, only plates sterilized by gamma-radiation were used in the assays (20,202). The methods of preparation of the reagents and media may be found in Appendix 1.

5.5.2 The Components of the Assay

5.5.2.1 The S9 Mix

In order to approximate intact mammalian metabolism in an *in vitro* microbial assay, it has been found necessary to add an NADPH-generating system (i.e., NADP and glucose-6-phosphate, which may be converted into NADPH by the glucose-6-phosphate dehydrogenase present in the cytosol) as well as a phosphate buffer (which stabilizes the S9 activity) (100,207).

The microsomal fraction was therefore incorporated into a buffered solution which contained these cofactors (the S9 mix). The S9 mix was prepared freshly for each assay as soon as the frozen S9 fraction had thawed (202).

For general mutagenicity screening, a standard S9 mix, resulting in 20 µℓ S9 per plate, is recommended (184,202,224); and this was the concentration employed for most of the assays of this investigation (see Table 5.4). A greater concentration of S9 (100 µℓ per plate) has been found necessary for the activation of DMN (202), and this 'high' S9 mix was also used to confirm the nonmutagenicity of the steroids, DMSO, and phenobarbital.

5.5.2.2 The Soft Agar

Before each assay, 100 mℓ of soft agar was melted, and 10 mℓ of a 0,5 mM histidine/biotin solution was thoroughly mixed into the molten agar (18,29,202).

The trace of histidine in the soft agar allows the plated bacteria to undergo several divisions; this DNA replication is necessary for mutagenesis (202), but it also results in a faintly visible 'background lawn' of bacterial growth.

This lawn is comprised of *his*$^-$ bacteria, which are able to utilize the trace of histidine in the soft agar (33,40,76,78). A minimal, or absent, lawn is indicative of bacterial toxicity, and colonies appearing in such a background are not scored (18,20,207).

An increase in the plated histidine levels results in enhanced mutagenesis but also causes heavy growth of the background lawn, obscuring the revertants (18,20,202). Care was therefore taken to accurately pipette soft agar, and to maintain consistency of technique in pouring the agar overlay, in order to eliminate variations in the histidine content of the plates (202).

5.5.2.3 The Tester Strain Cultures

An inoculum of tester strain, from a frozen culture, was incubated in 10 mℓ of nutrient broth for 10 hr in a shaking water-bath at 37°C. This procedure results in a culture density of approximately 1-2 x 10^9 cells/ml, which is considered ideal for the mutagenicity assay (40,76,78,202).

The culture density was determined spectrophotometrically by optical density at 650 nm (OD_{650}) and the titre was adjusted, if necessary, with sterile nutrient broth. The relationship between the OD_{650} and the viable cell count was previously determined by the serial dilution/plate count method. However, the actual number of cells plated, within broad limits, is not critical (40,202).

The culture bottles were wrapped in foil during incubation to protect them from light and were used immediately as there is reported to be some loss of viability in nutrient broth cultures of *rfa* strains if kept at room temperature for several hours (27,78, 202).

5.5.2.4 The Mutagen Solutions

It is a criterion of mutagenicity that there must be a range of mutagen concentrations within which the number of induced revertants increases linearly with increasing dose (202). All the mutagens chosen exhibit this relationship at subtoxic levels (184, 202,219). Most mutagens are also toxic to some extent, either to the bacteria or to the enzymes, so that the number of revertants usually decreases at higher concentrations. Mutagen concentrations were therefore chosen from the lower ranges of the dose-response curve.

Choosing low concentrations also meant that, on incubation, the revertant colonies were fewer and therefore easier to score accurately. In addition, if a mutagen concentration is high enough to induce as many as 1000 revertants/plate, the colony density may

become so great that mutual growth inhibition of the colonies may occur. This phenomenon results in the formation of many small 'microcolonies', which are difficult to count (54,201,202).

All mutagens were handled under a fume hood, and the incubator air outlet was led into the same. Latex gloves and a face mask were worn when manipulating mutagens, and all items in contact with mutagens were stored in double heavy-duty plastic bags for later incineration (202).

5.5.3 The Assay Procedure

Into a set of sterile test-tubes, held in an ice bath, was pipetted 0,5 mℓ of S9 mix (containing the relevant S9 type and concentration). Similar amounts of plain buffer were used in control assays (see Section 5.5.4) (13,18,20,202).

To the S9 mix was added, in order, 0,1 mℓ of a fresh overnight tester strain culture and 0,1 mℓ of the test mutagen solution (18, 20,40,202).

This mixture was vortexed gently (without allowing foaming) for 3 sec, and then incubated for 20 min at 37°C in a darkened water-bath under a fume hood (16,40,207).

After the preincubation was complete, a 2 mℓ aliquot of soft agar (containing the histidine/biotin solution) was added to each incubate, and the mixture vortexed for 3 sec before being poured onto the centre of a minimal glucose agar plate (40,184,207).

The assay mix was evenly distributed over the surface of the plate and left to set for a few minutes on a level surface. This procedure ensured an even distribution of the agar overlay, which is critical for uniform colony density (40,202,207). The pouring and distribution was performed within a strictly observed 20 sec time limit (20,202) to prevent the soft agar from setting in mid-operation. Should the agar set too early, a stippled overlay may result, which makes the scoring of revertants difficult.

The plates were then incubated at 37°C (20,202) in a Forma 3028 incubator (this specific model has been recommended for use in the Ames assay as it was found to have a maximum temperature gradient of only 0,25°C (40)). A relative humidity of 80% was employed as this has been found to retard the drying of the agar without causing excess condensation, which would permit the mobile *Salmonella* colonies to migrate (40).

The number of revertant colonies was counted manually after 48 hr, 63 hr, and 72 hr, until a plateau in colony count was reached (40, 76,78), and the presence of a background lawn was confirmed (see Section 5.5.2.2). The number of revertant colonies/plate was assumed to reflect the metabolic activation of the test mutagen by the various S9 preparations.

5.5.4 Protocol of Mutagenicity Assays Performed

The mutagen/tester strain combinations used are shown in Table 5.4, and the mutagenicity assays performed are summarized in Table 5.3. Experiments 17-28 of this investigation assayed the activation of the test mutagens, by the differently pretreated S9 preparations.

The balance of the experimental protocol consisted of controls. Tests 1-3 determined the spontaneous reversion frequency of each strain. Tests 4-6 tested DMSO for mutagenicity and tests 7-9, sodium phenobarbital. Tests 10-15 were used to confirm the nonmutagenicity of the two steroids. Each set of controls was performed with no S9 (phosphate buffer only), uninduced S9, and induced S9 (see Table 5.3). Test 16 was included in order to test the mutagenicity of the test compounds without activation by S9.

It is generally recommended that each assay be duplicated (56,76, 78); all the tests and controls in this study were repeated in triplicate.

Table 5.3 : Protocol for Mutagenicity Assays

Test	Test Compound	Dose/Plate	S9 Source
1	None	-	Buffer only
2	None	-	A
3	None	-	G
4	DMSO	0,10 mℓ	Buffer only
5	DMSO	0,10 mℓ	A
6	DMSO	0,10 mℓ	G
7	PB	5,00 mg	Buffer only
8	PB	5,00 mg	A
9	PB	5,00 mg	G
10	E_2	500,00 µg	Buffer only
11	E_2	500,00 µg	A
12	E_2	500,00 µg	G
13	PRG	500,00 µg	Buffer only
14	PRG	500,00 µg	A
15	PRG	500,00 µg	G
16	Test Mutagen	See Table 5.4	Buffer only
17	Test Mutagen	See Table 5.4	A
18	Test Mutagen	See Table 5.4	G
19	Test Mutagen	See Table 5.4	B
20	Test Mutagen	See Table 5.4	H
21	Test Mutagen	See Table 5.4	C
22	Test Mutagen	See Table 5.4	I
23	Test Mutagen	See Table 5.4	D
24	Test Mutagen	See Table 5.4	J
25	Test Mutagen	See Table 5.4	E
26	Test Mutagen	See Table 5.4	K
27	Test Mutagen	See Table 5.4	F
28	Test Mutagen	See Table 5.4	L

Table 5.4 : Tester-Strain / Mutagen Combinations

Tester Strain	Mutagen	Mutagen Dose	Solvent	S9 Conc. (µℓ/plate)
TA 97	BP	0,50 µg	DMSO	20
TA 98	AFB_1	0,10 µg	DMSO	20
TA 100	DMN	1,48 mg	H_2O	100
TA 102	MMC	0,25 µg	H_2O	20

Test compounds were compounded so that dose could be added to the mix in 0,1 mℓ solvent (see Appendix 1).

Each assay was performed in triplicate for each of the 4 tester strains (TA97, TA98, TA100 and TA102).

The S9 pretreatments are summarized in Table 5.1, reproduced here.

TREATMENT	RAT GROUP	
	UNINDUCED	PB-INDUCED
No Treatment	A	G
Ethyl Oleate Only	B	H
17β-Oestradiol 1 mg/kg	C	I
17β-Oestradiol 20 mg/kg	D	J
Progesterone 1 mg/kg	E	K
Progesterone 20 mg/kg	F	L

5.6 STATISTICAL ANALYSIS

Data analysis was performed by the Student-Newman-Keuls (SNK) procedure, described by Kirk (168), at the 95% level of significance (α = 0,05). The SNK procedure is an *a posteriori* contrast, which systematically compares all possible pairs of group data means, and divides the groups into homogeneous subsets, within which the difference between any two group means is not significant. Differences between two group means which exceed a critical value for any subset are significant. This critical value varies, depending on the number of means in the subset. The procedure is based on the test:

$$[x_i - x_j] < R(\alpha,g,f) \cdot S_x$$

where $R(\alpha,g,f)$ is a range based on a significance level (α), the number of groups in the subset (g), and the degrees of freedom (f) in the between-groups sum of squares. S_x is the standard error in the combined subset. The SNK procedure was performed using a subprogramme of the *Statistical Package for the Social Sciences (SPSS)* (Vogelback Computing Centre, Northwestern University). This subprogramme also generated various descriptive statistics on the data in addition to performing a one-way analysis of variance (F Ratio) and two tests for homogeneity of variance (Cochran's and Bartlett-Box) (see Appendix 2 for programmes).

Statistical models for the analysis of data in mutagenicity screening, using the Ames test, have been discussed in considerable detail in the literature, although no particular method is presently recommended (41,62,77,134,161,199,200,224,225,202,236,257,266, 274,293,298).

CHAPTER 6 : RESULTS AND DISCUSSION

6.1 EFFECTS OF PRETREATMENT ON THE RATS

6.1.1 Steroid Pretreatment

6.1.1.1 Effects on General Appearance

Pretreatment of female Sprague-Dawley rats with norethynodrel or ethinyl oestradiol (at 20 mg/kg/day for 2 days or more) has been reported to cause a change in coat-colour from white to cream, as well as a certain amount of hair-loss in some animals (135). Loss of hair has also been reported after protracted pretreatment with high doses of a norethynodrel/mestranol combination (61).

Although no changes in coat-colour were observed with the E_2 or PRG pretreatments of this study, slight alopecia was observed in rats receiving 20 mg PRG per kg (groups F and L).

Rats receiving the higher doses of both E_2 and PRG became listless and appeared unwell. These rat groups also showed the greatest loss in body weight after steroid treatment (see Section 6.1.1.2), and it is possible that these observations are interrelated.

A pronounced increase in general fat deposition was observed, on dissection, in rats treated with E_2. Tissues affected were the breasts and several abdominal organs, including the liver, spleen, kidneys, and the gastrointestinal tract. These clearly visible fatty deposits were 'patchy' at 1 mg/kg, but were more extensive in rats receiving the higher dose.

This phenomenon was not observed in either the untreated or vehicle-injected control rats, or in rats receiving the lower doses of PRG. A certain amount of fat deposition was evident in the breast tissue of rats receiving PRG at the higher dose level.

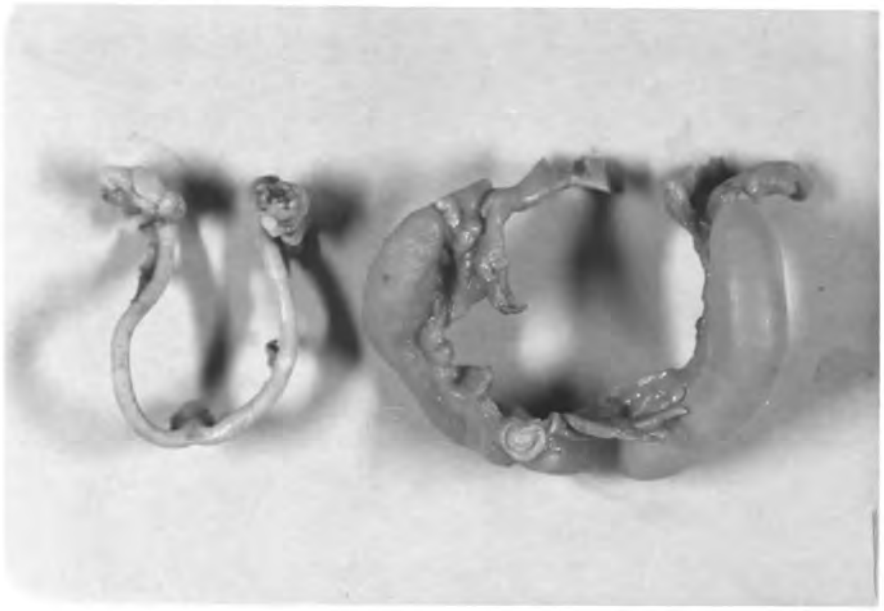


Figure 6.1 : Effect of Pretreatment with E_2 (20 mg/kg/day) on the Uterine Horns. Untreated control on left.

Figure 6.2 : Effect of Pretreatment with E_2 (20 mg/kg/day) on the Liver. Untreated control on left.

The uterine horns of rats pretreated with E_2 were considerably enlarged and filled with clear fluid (see Figure 6.1); this uterotropic effect was more pronounced in those rats receiving the higher E_2 dose. Welch *et al.* (300) have also reported an increase in uterine mass after E_2 pretreatment.

The livers of rats pretreated with E_2 were also visibly enlarged, and the lobes of the liver were more 'rounded' in shape than those of either control or vehicle-injected rats (Figure 6.2). Post-mortem tests, performed by the State Veterinary Laboratory, Grahamstown, confirmed the absence of any infection; thus, the observed changes were probably the result of the steroid pretreatments.

6.1.1.2 Effects on Body Mass

It has been reported that pretreatment with E_2 (1 mg/kg/day for 14 days) (52), or with various synthetic oestrogens and progestins (61,96,255), cause a decrease in the body mass of experimental rats. The effects of the steroid pretreatments on body mass in this study conform to the reported findings, and are shown in Table 6.1 and represented graphically in Figure 6.3.

Control rats (group A) weighed 109% of their original mass after two weeks, a percentage increase which is in agreement with the reported findings of other investigators (61,135). The mass gain in vehicle-injected control rats (group B) was not significantly different from that of the rat group which was untreated.

After two weeks of E_2 pretreatment at 1 mg/kg (group C) or 20 mg/kg (group D), rats weighed 97% and 93% of their original mass respectively. Although mass loss at the higher dose of E_2 was greater than that at the lower dose, the difference was not statistically significant.

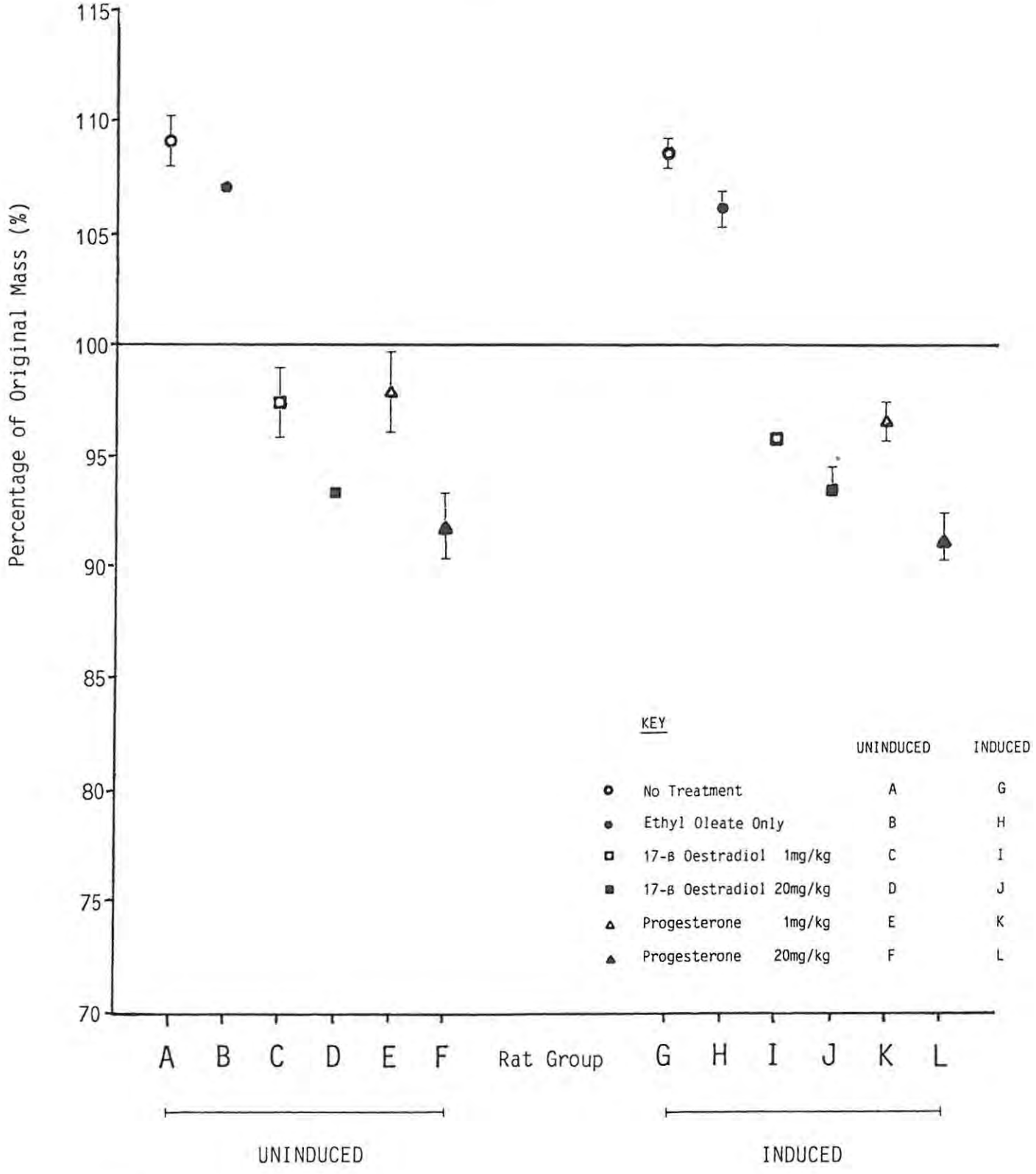
Progesterone pretreatment also caused a significant loss of body mass. Rats in group E (1 mg/kg) weighed an average of 98% of their original mass after two weeks. Those in group F (20 mg/kg) weighed 92% of their original mass, a significantly greater loss than that observed with the lower dose of PRG.

Table 6.1 : Effect of Pretreatment on Mass Gain, Liver Mass and Liver/Body Mass Ratio.

Data represent means ± S.E. for groups of 2 rats. See also Figure 6.3 and 6.4

Rat Group	Initial Mass (I.M.)	Final Mass (F.M.)	F.M./I.M. x 100	Liver Mass	Liver/Body Mass Ratio
	(g)	(g)	(%)	(g)	(g/100 g)
A	204,68 ± 13,68	223,58 ± 17,08	109,17 ± 1,04	8,30 ± 0,53	3,72 ± 0,04
B	205,78 ± 1,23	220,49 ± 0,91	107,16 ± 0,19	8,56 ± 0,03	3,88 ± 0,03
C	219,47 ± 7,99	213,80 ± 10,80	97,37 ± 1,38	10,48 ± 0,62	4,90 ± 0,04
D	209,75 ± 2,75	195,75 ± 2,85	93,29 ± 0,17	10,45 ± 0,15	5,34 ± 0,00
E	205,48 ± 2,88	201,08 ± 0,41	97,88 ± 1,57	9,01 ± 0,10	4,48 ± 0,04
F	218,50 ± 13,50	200,65 ± 15,20	91,75 ± 1,29	10,17 ± 0,83	5,07 ± 0,03
G	218,25 ± 10,95	236,79 ± 10,69	108,52 ± 0,55	11,47 ± 0,64	4,84 ± 0,05
H	200,45 ± 4,75	212,54 ± 6,54	106,01 ± 0,75	10,66 ± 0,30	5,02 ± 0,01
I	217,60 ± 8,40	208,14 ± 8,23	95,65 ± 0,09	12,43 ± 0,59	5,97 ± 0,05
J	217,58 ± 6,73	203,16 ± 4,33	93,40 ± 0,90	13,16 ± 0,20	6,48 ± 0,04
K	220,50 ± 8,50	212,63 ± 6,50	96,46 ± 0,77	11,77 ± 0,22	5,54 ± 0,09
L	207,75 ± 7,55	189,19 ± 4,85	91,10 ± 0,98	11,65 ± 0,24	6,16 ± 0,03

Figure 6.3 : Effect of Pretreatments on Mass Gain



For each observation, n = 2
Graph shows both data measurements and means of each rat group

6.1.1.3 Effects on Liver Mass and Liver Somatic Index

There was an increase in liver size, both in actual terms and in relation to the body mass, in all the steroid-pretreated groups, when compared with control values.

Due to the concomitant variations in body mass, statistical analysis was not performed on the changes in liver mass but on the relationship between liver mass and total body mass. This relationship is expressed as the liver mass/body mass ratio, or as the liver somatic index (LSI) (units : g/100g, or %) (73,92). The effects of the steroid pretreatments on the LSI are shown in Table 6.1 and Figure 6.4.

The mean LSI of uninduced control rats (group A) was 3,72, a value similar to those reported for untreated rats (52,73,135,235).

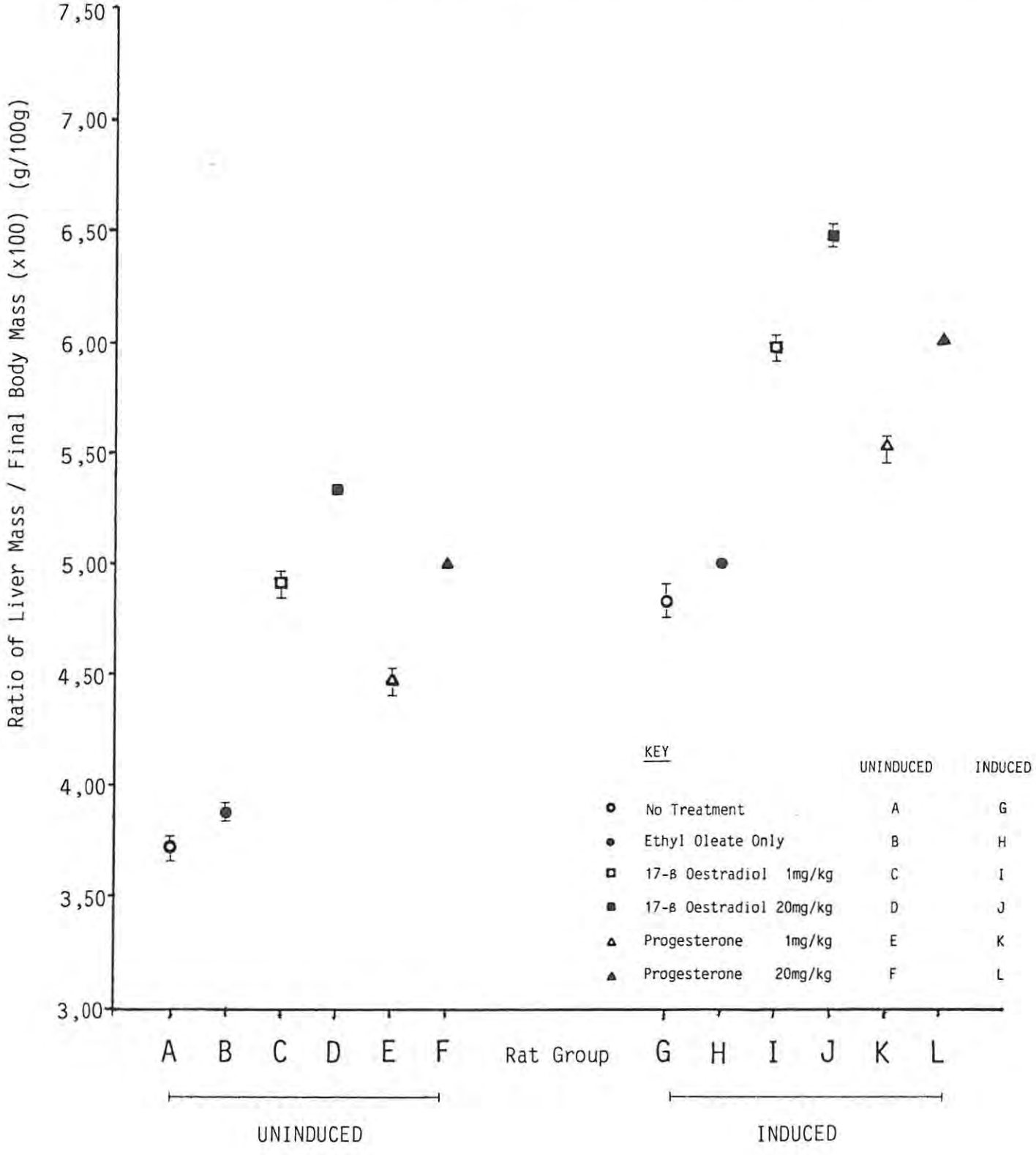
A significant and dose-dependent increase in LSI over control values was observed with both E_2 and PRG pretreatment. The mean LSI of rats receiving 20 mg/kg of E_2 was significantly higher than that of rats receiving 1 mg/kg. Similarly, the mean LSI of the rats pretreated with PRG at a level of 20 mg/kg was significantly higher than that observed after pretreatment at 1 mg/kg.

The observed increases in the LSI following pretreatment with E_2 were in agreement with the reported effects of similar doses of the steroid (4,52). In these other studies, no change in liver mass was found and, therefore, the increase in LSI was attributed to decreased body mass alone.

In this study however the increased LSI, which followed steroid pretreatment was associated with both a decrease in body mass and a real increase in liver mass.

Similar findings have been reported after the pretreatment of mice with E_2 and PRG (82), and of rats with several synthetic sex-steroids (61,135,255,290). A significant increase in liver mass of between 33% and 50% has also been observed in pregnant rats (72,73,226,294).

Figure 6.4 : Effect of Pretreatment on Liver/Body Mass Ratio



For each observation, n = 2
Graph shows both data measurements and means of each rat group

6.1.2 Effect of Induction by PB

Each of the control and steroid pretreated groups were duplicated, one set of groups (A-F) being uninduced while a second (G-L) were induced by PB.

Rats which were induced by PB, but which otherwise were either untreated (Group G) or injected with vehicle only (Group H) experienced an increase in mass, which was similar to that of the equivalent uninduced control groups (Figure 6.3). This is in agreement with the findings of McLean and Marshall (217) and Peraino *et al.* (235).

Induction by PB also did not alter any of the changes induced by the various steroid-pretreatments, in mass gain (Figure 6.3), fat deposition, or uterine size.

Induction by PB increased both the liver mass and, since the body mass was unaffected, the LSI of all the rat groups (both control and treatment). The increased liver size, which was observed after induction by PB, was of the same order of magnitude as the increases (20%-60%) reported in the literature (66,120,248,296). Rat groups which were induced by PB responded to the steroid pretreatments in a very similar way to the equivalent uninduced groups, but there was a pronounced upward 'shift' of the pattern (Figure 6.4).

A strongly positive relationship has been found between the liver hypertrophy produced by PB (and other inducing agents) and the level of enzyme induction produced (66). It is possible therefore that the hypertrophy of the liver produced by the steroid pretreatments of this study may be indicative of the increased synthesis of drug-metabolizing enzymes, among other proteins.

6.2 THE MUTAGENICITY ASSAYS

6.2.1 Genotype Confirmations

Genotype tests were performed, as detailed in Section 5.4, on arrival of the tester strains, and all four of the strains were found to have retained their genetic characteristics in transit.

Genotypic confirmations were also incorporated into each mutagenicity assay, and, in the very few instances when these were inconclusive, the assays were repeated with fresh cultures.

6.2.2 Mutagenicity Controls

The results of all the mutagenicity assays, both tests and controls, for the four tester strain/mutagen combinations are represented as summarized here :

Strain/Mutagen	Table	Figure
TA97/BP	6.2	6.5
TA98/AFB_1	6.3	6.7
TA100/DMN	6.4	6.10
TA102/MMC	6.5	6.12

6.2.2.1 Spontaneous Reversion

In this study, the SRF, without S9, was determined by test 1, while tests 2 and 3 corresponded to the SRF with uninduced S9 (A) and PB-induced S9 (G), respectively.

The mean SRFs of the three *uvrB*$^-$ tester strains, TA97, TA98, and TA100 were found to lie well within the published limits (Tables 6.2, 6.3, and 6.4), while the mean SRF of TA102 was just below the published lower limit (Table 6.5).

Maron (201) has confirmed that, despite the low SRF observed for TA102 in this study, the extent of reversion by the standard mutagen (MMC) implies that this SRF is still acceptable. Also, the SRF of all four strains was consistent within the ranges established in this laboratory, a fact which is another important consideration (76). Apparently, difficulty in the establishment of a good SRF range is a problem inherent with a strain carrying a multicopy plasmid (201), and several laboratories have experienced this problem (7).

There was no change in the SRF of any of the tester strains when S9 (either uninduced or PB-induced) was added to the incubate.

6.2.2.2 Mutagenicity of DMSO

The mutagenicity of the solvent DMSO, used for BP and AFB_1, was investigated in tests 4, 5 and 6. For all four tester strains, no increase in reversion frequency was observed in these tests, and, therefore, the reported nonmutagenicity of DMSO, and its suitability as a solvent for nonpolar mutagens in the Ames test (74,174,202, 203) was confirmed.

6.2.2.3 Mutagenicity of PB

Although PB, administered in the drinking water to induce the enzymes of groups G-L, is reported to be nonmutagenic (127,213,215, 217,284), tests 7, 8, and 9 were included to confirm these findings. PB was found to be nonmutagenic, both with and without the incorporation of S9, when tested with the standard tester strain set (Tables 6.2, 6.3, 6.4 and 6.5).

6.2.2.4 Mutagenicity of the Steroids

As described in Section 3.5, E_2 and PRG have been found to be nonmutagenic in *Salmonella*, at concentrations below those which were toxic to the bacteria. Neither E_2 (test 10, 11 and 12) nor PRG (tests 13, 14 and 15), at the highest concentration which is still

considered to be subtoxic (500 μg/plate), caused any increase in the number of revertant colonies/plate with any of the tester strains (Figures 6.5, 6.7, 6.10, and 6.12). These results confirmed the reported nonmutagenicity of the sex-steroids.

6.2.2.5 Mutagenicity Without Metabolic Activation

Each mutagen was tested with the strain chosen to detect it in the absence of a microsomal (S9) fraction (test 16).

DMN, BP and AFB_1 failed to increase the number of *his*$^+$ revertants per plate when tested with their respective tester strains (Figures 6.5, 6.7, and 6.10), a finding which confirmed their reported requirement for metabolic activation to mutagenic metabolites (Chapter 2).

MMC however caused a two-fold increase in the number of revertants per plate over the SRF of TA102. (Figure 6.12). This was not unexpected as MMC is known to cause mutation in TA102 without S9 activation (Section 2.5).

6.2.3 Effects of Sex-Steroids on Mutagen Activation

6.2.3.1 Benzo(a)pyrene

The results of the mutagenicity assays using TA97 and different liver microsomal fractions to activate BP are shown in Table 6.2, and graphically represented in Figures 6.5 and 6.6.

The incubation of BP with S9 from untreated rats (test 17; S9:A) or from rats injected with vehicle only (test 19; S9:B) caused no increase in reversion frequency over controls.

S9 pretreated with either dose of E_2 (test 21; S9:C and test 23; S9:D) also failed to increase the number of *his*$^+$ revertants per plate.

Table 6.2 : Results of TA97 / BP Mutagenicity Assays

Tester Strain : TA 97 Mutagen : BP Mutagen Dose : 0,5 µg S9 Concentration : 20 µℓ

Spontaneous Reversion Limits : 90 - 180

Data represent 3 plate counts (visible revertant colonies / plate), listed from smallest to largest count; count means, standard errors (S.E.), and 95% confidence intervals (C.I.) of means, the latter calculated from antilogs of transformed data. Also shown are the logarithmic transformations (base 10) of the original data.

Test	Plate			Means	S.E.	95% C.I.of Means		Log_{10} Transformations	
	A	B	C			Lower	Upper	Means	S.E.
	Revertant Colonies/Plate				±				±
01	143	144	153	146,67	3,18	133,66	160,77	2,166	0,009
02	147	157	163	155,67	4,67	136,58	177,09	2,192	0,013
03	139	146	154	146,33	4,33	128,74	166,07	2,165	0,013
04	143	151	152	148,67	2,85	136,74	161,51	2,172	0,008
05	140	142	151	144,33	3,38	130,56	159,40	2,159	0,010
06	141	148	157	148,67	4,63	129,93	169,79	2,172	0,014
07	138	145	149	144,00	3,21	130,68	158,53	2,158	0,010
08	144	145	153	147,33	2,85	135,61	159,92	2,168	0,008
09	138	145	147	143,33	2,73	131,92	155,60	2,156	0,008
10	143	151	154	149,33	3,28	135,68	164,21	2,174	0,010
11	141	149	154	148,00	3,79	132,40	165,20	2,170	0,011
12	141	153	155	149,67	4,37	131,64	169,86	2,175	0,013
13	143	150	153	148,67	2,96	136,33	161,99	2,172	0,009
14	136	139	148	141,00	3,61	126,36	157,14	2,149	0,011
15	139	149	154	147,33	4,41	129,27	167,61	2,168	0,013
16	151	157	162	156,67	3,18	143,48	170,92	2,195	0,009
17	144	156	157	152,33	4,18	135,05	171,55	2,183	0,012
18	450	459	465	458,00	4,36	439,54	477,09	2,661	0,004
19	155	167	169	163,67	4,37	145,58	183,74	2,214	0,012
20	455	468	471	464,67	4,91	443,92	486,30	2,667	0,005
21	152	156	164	157,33	3,53	142,86	173,10	2,197	0,010
22	441	453	462	452,00	6,08	426,48	478,96	2,655	0,006
23	147	155	161	154,33	4,06	137,69	172,78	2,188	0,012
24	457	470	472	466,33	4,70	446,38	487,08	2,669	0,004
25	274	285	290	283,00	4,73	263,21	304,09	2,452	0,007
26	470	479	489	479,33	5,49	456,25	503,50	2,681	0,005
27	251	255	266	257,33	4,48	238,78	277,14	2,410	0,008
28	460	461	479	466,67	6,17	440,96	493,74	2,669	0,006

Significance of F in One-way Analysis of Variance

Log_{10} Data : F-Prob = 0,0000

P Values for 2 Data Homoscedasticity Tests

1. Cochran's Test (Max.variance/sum of variances)

Raw Data : P = 1,000 (approx.)

Log_{10} Data : P = 1,000 (approx.)

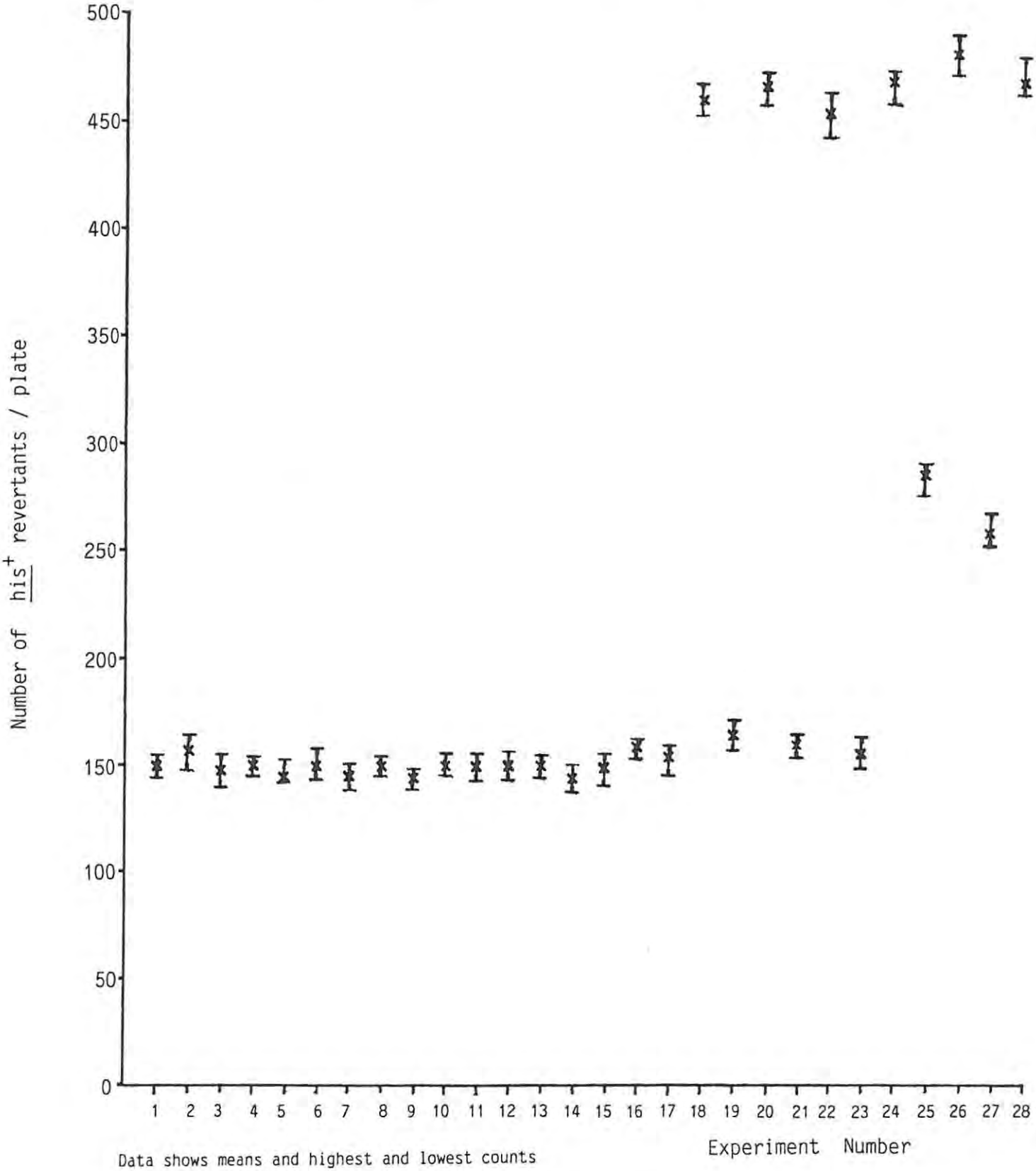
2. Bartlett-Box Test (Max.variance/Min.variance)

Raw Data : P = 1,000

Log_{10} Data : P = 0,999

See also Figure 6.5 and 6.6

Figure 6.5 : Results of TA97/BP Mutagenicity Assays



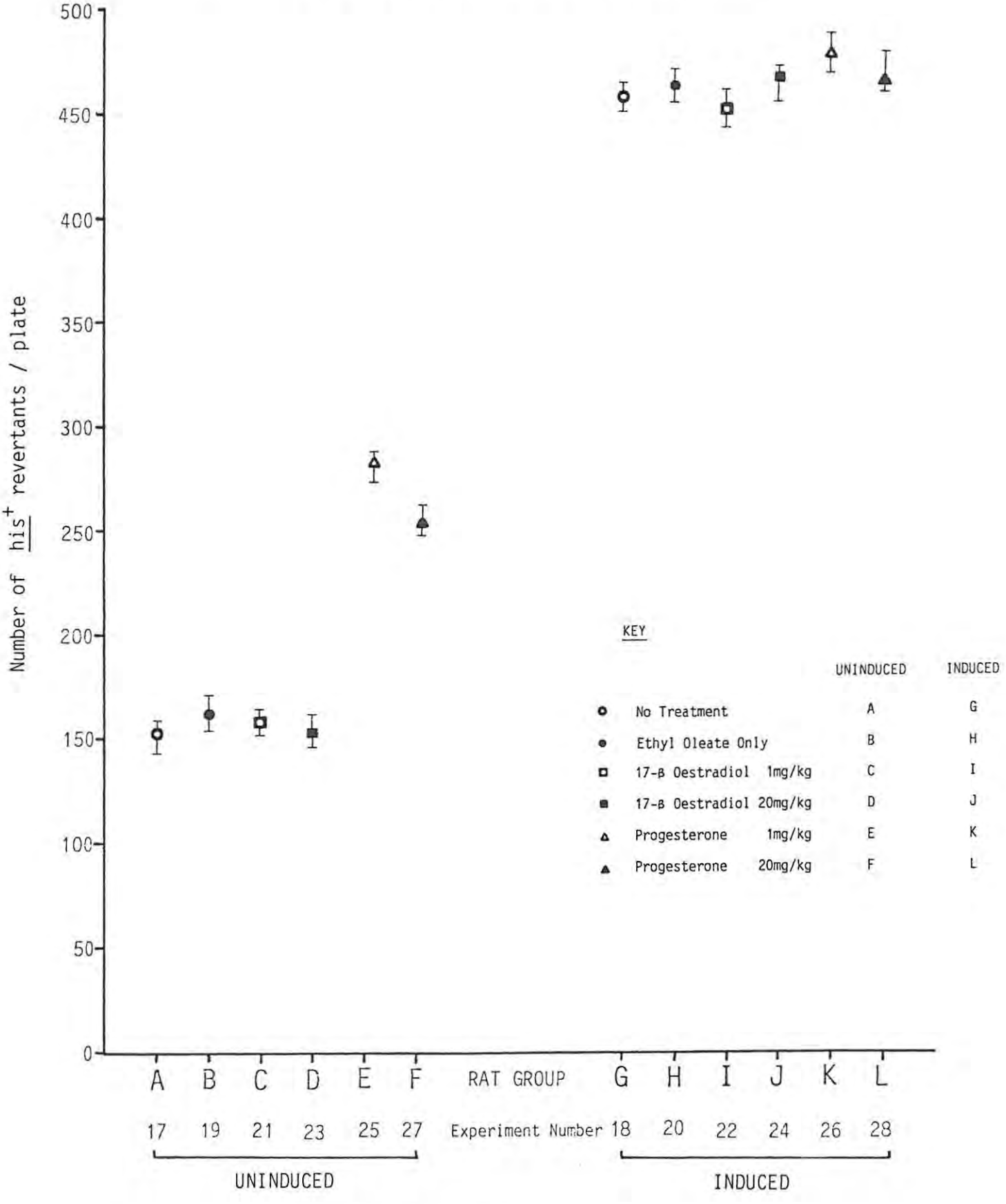
Data shows means and highest and lowest counts

For each observation, n = 3

S9 is from the pooled livers of 2 similarly treated rats

Spontaneous revertants not subtracted

Figure 6.6 : Effect of the S9-pretreatments on the Activation of BP to mutagenic metabolites with TA97



Data shows means and highest and lowest counts

For each observation, n = 3

S9 is from the pooled livers of 2 similarly treated rats

Spontaneous revertants not subtracted

A significant increase in the number of colonies per plate over control values was observed when S9 pretreated with PRG was used. The lower PRG dose (1 mg/kg, test 25; S9:E) caused significantly more revertants than the higher dose (20 mg/kg, test 27; S9:F).

S9 prepared from each of the rat groups which were induced by PB (G-L) (tests 18, 20, 22, 24, 26 and 28) showed a three-fold increase in the reversion rate over uninduced untreated controls (test 17; S9:A). No statistical differences were detected among any of these groups.

The metabolism of BP (Section 2.4) is complex , involving pathways of mutagenic activation and detoxification catalysed by different groups of enzymes.

The hepatic microsomal fraction in the assay contains both the enzymes involved in BP epoxidation, cyt P-450 and cyt P-448, and EH, which hydrolyses the BP epoxides to the corresponding dihydrodiols (281). Therefore, the resultant mutagenicity of BP in *Salmonella* is dependent on the ratio of the activities of the different enzymes (including level of induction) involved (281).

The metabolic activation of BP to mutagenic intermediates in *Salmonella* is reportedly enhanced by the induction of the S9 fraction by 3MC, a phenomenon which suggests that the mutagenicity of BP may be preferentially mediated by a cyt P-448-dependent monooxygenase (132,267). However, the K-region epoxidation of BP is supported by a cyt P-450 inducible by PB, as is the activity of EH, which facilitates the secondary activation of BP-7,8-dihydrodiol to another mutagenic metabolite.

Therefore, the enhanced mutagenic activation of BP by an S9 fraction which was induced by PB is not inexplicable. These findings would seem to reflect that the active metabolites mentioned above contribute significantly to the *in vitro* mutagenicity of BP when the liver is induced by PB.

Tauc *et al.* (281) have also shown an increase in the mutagenicity of BP when the S9 was induced by PB, yet far greater mutagenic activity was observed after induction with 3MC.

Although it is not clear why the lower dose of PRG caused a greater response than the higher dose, the results at both dose levels indicate that PRG is capable of the induction of the enzymes involved in the mutagenic activation of BP.

A possible explanation for this phenomenon might be that the higher dose of PRG enhances the activity of one or more of the enzymes involved in the detoxification of the mutagenic metabolites of BP.

Since the microsomal fraction contained a full complement of metabolic enzymes, it is not possible to characterize the particular haemoprotein P-450 (or other enzyme) induced by PRG.

6.2.3.2 Aflatoxin B_1

The effects of the different S9 fractions on the metabolic activation of AFB_1 to mutagenic metabolites (as detected by TA98) are shown in Table 6.3, and Figures 6.7 and 6.8.

The reversion produced by the S9 of the untreated (test 17; S9:A) and vehicle-injected (test 19; S9:B) control groups was unchanged from the spontaneous reversion rate for this strain.

No increase in the number of *his*$^+$ colonies/plate over controls occurred with E_2-pretreatment at 1 mg/kg (test 21; S9:C). When administered at 20 mg/kg (test 23; S9 D) however, significant increases over controls were observed.

Both doses of PRG enhanced AFB_1 activation considerably. Administration at 20 mg/kg (test 27; S9:F) resulted in significantly greater induction than at 1 mg/kg (test 25; S9:E).

Table 6.3 : Results of TA98 / AFB_1 Mutagenicity Assays

Tester Strain : TA 98 Mutagen : AFB_1 Mutagen Dose : 0,1 µg S9 Concentration : 20 µl

Spontaneous Reversion Limits : 20 - 50

Data represent 3 plate counts (visible revertant colonies / plate), listed from smallest to largest count; count means, standard errors (S.E.), and 95% confidence intervals (C.I.) of means, the latter calculated from antilogs of transformed data. Also shown are the logarithmic transformations (base 10) of the original data.

Test	Plate			Means	S.E.	95% C.I.of Means		Log_{10} Transformations	
	A	B	C			Lower	Upper	Means	S.E.
	Revertant Colonies/Plate				±				±
01	29	33	37	33,00	2,31	24,26	44,45	1,516	0,031
02	31	32	40	34,33	2,85	24,15	48,16	1,533	0,035
03	29	31	37	32,33	2,40	23,52	43,96	1,507	0,032
04	26	30	34	30,00	2,31	21,36	41,62	1,475	0,034
05	33	37	42	37,33	2,60	27,53	50,13	1,570	0,030
06	33	34	41	36,00	2,52	26,75	48,00	1,554	0,030
07	27	29	37	31,00	3,06	20,38	46,29	1,487	0,041
08	29	34	35	32,67	1,86	25,32	41,87	1,513	0,025
09	26	31	35	30,67	2,60	21,00	44,13	1,484	0,038
10	28	32	37	32,33	2,60	22,72	45,42	1,507	0,035
11	28	35	35	32,67	2,33	23,59	44,75	1,512	0,032
12	26	29	33	29,33	2,03	21,70	39,27	1,465	0,030
13	25	28	32	28,33	2,03	20,74	38,32	1,450	0,031
14	32	37	39	36,00	2,08	27,82	46,27	1,555	0,026
15	28	29	35	30,67	2,19	22,65	41,11	1,485	0,030
16	26	27	33	28,67	2,19	20,73	39,19	1,455	0,032
17	29	35	40	34,67	3,18	23,01	51,35	1,536	0,041
18	361	374	392	375,67	8,99	338,84	416,01	2,575	0,010
19	32	39	39	36,67	2,33	27,49	48,48	1,562	0,029
20	374	393	407	391,33	9,56	351,88	434,61	2,592	0,011
21	32	40	41	37,66	2,85	26,67	52,55	1,573	0,034
22	373	417	425	405,00	16,17	339,39	481,73	2,607	0,018
23	59	73	81	71,00	6,43	47,13	105,15	1,848	0,041
24	367	375	384	375,33	4,91	354,73	397,01	2,574	0,006
25	97	109	117	107,67	5,81	84,86	135,80	2,031	0,024
26	377	398	413	396,00	10,44	353,10	443,51	2,597	0,012
27	263	271	296	276,67	9,94	237,19	321,88	2,441	0,015
28	383	420	427	410,00	13,65	354,08	473,70	2,612	0,015

Significance of F in One-way Analysis of Variance

Log_{10} Data : F-Prob = 0,000

P Values for 2 Data Homoscedasticity Tests

1. Cochran's Test (Max.variance/sum of variances)

Raw Data : P = 0,012 (approx.)

Log_{10} Data : P = 1,000 (approx.)

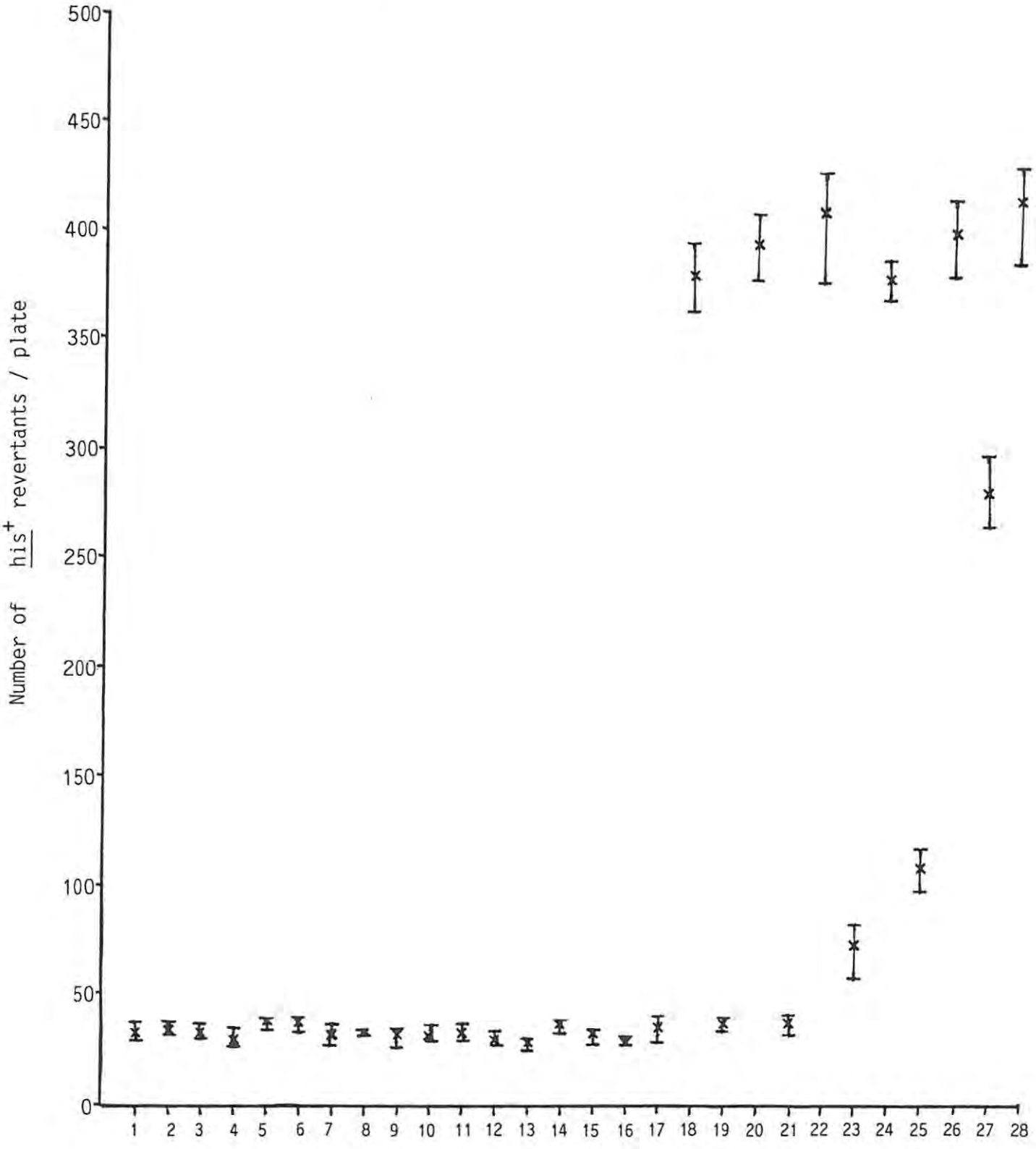
2. Bartlett-Box Test (Max.variance/Min.variance)

Raw Data : P = 0,006

Log_{10} Data : P = 0.973

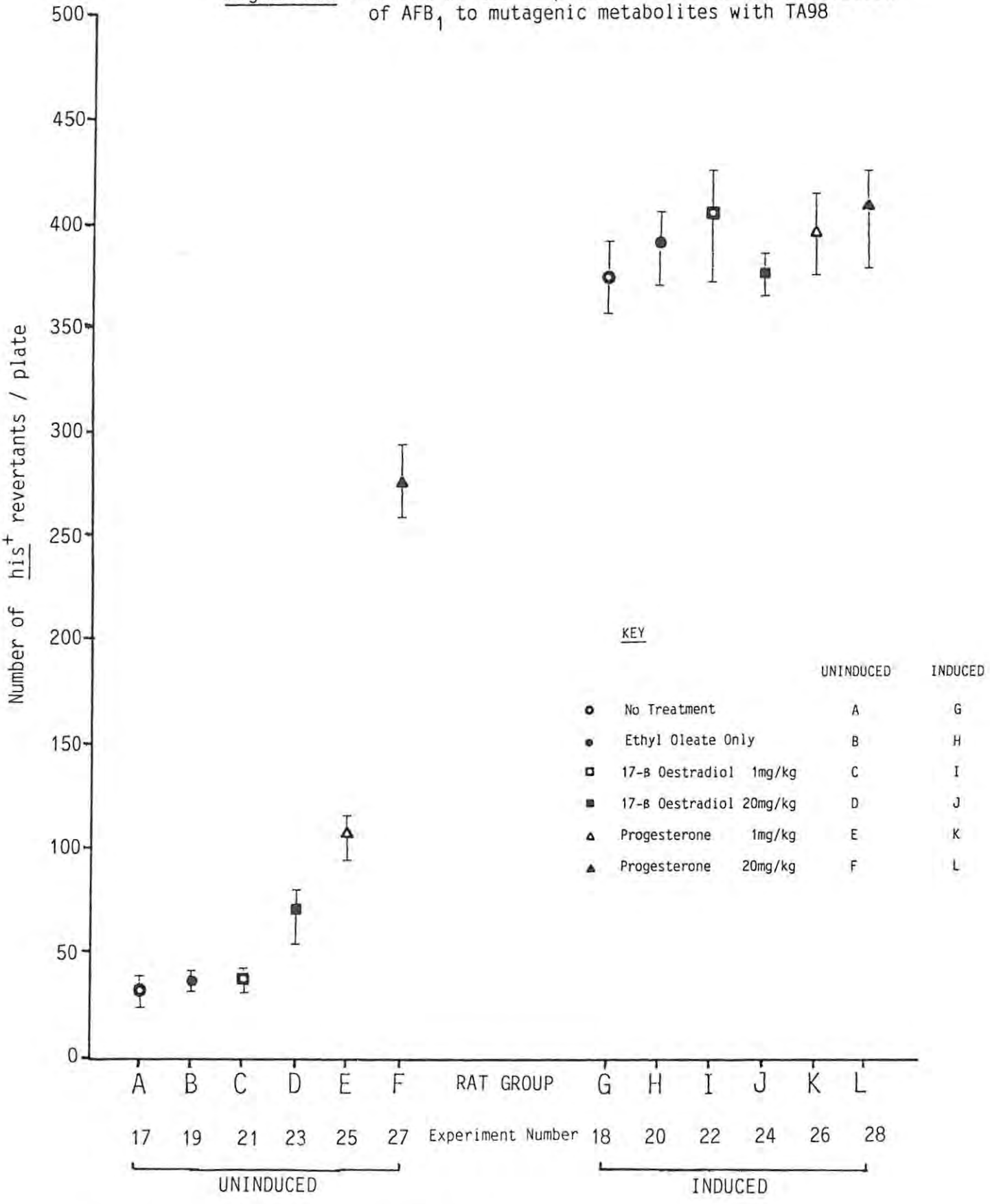
See also Figure 6.7 and 6.8

Figure 6.7 : Results of TA98/AFB_1 Mutagenicity Assays

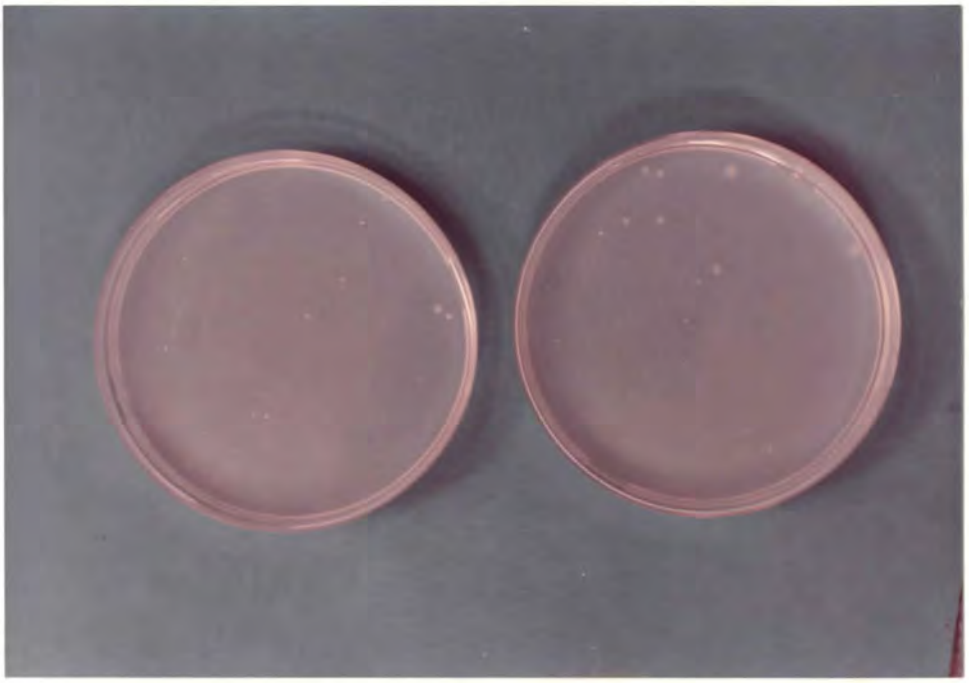


Data shows means and highest and lowest counts
For each observation, n = 3
S9 is from the pooled livers of 2 similarly treated rats
Spontaneous revertants not subtracted

Figure 6.8 : Effect of the S9-pretreatments on the Activation of AFB_1 to mutagenic metabolites with TA98



Data shows means and highest and lowest counts
For each observation, n = 3
S9 is from the pooled livers of 2 similarly treated rats
Spontaneous revertants not subtracted



6.9 a) *His*$^+$ Revertant TA98 Colonies Produced After Incubation of AFB_1 with control (untreated) S9 (Test 17; S9:A)

6.9 b) *His*$^+$ Revertant TA98 Colonies Produced After Incubation of AFB_1 with S9 induced by PB (Test 18; S9:G)

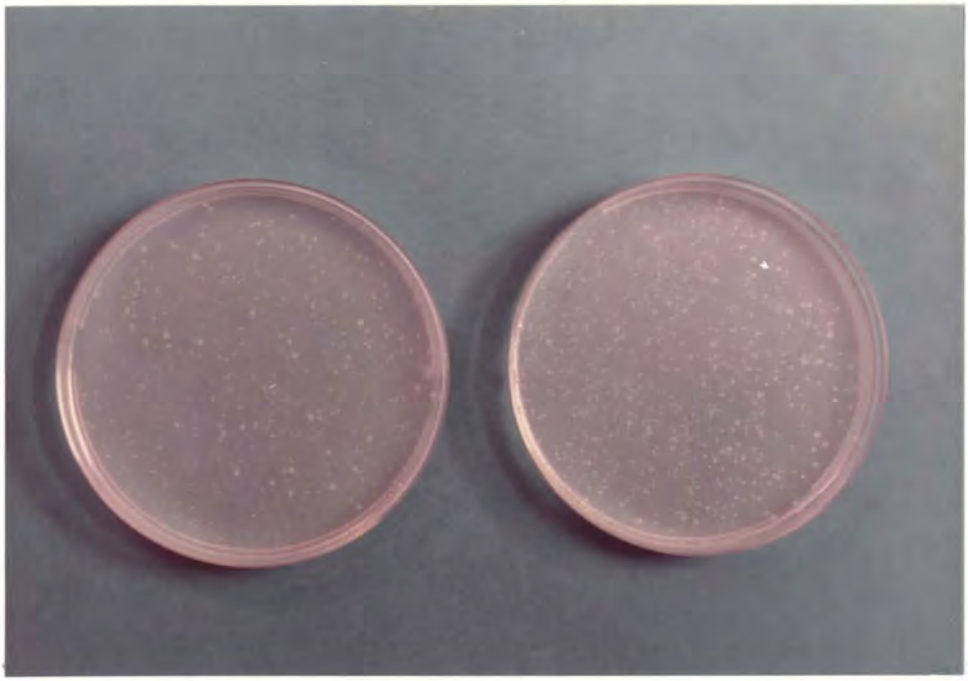


Figure 6.9 : Effect of Induction by PB on Activation of AFB_1 to mutagenically active metabolites, using *S.typhimurium* TA98.

Figure 6.9 shows the ten-fold increase in reversion rates (over controls) produced by S9 induced by PB. No significant differences were found among any of the groups which were induced by PB (tests 18, 20, 22, 24, 26 and 28).

The toxicity of AFB_1, like that of BP, depends on the balance between activation and detoxification reactions, specifically the relative rates of the epoxidation reaction giving rise to AFB_1-2,3-epoxide and on the subsequent conjugation with glutathione (Section 2.3) (267). However, unlike the metabolic activation of other polycyclic hydrocarbons, the epoxidation of AFB_1 is more definitively characterized as being dependent on a cyt P-450 isozyme which is induced by PB-type inducers (165,267).

Hence, the finding that induction by PB enhances the metabolic activation of AFB_1 is consistent with what is known about the metabolism of the mutagen. Also, since metabolic activation has been characterized as being preferentially induced by PB-type inducers, it may be justifiable to presume that the steroids are PB-type inducers, and that the enhancement of AFB_1 mutagenesis, by both PRG and E_2, reflects the induction of a cyt P-450-dependent mono-oxygenase (see also Chapter 7).

6.2.3.3 Dimethylnitrosamine

The effects of the different S9-pretreatments on the metabolic activation of DMN to an alkylating metabolite(s) (as detected by TA100) are shown in Table 6.4 and Figures 6.10 and 6.11.

DMN, like BP and AFB_1, was not detected as mutagenic with uninduced S9 (in either control groups) (tests 17 and 19).

S9 from E_2-pretreated rats, at either dose, did not cause any induction of the enzyme(s) responsible for the activation of DMN to metabolites mutagenic to TA100 (tests 21 and 23).

Table 6.4 : Results of TA100 / DMN Mutagenicity Assays

Tester Strain : TA 100 Mutagen : DMN Mutagen Dose : 1,48 mg S9 Concentration : 100 µℓ

Spontaneous Reversion Limits : 100 - 200

Data represent 3 plate counts (visible revertant colonies / plate), listed from smallest to largest count; count means, standard errors (S.E.), and 95% confidence intervals (C.I.) of means, the latter calculated from antilogs of transformed data. Also shown are the logarithmic transformations (base 10) of the original data.

Test	Plate			Means	S.E.	95% C.I.of Means		Log_{10} Transformations	
	A	B	C			Lower	Upper	Means	S.E.
	Revertant Colonies/Plate				±				±
01	124	129	135	129,33	3,18	116,31	143,65	2,111	0,011
02	124	130	132	128,67	2,40	118,60	139,48	2,109	0,008
03	120	130	139	129,67	5,49	107,82	155,38	2,112	0,019
04	119	127	130	125,33	3,28	111,76	140,35	2,098	0,012
05	124	129	137	130,00	3,79	114,66	147,13	2,114	0,013
06	119	128	139	128,67	5,78	105,85	155,74	2,109	0,020
07	122	128	132	127,33	2,91	115,32	140,44	2,105	0,010
08	120	127	129	125,33	2,73	113,97	137,69	2,098	0,010
09	119	128	130	125,67	3,38	111,69	141,19	2,099	0,012
10	119	128	136	127,67	4,91	107,97	150,52	2,105	0,017
11	119	130	136	128,33	4,98	108,24	151,71	2,108	0,017
12	126	128	134	129,33	2,40	119,43	139,99	2,112	0,008
13	116	125	132	124,33	4,63	105,68	145,88	2,094	0,016
14	134	135	135	134,67	0,33	133,23	136,11	2,129	0,001
15	129	134	139	134,00	2,89	122,07	146,96	2,127	0,009
16	116	128	137	127,00	6,08	102,92	155,99	2,103	0,021
17	119	138	139	132,00	6,51	105,90	163,72	2,120	0,022
18	283	310	339	310,67	16,17	247,57	387,70	2,491	0,023
19	117	129	140	128,67	6,64	102,66	160,40	2,108	0,023
20	297	343	352	330,67	17,03	262,66	414,00	2,518	0,023
21	118	138	143	133,00	7,64	102,83	170,88	2,122	0,026
22	274	312	331	305,67	16,76	239,66	387,44	2,484	0,024
23	112	126	133	123,67	6,17	99,15	153,43	2,091	0,022
24	291	307	312	303,33	6,33	276,95	331,97	2,482	0,009
25	237	253	264	251,33	7,84	219,38	287,41	2,400	0,014
26	271	298	324	297,67	15,30	237,79	370,68	2,473	0,022
27	281	295	306	294,00	7,23	264,24	326,74	2,468	0,011
28	337	379	380	365,33	14,17	307,61	432,51	2,562	0,017

Significance of F in One-way Analysis of Variance

Log_{10} Data : F-Prob = 0,0000

P Values for 2 Data Homoscedasticity Tests

1. Cochran's Test (Max.variance/sum of variances)

Raw Data : P = 0,286 (approx.)

Log_{10} Data : P = 1,000 (approx.)

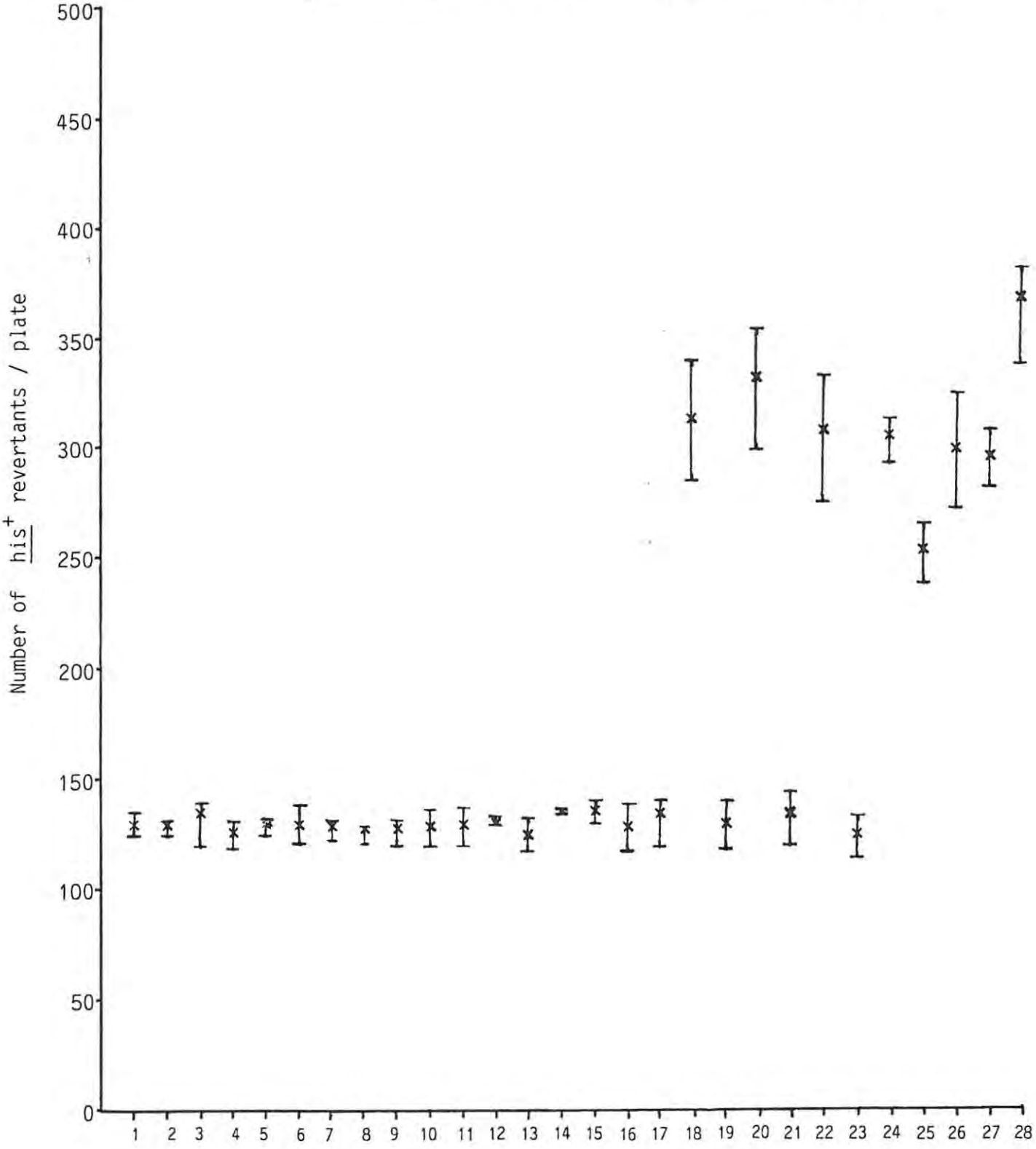
2. Bartlett-Box Test (Max.variance/Min.variance)

Raw Data : P = 0,022

Log_{10} Data : P = 0,868

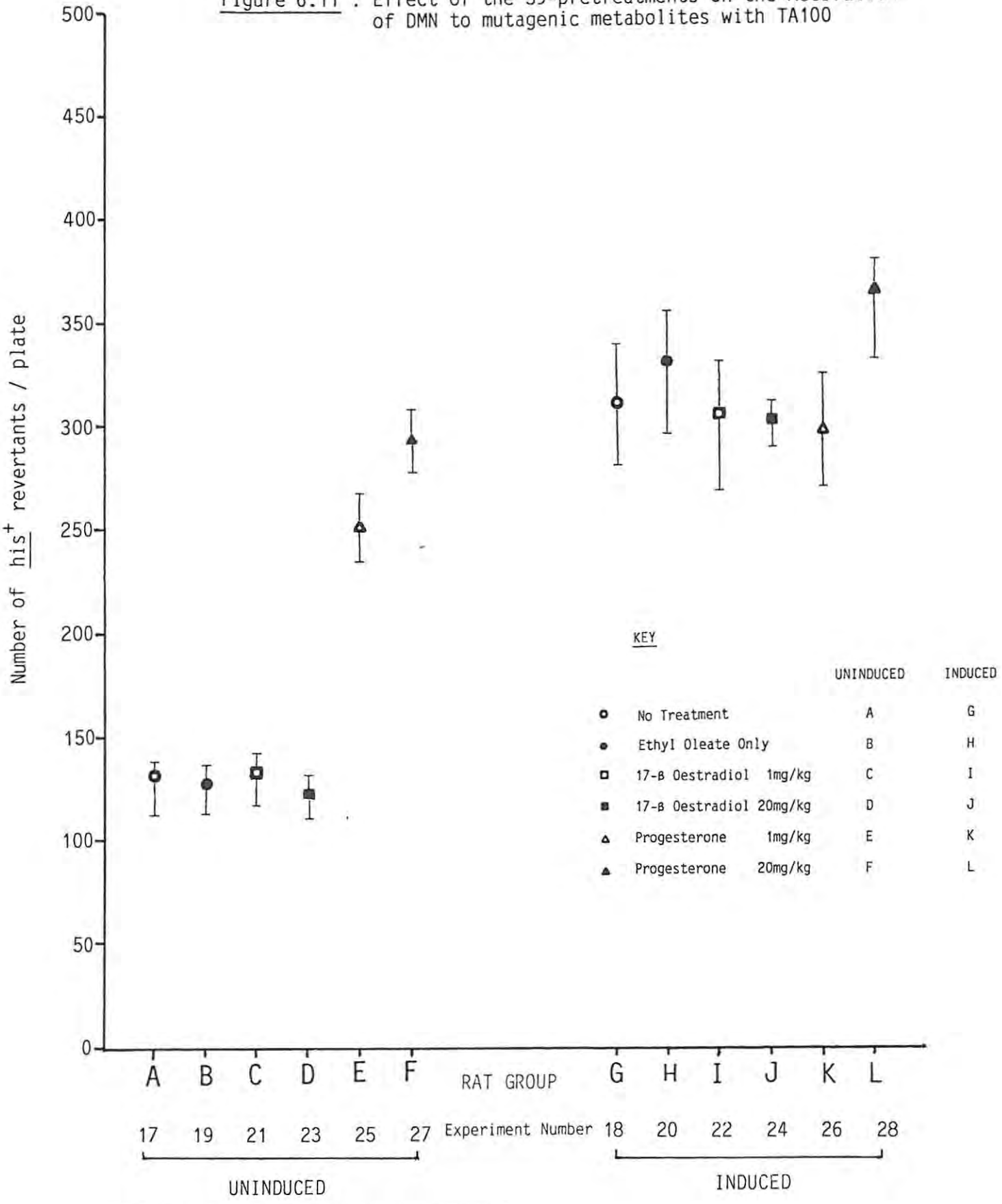
See also Figure 6.10 and 6.11

Figure 6.10 : Results of TA100/DMN Mutagenicity Assays



Data shows means and highest and lowest counts
For each observation, n = 3
S9 is from the pooled livers of 2 similarly treated rats
Spontaneous revertants not subtracted

Figure 6.11 : Effect of the S9-pretreatments on the Activation of DMN to mutagenic metabolites with TA100



Data shows means and highest and lowest counts

For each observation, n = 3

S9 is from the pooled livers of 2 similarly treated rats

Spontaneous revertants not subtracted

The administration of PRG at 1 mg/kg (test 25; S9:E) caused a two-fold increase in reversion rate over controls.

An even greater increase occurred when PRG was administered at 20 mg/kg (test 27; S9:F). In fact, the increase in reversion rate at the higher PRG level was not statistically dissimilar to the increase caused by the induction of S9 by PB.

As with the other mutagens tested, the induction of S9 with PB increased considerably the number of *his*$^+$ revertants/plate produced by DMN, and no other statistically significant differences were observed among the PB-induced groups (tests 18, 20, 22, 24, 26 and 28).

Two forms of DMN demethylase, controlled independently by the enzyme inducers 3MC and PB, are thought to exist (see Section 2.2).

Induction of the rat microsomal MFO system by either PB or 3MC has been reported to cause a two-fold increase in the activation of DMN to mutagenic metabolites (38,71,195). In fact, an increase of this magnitude was observed in this study, with S9 induced by PB using the tester strain TA100, which detects base-pair substitution and/or frameshift mutagens.

In a very similar pattern to that observed with AFB_1 activation, pretreatment with PRG produced a dose-dependent induction of DMN activation, with the magnitude of induction at the higher dose being of the same order as that achieved by induction by PB.

6.2.3.4 Mitomycin C

The results of the mutagenicity assays using MMC and TA102 are shown in Table 6.5 and Figures 6.12 and 6.13.

The inclusion of untreated S9 caused no change in the reversion rates observed when MMC was tested without S9 (test 18, 20, 22, 24, 26 and 28).

Table 6.5 : Results of TA102 / MMC Mutagenicity Assays

Tester Strain : TA 102 Mutagen : MMC Mutagen Dose : 0,25 µg S9 Concentration : 20 µl

Spontaneous Reversion Limits : 240 - 360

Data represent 3 plate counts (visible revertant colonies / plate), listed from smallest to largest count; count means, standard errors (S.E.), and 95% confidence intervals (C.I.) of means, the latter calculated from antilogs of transformed data. Also shown are the logarithmic transformations (base 10) of the original data.

Test	Plate			Means	S.E.	95% C.I.of Means		Log_{10} Transformations	
	A	B	C			Lower	Upper	Means	S.E.
	Revertant Colonies/Plate				±				±
01	175	203	250	209,33	21,88	132,71	323,22	2,316	0,045
02	182	192	227	200,33	13,64	149,76	265,58	2,300	0,029
03	171	183	197	183,67	7,51	153,78	218,63	2,263	0,018
04	179	231	239	216,33	18,81	144,94	317,76	2,332	0,040
05	173	184	235	197,33	19,10	130,77	292,48	2,291	0,041
06	211	216	273	233,33	19,89	162,67	330,14	2,365	0,036
07	171	213	248	210,67	22,26	130,92	331,28	2,319	0,047
08	177	233	245	218,33	20,95	139,96	333,89	2,335	0,044
09	166	179	218	187,67	15,62	131,43	264,42	2,271	0,035
10	194	257	266	239,00	22,65	153,96	363,92	2,374	0,043
11	174	182	252	202,67	24,77	120,95	330,14	2,301	0,051
12	184	189	205	192,67	6,33	167,34	221,36	2,284	0,014
13	168	179	202	183,00	10,02	144,61	230,25	2,261	0,024
14	182	191	260	211,00	24,64	128,80	336,90	2,319	0,049
15	167	180	223	190,00	16,92	129,87	273,72	2,275	0,038
16	408	478	507	464,33	29,38	349,78	611,22	2,665	0,028
17	334	387	463	394,67	37,44	260,56	587,22	2,592	0,041
18	1060	1130	1300	1163,33	71,26	894,33	1502,10	3,064	0,026
19	581	590	674	615,00	29,61	500,96	751,62	2,788	0,021
20	981	982	1020	994,33	12,84	940,81	1050,51	2,998	0,006
21	464	467	583	504,67	39,18	363,33	692,95	2,701	0,033
22	990	1130	1200	1106,67	61,73	863,97	1408,64	3,043	0,025
23	377	395	459	410,33	24,88	316,66	527,84	2,612	0,026
24	1050	1250	1350	1216,67	88,19	878,82	1666,10	3,083	0,032
25	382	420	437	413,00	16,26	347,30	489,55	2,615	0,017
26	913	960	1010	961,00	28,01	847,03	1088,43	2,982	0,013
27	460	512	540	504,00	23,44	410,50	616,03	2,702	0,021
28	990	1020	1250	1086,67	82,12	788,50	1481,15	3,034	0,032

Significance of F in One-way Analysis of Variance

Log_{10} Data : F-Prob = 0,0000

P Values for 2 Data Homoscedasticity Tests

1. Cochran's Test (Max.variance/sum of variances)

Raw Data : P = 0,036 (approx.)

Log_{10} Data : P = 1,000 (approx.)

2. Bartlett-Box Test (Max.variance/Min.variance)

Raw Data : P = 0,049

Log_{10} Data : P = 0,964

See also Figure 6.12 and 6.13

Figure 6.12 : Results of TA102/MMC Mutagenicity Assays

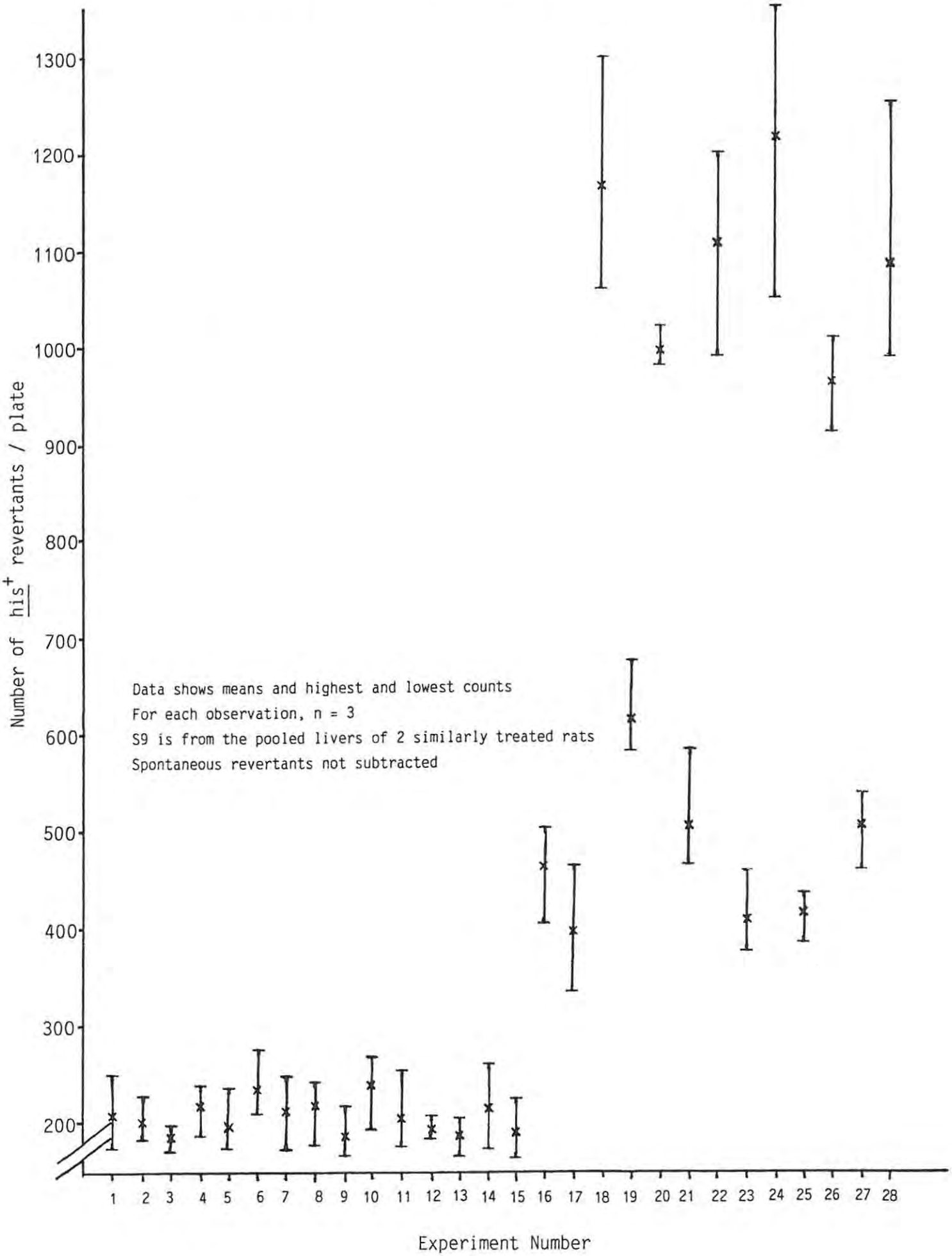
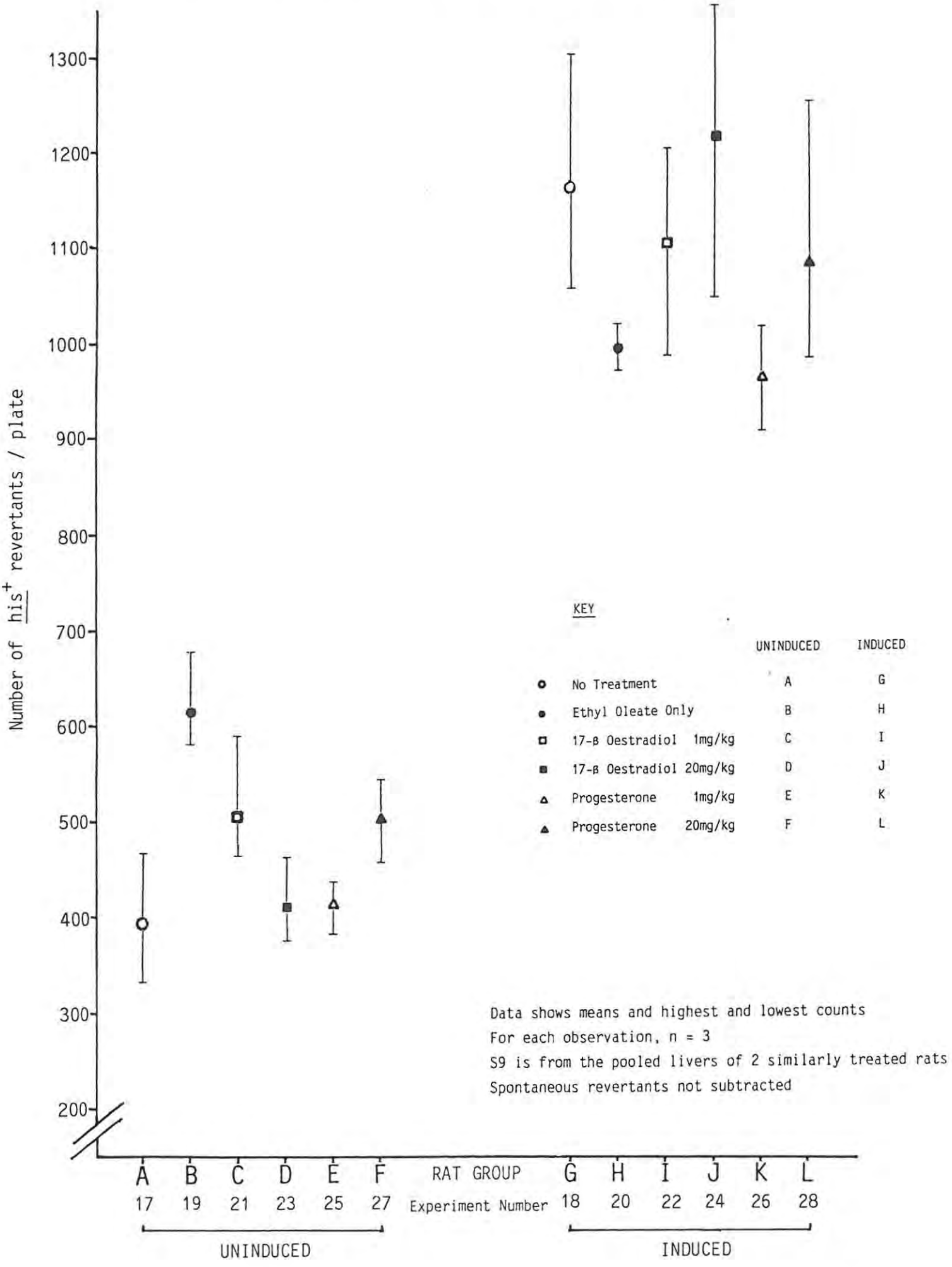


Figure 6.13 : Effect of the S9-pretreatments on the Activation of MMC to mutagenic metabolites with TA102



Significantly more *his*$^+$ revertants/plate were scored on plates which incorporated S9 from vehicle-injected rats (test 19; S9:B)than with untreated S9 (test 17; S9:A). This result is unusual when compared both with the results of similar tests with other strains and with the other steroid-pretreated groups with the same strain.

The administration of either dose of E_2 to rats caused no significant increase in reversion rates over the untreated S9 control groups (tests 21 and 23).

Although PRG administration at 20 mg/kg (test 27; S9:F) caused increased reversion over controls, this increase was not significant. Therefore, unlike the findings with the other three tester strains, neither dose of PRG (see also test 25) caused an increase in mutagenic activation.

Very high numbers of revertants/plate were observed when S9 induced with PB was incubated with MMC. As with the other mutagen/strain combinations, no significant differences were observed among the PB-induced groups (test 18, 20, 22, 24, 26 and 28).

The metabolism of MMC to mutagenically active species, described in Section 2.5, is essentially different to that of the other test mutagens.

Alkylating species are produced by NADPH-dependent enzymatic reduction of MMC, and DNA cleavage can result from the formation of free radicals, in cyclic redox processes (147,192). Although MMC was recommended as a positive control for TA102 in the 1983 'methods' paper of Maron and Ames (202), correspondence received since that publication has advised that MMC no longer be so utilized as it causes extragenic suppressor mutations (7,201). These mutations result in slow-growing pin-point colonies associated with both scoring difficulties and wide variations in the exposure time of the bacteria to the mutagen (7,201). Danthron, an anthracene quinone, is now recommended as a positive control requiring S9 activation (7,201).

It may be then that the somewhat unusual results observed with MMC and TA102 are related to the use of both difficult substrate and strain.

Even so, a similar result to those observed with the other tester strain/mutagen combinations was observed on induction by PB. Thus it would appear that an enzyme inducible by PB plays an important role in MMC activation. Although the inclusion of a microsomal S9 fraction was found to increase the mutagenic response several-fold, no details of the inducibility of the enzymes involved in MMC activation have been reported. The observed increase in the metabolic activation of MMC, by microsomes induced by PB, to mutagenic metabolites in this study cannot therefore be corroborated by the literature.

CHAPTER 7 : CONCLUSIONS

Species- and sex-related differences in the metabolism of promutagens may be related to heterogeneity of the structural or regulatory genes associated with the synthesis of metabolic enzymes. Alternatively, they may result from the influence of secondary factors, such as environmental or hormonal parameters, on the activity of these enzymes.

Because several of the natural and synthetic female sex-steroids (including components of the oral contraceptive formulations) have been implicated in the carcinogenic process, the elucidation of hormonal control of mutagen metabolism is considered to be of some importance.

This study has confirmed, in *Salmonella*, the reported nonmutagenicity of the steroids, but on the other hand it has also indicated that the steroids may influence the metabolism of mutagens. These findings support the hypothesis that the sex-steroids may act as 'promoters', rather than 'initiators' of tumours.

Specifically, the results of this study suggest that supraphysiological doses of PRG may cause a definite dose-related inductive effect on the activation of mutagens. With DMN, the degree of induction produced by PRG was as high as that produced by the classical inducer, PB.

Pretreatment with high levels of E_2 only induced the activation of one of the four test mutagens, AFB_1 (and this only at the higher dose), and, therefore, it appears that pretreatment with E_2 has little or no inductive effect on the metabolism of the other test mutagens.

The present results are in agreement with those of Bakshi *et al.* (31), who showed that pretreatment of mice with sex-steroids enhanced the *in vitro* mutagenic activation of DMN and 2-acetylaminofluorene in the Ames test. Both PRG and E_2, when administered at similar levels to those of this investigation, have also been reported to induce the activities of several of the mono-oxygenases of drug metabolism and to enhance the hepatic levels of cyt P-450 (Chapter 4).

A bacterial mutagenicity assay, coupled to a microsomal metabolic activation system, was used to monitor the formation of active metabolites. The rate of mutagenic activation, as measured by the reversion to prototrophy of histidine auxotrophes of *Salmonella*, was assumed to reflect the metabolic capacity of the liver in the intact animal.

The Ames test, employed in this manner, does not constitute a specific assay for any of the enzymes involved in mutagen activation. The hepatic microsomal fraction used in the assay contains the complex array of enzymes involved in both the activation and detoxification of mutagens, including the cyt(s) P-450-dependent mono-oxygenases, EH, and the conjugating enzymes of detoxification (267,281). Therefore, the assay measures the overall result, under the different conditions of each test, of the set of complex reactions involved in the activation and detoxification of each mutagen (13,267,281).

For the purposes of this investigation, this situation may represent a more faithful reproduction of the *in vivo* situation than would assays for specific enzymes, although these have great value in the characterization of the activities of the enzymes of mutagen metabolism (281).

It does follow however that the *Salmonella*/microsome test does not differentiate between the discrete cytochrome(s) species and will detect the induction of mono-oxygenases which are dependent on either cytochromes P-450 or cytochromes P-448.

The fact that the induction of the microsomal enzymes with PB was found to enhance the activation of all four test mutagens indicates that cytochrome P-450-dependent mono-oxygenases are important to the metabolism of each of the mutagens. In addition, no additive effects on the induction of mutagen metabolism were observed when steroid-pretreated rats were also induced by PB. These observations may indicate that the steroids induce the same cyt P-450-dependent isozymes which are inducible by PB.

Similar findings were noted by other investigators (135,231), who have reported that combined treatment with PB and the sex-steroids enhance the levels of both cyt P-450 and CcR in the liver of the rat to levels similar to those observed after pretreatment with PB alone.

Finally, in any appraisal of an *in vitro* investigation, such as the one described here, a number of factors must be considered :

First and foremost is the relevance of an *in vitro* assay which utilizes an homogenate of rat liver as an approximation of the *in vivo* metabolism of the intact organism (Section 1.5.3).

Also of considerable importance is the extent of the correlation between *in vitro* mutagenicity and the *in vivo* risk of carcinogenicity (Section 1.5.3). In particular, there have been reports that, while enzyme induction has enhanced the *in vitro* activation of mutagens, it has provided protection, *in vivo*, from the carcinogenic effects of these same compounds (Section 2.2).

Although this investigation confirms the reported inductive effects of high doses of steroids, the levels of administration were greater than those physiologically experienced by either the rat, or the human. This having been stated, it is possible that supra-physiological doses administered over a short period of time may effect similar changes qualitatively, if not quantitatively, as does protracted treatment with lower doses.

Although high doses of the female sex-steroids appear to have a specific regulatory action on the metabolism of mutagens, hasty conclusions should therefore not be drawn concerning any physiological regulation of carcinogenic risk by the endogenous steroids in animals exposed to environmental mutagens.

Further studies, into the effects of both physiological and supra-physiological levels of the sex-steroids, perhaps using the intact animal, may provide valuable clues to further the elucidation of hormonal regulation of mutagen metabolism.

Appendix 1 : Formulae

All reagents used were of analytical grade wherever possible, and all water was double-distilled. Where sterilization was by autoclaving, 20 min at 121°C and 15 psi was employed. Bottle-tops were loosened prior to autoclaving, and retightened on cooling. All media and solutions were stored in amber glass bottles at 4°C unless otherwise stated. All media plates were prepared one week before required (and stored at 4°C), during which time microbial contamination could manifest itself, and any spoiled plates be discarded. Formulae used in the mutagenicity assays were largely those of the revised 'methods' paper of Maron and Ames (202).

A. Rat Pretreatment and S9 Preparation

A.1 Steroid Pretreatment

	Per 20 mℓ	
	1 mg/kg (0,4 mg/mℓ)	20 mg/kg (8,0 mg/mℓ)
E_2 or PRG	8 mg	160 mg
ethyl oleate	to 20 mℓ	20 mℓ

Both steroids were dissolved in ethyl oleate, in the concentrations indicated, and distributed into 20 mℓ amber injection vials, then sealed with multidose closures, and sterilized by maintaining at 150°C for one hour (206). Control ethyl oleate injections were similarly prepared and sterilized.

B. Genotype Confirmations

B.1 Crystal Violet Sensitivity Discs

Small discs were punched from filter paper and autoclaved. These discs were impregnated with a sterile 0,1% (1 mg/mℓ) crystal violet solution.

B.2 Nutrient Agar Plates

	Per litre
Oxoid nutrient broth no. 2	25 g
Difco-Bacto agar	15 g
distilled water	to 1000 mℓ

All the ingredients were dissolved in the water in a 2ℓ flask, which was then autoclaved. When the agar was cooler, petri-plates were poured.

B.3 0,1 M Histidine Solution

	Per 10 mℓ
L-histidine (mol.mass = 209,6)	209,6 mg
distilled water	to 10,0 mℓ

Solution was prepared and sterilized by autoclaving.

B.4 0,5 mM Biotin Solution

	Per litre
D-biotin (mol.mass = 244,3)	122,15 mg
distilled water	to 1000 mℓ

Solution was prepared and sterilized by autoclaving.

C. Mutagenicity Assays

C.1 Nutrient Broth

	Per litre
Oxoid nutrient broth no. 2	25 g
distilled water	to 1000 mℓ

Solution was prepared and sterilized by autoclaving.

C.2 Soft Agar

		Per litre
Difco-Bacto agar		6 g
sodium chloride (NaCl)		5 g
distilled water	to	1000 mℓ

Ingredients were dissolved, and 100 mℓ aliquots were transferred to 250 mℓ screw-cap bottles, and then autoclaved.

C.3 Vogel-Bonner Medium E (50 x)

		Per litre
magnesium sulphate ($MgSO_4.7H_2O$)		10 g
citric acid monohydrate ($C_6H_8O_7.H_2O$)		100 g
dipotassium hydrogen phosphate (anhyd.) (K_2HPO_4)		500 g
sodium ammonium phosphate ($NaHNH_4(PO_4.4H_2O)$)		175 g
distilled water	to	1000 mℓ

The salts were added, in the order indicated, to some warm water in a 2 ℓ flask on a magnetic stirring hot plate. Each salt was allowed to dissolve completely before adding the next. After adjusting to volume, the solution was sterilized by autoclaving (292).

C.4 0,5 mM Histidine / Biotin Solution

		Per 250 mℓ
D-biotin (mol.mass = 244,3)		30,54 mg
L-histidine.HCl (mol.mass = 209,6)		26,20 mg
distilled water	to	250,0 mℓ

Solution was prepared and sterilized by autoclaving.

C.5 1,65 M Potassium Chloride/0,4 M Magnesium Chloride Solution

		Per 100 mℓ
potassium chloride (KCl)		12,30 g
magnesium chloride ($MgCl_2.6H_2O$)		8,14 g
distilled water	to	100,0 mℓ

Solution was prepared and sterilized by autoclaving.

C.6 0,2 M Sodium Phosphate Buffer (pH 7,4)

	Per 100 mℓ
0,2 M sodium dihydrogen phosphate ($NaH_2PO_4.H_2O$) (2,76 g/100 mℓ)	12 mℓ *
0,2 M disodium hydrogen phosphate (Na_2HPO_4) (2,84 g/100 mℓ)	88 mℓ *

* These are approximate values. The pH was adjusted to pH 7,4. The solution was sterilized by autoclaving.

C.7 0,1 M Nicotinamide Adenine Dinucleotide Phosphate Solution

		Per 5 mℓ
NADP (mol.mass = 853,5)		426,75 mg
sterile distilled water	to	5,00 mℓ

The solution was prepared and stored at -20°C.

C.8 1 M Glucose-6-Phosphate Solution

		Per 5 mℓ
D-glucose-6-phosphate (mol.mass = 282,1)		1,41 g
sterile distilled water	to	5,00 mℓ

Solution was prepared and stored at -20°C.

C.9 Minimal Glucose Agar Plates

	Per litre
Difco-Bacto agar	15 g
* sterile 50 x Vogel-Bonner E salts	20 mℓ
* sterile 40% glucose solution	50 mℓ
distilled water	930 mℓ

* These were autoclaved separately.

Agar was added to 930 mℓ distilled water in a 2 ℓ flask containing a magnetic stirring bar. The flask was autoclaved, and when the solution had cooled slightly, the 50 x V-B salts and then the 40% glucose solution were added. The solution was stirred thoroughly, and then 25 mℓ aliquots were poured into sterile petri-plates.

C.10 S9 Mix (Rat liver microsomal fraction and cofactors)

	Per 50 mℓ		
	Buffer only (no S9)	Standard S9 Mix (20 µℓ S9/plate) (4%)	High S9 Mix (100 µℓ S9/plate) (10%)
	mℓ	mℓ	mℓ
rat liver S9	N/A	2,00	10,00
$MgCl_2$-KCl salts	1,00	1,00	1,00
1 M glucose-6-phosphate	0,25	0,25	0,25
0,1 M NADP	2,00	2,00	2,00
0,2 M buffer pH 7,4	25,00	25,00	25,00
sterile distilled water	21,75	19,75	11,75

Ingredients were added in the reverse order indicated above, so that the liver was added to a buffered solution. Since thawed S9 preparations should not be refrozen (202), any remaining S9 or S9 mix was discarded.

C.11 The Mutagen Solutions

All mutagen solutions were prepared with the required amount of mutagen in 0,1 mℓ solution (40). All were prepared freshly and stored at 4°C in amber bottles.

a) AFB_1 - 1 µg/mℓ

i) Dilution I - 100 µg/mℓ

AFB_1 (mol.mass = 312,28)		1,00 mg
sterile DMSO	to	10,00 mℓ

ii) Dilution II - 1 µg/mℓ

dilution I		0,50 mℓ (equivalent to 50 µg)
sterile DMSO	to	50,0 mℓ

Contained 0,1 µg/0,1 mℓ.

b) DMN - 14,81 mg/mℓ

Reagent solution contained 1,01 g/mℓ.

DMN reagent		0,50 mℓ
sterile distilled water	to	34,10 mℓ

Contained 1,481 mg/0,1 mℓ.

c) BP - 5 µg/mℓ

i) Dilution I - 2,5 mg/mℓ

BP (mol.mass = 252,3)		25,0 mg
sterile DMSO	to	10,0 mℓ

ii) Dilution II - 5 µg/mℓ

dilution I		0,10 mℓ (equivalent to 0,25 mg)
sterile DMSO	to	50,00 mℓ

Contained 0,5 µg/0,1 mℓ.

d) MMC - 2,5 µg/mℓ

1 reagent vial was equivalent to 2 mg MMC (Mol.mass = 334,3)

MMC (1 vial)		2,00 mg
sterile distilled water	to	800,00 mℓ

Contained 0,25 µg/0,1 mℓ.

e) E_2 or PRG - 5 mg/mℓ

E_2 or PRG		5,00 mg
sterile DMSO	to	1,00 mℓ

Contained 500 µg/0,1 mℓ.

f) Sodium Phenobarbital - 50 mg/mℓ

sodium phenobarbital		500,00 mg
sterile distilled water	to	10,00 mℓ

Contained 5 mg/0,1 mℓ.

Appendix 2 : Statistics Programmes

1. Programme used to Generate Statistical Analysis, by SPSS, of the Rat Pretreatment Data.

```
FILE NAME       NORMRAT
COMMENT         EFFECTS OF STEROID PRETREATMENT AND INDUCTION ON RATS
                DUNCAN MULTIPLE RANGE TEST AT P=0,05
                SNK MULTIPLE RANGE TEST AT P=0,05
                RATGP REPRESENTS THE DIFFERENT RAT PRETREATMENT GROUPS
                Y = PERCENTAGE OF ORIGINAL MASS OR LIVER/SOMATIC INDEX
                NAME OF DATA FILE :_____________
VARIABLE LIST   RATGP  Y
INPUT FORMAT    FIXED (F2.0,1X,F6.2)
READ INPUT DATA
ONEWAY          Y BY RATGP (1,12)/
                RANGES=DUNCAN (.05)/
                RANGES=SNK/
STATISTICS      ALL
FINISH
```

2. Programme used to Generate Statistical Analysis, by SPSS, of the Mutagenicity Assay Data.

```
FILE NAME       LPLATES
COMMENT         AMES TEST DATA ANALYSIS
                LOG (BASE 10) DATA TRANSFORMATIONS USED
                DUNCAN MULTIPLE RANGE TEST AT P=0,05
                SNK MULTIPLE RANGE TEST AT P=0,05
                TESTGP IS THE EXPERIMENT NUMBER
                Y IS NO. OF REVERTANT S.TYPHIMURIUM COLONIES PER PLATE
                Z IS THE LOG (BASE 10) OF Y
                NAME OF DATA FILE :_____________
VARIABLE LIST   TESTGP  Y
INPUT FORMAT    FIXED (F2.0,1X,F4.0)
READ INPUT DATA
*COMPUTE        Z=LG10(Y)
ONEWAY          Y BY TESTGP (1,28)/
                RANGES=DUNCAN (.05)/
                RANGES=SNK/
STATISTICS      ALL
FINISH
```

Appendix 3 : Sources

A. Reagents

Merck (Darmstadt) :

1. Calcium chloride, dihydrate (2382) ($CaCl_2.2H_2O$)
2. Citric acid, monohydrate (242) ($C_6H_8O_7.H_2O$)
3. Dimethyl sulphoxide, spectrophotometric grade (2950) (C_2H_6OS)
4. Dipotassium hydrogen phosphate, anhydrous (5101) (K_2HPO_4)
5. Disodium hydrogen phosphate, anhydrous (6586) (Na_2HPO_4)
6. Magnesium chloride (5833) ($MgCl_2.6H_2O$)
7. Magnesium sulphate (5882) ($MgSO_4.7H_2O$)
8. Potassium chloride (4936) (KCl)
9. Potassium dihydrogen phosphate (4873) (KH_2PO_4)
10. Sodium ammonium phosphate (6682) ($NaNH_4HPO_4.4H_2O$)
11. Sodium dihydrogen phosphate, dihydrate (6343) ($NaH_2PO_4.2H_2O$)

Sigma (St.Louis, Mo.) :

1. Aflatoxin B_1 (A-6636) ($C_{17}H_{12}O_6$)
2. D-Biotin (B-4501) ($C_{10}H_{16}N_2O_3S$)
3. D-Glucose-6-phosphate, monosodium salt (G-7879) ($C_6H_{13}O_9P$)
4. Dimethylnitrosamine (N-7756) ($C_2H_6N_2O$)
5. Nicotinamide adenine dinucleotide phosphate (NADP) (N-0505) ($C_{21}H_{27}N_7O_{17}P_3Na$)
6. 17β-Oestradiol (E-8875) ($C_{18}H_{24}O_2$)
7. Progesterone (P-0130) ($C_{21}H_{30}O_2$)

Other :

1. Ampicillin and Tetracycline sensitivity discs - BBL, Maryland.
2. Benzo(a)pyrene ($C_{20}H_{12}$) - Nutritional Biochemical Corp., Cleveland, Ohio.
3. Crystal Violet - BDH reagents, Poole.
4. Difco-Bacto agar - Difco, Detroit, Michigan.
5. Ethyl oleate ($C_{20}H_{38}O_2$) - Hopkins & Wilkins, London.
6. D-Glucose, anhydrous ($C_6H_{12}O_6$) - BDH reagents, Poole.
7. L-Histidine.HCl, monohydrate ($C_6H_9N_3O_2.HCl.H_2O$) Calbiochem, San Diego, California.
8. Mitomycin C ($C_{15}H_{18}N_4O_5$) - Kyowa Hakko Kogya Co., Tokyo.
9. Oxoid Nutrient Broth no.2 - Oxoid, London.
10. Sodium phenobarbital ($C_{12}H_{11}N_2NaO_3$) - SA Druggists, Jhb., SA.

B. Equipment

1. FORMA Scientific Incubator Model 3028 - Forma Scientific, Ohio.
2. BRAUNSONIC 1510 Ultrasonicator, B. Braun, Melsungen.
3. MSE 18 Centrifuge, MSE, London.
4. UNICAM SP600 Series 2 Spectrophotometer, Unicam, Cambridge, UK.

LITERATURE CITED

1. Abul-Hajj, Y.J. (1978) *Steroids* **31(6)**, 841-847.
2. Aeschbaucher, H.U., Friederich, U. & Seiler, J.P. (1983) *Teratogenesis Carcinog.Mutagen.* **3(2)**, 195-203.
3. Agnese, G., Risso, D. & De Flora, S. (1984) *Mutat.Res.* **130(1)**, 27-44.
4. Al-Turk, W.A., Stohs, S.J. & Roche, E.B. (1980) *Drug Metab.Dispos.* **8**, 143-146.
5. Al-Turk, W.A., Stohs, S.J. & Roche, E.B. (1980) *Horm.Metab.Res.* **12(8)**, 404-409.
6. Alvares, A.P., Bickers, D.R. & Kappas, A. (1973) *Proc.Nat.Acad.Sci. USA* **70(5)**, 1321-1325.
7. Ames, B.N., personal communication.
8. Ames, B.N. (1972) in *Mutagenic Effects of Environmental Contaminants*, eds. Sutton, H.E. & Harris, M.I. (Academic Press, N.Y.), pp57-66.
9. Ames, B.N. (1976) *Science* **191**, 241-245.
10. Ames, B.N. (1977) *Fed.Proc.* **36(5)**, 1630.
11. Ames, B.N. (1979) *Science* **204**, 587-593.
12. Ames, B.N. (1982) in *Mutagens in our Environment*, eds. Sorsa, M. & Vainio, H. (A.R.Liss, N.Y.), pp3-19.
13. Ames, B.N., Durston, W.E., Yamasaki, E. & Lee, F.D. (1973) *Proc. Nat.Acad.Sci.USA* **70(8)**, 2281-2285.
14. Ames, B.N., Gurney, E.G., Miller, J.A. & Bartsch, H. (1972) *Proc. Nat.Acad.Sci.USA* **69(11)**, 3128-3132.
15. Ames, B.N. & Haroun, L. (1979) *Mutat.Res.* **62**, 393-395.
16. Ames, B.N. & Haroun, L. (1980) in *Microsomes, Drug Oxidations and Chemical Carcinogenesis* , eds. Coon, M.J., Conney, A.H., Estabrook, R.W., Gelboin, H.V., Gillette, J.R. & O'Brien, P.J., (Academic Press, N.Y.), Vol.2, pp1025-1040.
17. Ames, B.N. & Hooper, K. (1978) *Nature* **274(5666)**, 19-20.
18. Ames, B.N., Lee, F.D. & Durston, W.E. (1973) *Proc.Nat.Acad.Sci.USA* **70(3)**, 782-786.
19. Ames, B.N. & McCann, J. (1981) *Cancer Res.* **41**, 4192-4196.
20. Ames, B.N., McCann, J. & Yamasaki,E. (1975) *Mutat.Res.* **31(6)**, 347-364.

21. Ames, B.N., Sims, P. & Grover, P.L. (1972) *Science* **176(4030)**, 47-49.
22. Ames, B.N. & Yanofsky, C. (1971) in *Chemical Mutagens - Principles and Methods for Their Detection*, ed. Hollaender, A. (Plenum Press, N.Y.), Vol.1, pp267-282.
23. Anderson, D., Green, M.H.L., Mattern, I.E. & Godley, M.J. (1984) *Mutat.Res.* **130**, 1-10.
24. Anderson, K.E., Freddara, U. & Kappas, A. (1982) *Arch.Biochem. Biophys.* **217**, 597-608.
25. Anderson, K.E., Kappas, A., Conney, A.H., Bradlow, H.L. & Fishman, J. (1984) *J.Clin.Endocrinol.Metab.* **59(1)**, 103-107.
26. Arcos, J.C., Davies, D.L., Brown, Ch.E.L. & Argus, M.F. (1977) *Z. Krebsforsch.* **89**, 181-199.
27. Ashby, J. & Styles, J.A. (1978) *Nature* **271**, 452-455.
28. Ashby, J. & Styles, J.A. (1978) *Nature* **274**, 20-22.
29. Ashwood-Smith, M.J. (1980) *Mutat.Res.* **69**, 199-200.
30. Aufrère, M.B. & Benson, H. (1976) *J.Pharm.Sci.* **65**, 783-800.
31. Bakshi, K., Brusick, D., Bullock, L. & Bardin, C. (1977) in *Origins of Human Cancer*, eds. Hiatt, H.H., Watson, J.D., & Winsten, J.A. (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.), Bk.B, pp683-695.
32. Ball, P., Gelbke, H.P. & Knuppen, R. (1975) *J.Clin.Endocr.Metab.* **40**, 406-408.
33. Barber, E.D., Donish, W.H. & Mueller, K.R. (1983) *Mutat.Res.* **113(2)**, 89-101.
34. Baron, J.A. (1984) *Am.J.Epidemiol.* **119(1)**, 9-22.
35. Bartsch, H. (1983) *Ann.N.Y.Acad.Sci.* **407**, 351-361.
36. Bartsch, H., Camus, A. & Malaveille, C. (1976) *Mutat.Res.* **37**, 149-162.
37. Bartsch, H., Kuroki, T., Roberfroid, M. & Malaveille, C. (1982) in *Chemical Mutagens - Principles and Methods for Their Detection*, eds. De Serres, F.J. & Hollaender, A. (Plenum, N.Y.), Vol.7, pp95-161.
38. Bartsch, H., Malaveille, C. & Montesano, R. (1975) *Cancer Res.* **35**, 644-651.
39. Bartsch, H., Malaveille, C. & Montesano, R. (1976) in *Screening Tests in Chemical Carcinogenesis*, eds. Montesano, R., Bartsch, H., Tomatis, L. & Davis, W. (IARC publication no. 12, Lyons), pp467-491.

40. Belser, W.L., Shaffer, S.D., Bliss, R.D., Hynds, P.M., Yamamoto, L., Pitts, J.N. & Winer, J.A. (1981) *Environ.Mutagen.* **3**, 123-139.
41. Bernstein, L., Kaldor, J., McCann, J. & Pike, M.C. (1982) *Mutat.Res.* **97**, 267-281.
42. Bigger, C.A.H., Tomaszewski, J.E., Dipple, A. & Lake, R.S. (1980) *Science* **209**, 503-505.
43. Blackham, A. & Spencer, P.S.J. (1969) *Br.J.Pharmacol.* **37**, 129-139.
44. Bolt, H.M. (1979) *Clin.Pharmacol.Ther.* **4**, 155-181.
45. Booth, J. & Gillette, J.R. (1962) *J.Pharmacol.Exp.Ther.* **137**, 374-379.
46. Booth, J., Keysell, G.R. & Sims, P. (1974) *Biochem.Pharmacol.* **23**, 735-744.
47. Bos, R.P., Neis, J.M., van Gemert, P.J. & Henderson, P.T. (1983) *Mutat.Res.* **124(2)**, 103-112.
48. Bowman, W.C. & Rand, M.J. (1980) *Textbook of Pharmacology*, 2nd Edit., (Blackwell Scientific Publications, London), pp39.68-39.69.
49. Breuer, H. (1972) in *Methods in Steroid Toxicology*, eds. Plotz, E.J. & Haller, J. (Geron X, Los Altos, California), pp100-116.
50. Bridges, B.A. (1976) *Nature* **261**, 195-200.
51. Briggs, N.H. & Brotherton, J. (1970) *Steroid Biochemistry and Pharmacology* (Academic Press, London), pp52-138.
52. Brock, W.J. & Vore, M. (1982) *Drug Metab.Dispos.* **10(4)**, 336-343.
53. Brooks, S.C. (1976) in *Steroid Hormone Action and Cancer*, eds. Menon, K.M.J. & Reel, J.R. (Plenum Press, N.Y.), pp36-49.
54. Brusick, D.J. (1978) *Pharm.Technol.* **2**, 37-40, 65.
55. Brusick, D.J. (1983) *Ann.N.Y.Acad.Sci.* **406**, 68-73.
56. Brusick, D.J. (1983) *Ann.N.Y.Acad.Sci.* **407**, 164-176.
57. Bulger, W.H. & Kupfer, D. (1983) *Biochem.Pharmacol.* **32(6)**, 1005-1010.
58. Burke, M.D., Vadi, H., Jernström, B. & Orrenius, S. (1977) *J.Biol.Chem.* **252(18)**, 6424-6431.
59. Capdevila, J. & Orrenius, S. (1980) *FEBS letters* **119(1)**, 33-37.
60. Carlson, S.E., Mitchell, A.D., Carter, M.L. & Goldfarb, S. (1980) *Biochim.Biophys.Acta* **633(2)**, 154-161.
61. Carter, D.E., Goldman, J.M., Bressler, R., Huxtable, R.J., Christian, C.D. & Heine, M.W. (1974) *Clin.Pharmacol.Ther.* **15(1)**, 22-31.
62. Cheli, C., De Francesco, D., Petrullo, L.A., McCoy, E. & Rosenkranz, H.S. (1980) *Mutat.Res.* **74**, 145-150.
63. Colby, H.D. (1980) *Adv.Sex Horm.Res.* **4**, 27-71.

64. Commoner, B., Vithayathil, A.J. & Henry, J.I. (1974) *Nature* **249**, 850-852.
65. Commoner, B., Vithayathil, A.J., Henry, J.I., Gold, J.C. & Reding, M.J. (1976) *U.S. Environmental Protection Agency Report EPA-600 (1-76-022)*, (E.P.A.publication, Springfield, Va.).
66. Conney, A.H. (1967) *Pharmacol.Rev.* **19(3)**, 317-366.
67. Conney, A.H. (1971) in *Fundamentals of Drug Metabolism and Drug Distribution* eds. La Du, B.N., Mandel, H.G. & Way, E.L. (Williams & Wilkins, Baltimore), pp253-278.
68. Conney, A.H., Levin, W., Jacobson, M. & Kuntzman, R. (1973) *Clin. Pharmacol.Ther.* **14(4)**, 727-741.
69. Crawford, J.S. & Rudofsky, S. (1966) *Brit.J.Anaesth.* **38**, 446-454.
70. Crooke, S.T. (1979) in *Mitomycin C - Current Status and New Developments* eds. Carter, S.K., Crooke, S.T. & Alder, N.A. (Academic Press, N.Y.), pp1-4.
71. Czygan, H.G., Greim, H., Garro, A.J., Hutterer, F., Schaffner, F. Popper, H., Rosenthal, D. & Cooper, D.Y. (1973) *Cancer Res.* **33**, 2983-2986.
72. Davis, M., Simmons, C.J., Dordoni, B., Maxwell, J.D. & Williams, R. (1973) *J.Obstet.Gynaec.Brit.Cmwlth.* **80(8)**, 690-694.
73. Dean, M.E. & Stock, B.H. (1975) *Drug Metab.Dispos.* **3(5)**, 325-331.
74. De Flora, S., Zanacchi, P., Camoirano, A., Bennicelli, C. & Badolati, G.S. (1984) *Mutat.Res.* **133**, 161-198.
75. De Serres, F.J. (1972) in *Mutagenic Effects of Environmental Contaminants* eds. Sutton, H.E. & Harris, M.I. (Academic Press, N.Y.), pp41-55.
76. De Serres, F.J. & Shelby, M.D. (1979) *Science* **203**, 563-565.
77. De Serres, F.J. & Shelby, M.D. (1979) *Environ.Mutagen.* **1**, 87-92.
78. De Serres, F.J. & Shelby, M.D. (1979) *Mutat.Res.* **64**, 159-165.
79. Deveret, R. (1979) *Sci.Am.* **241(2)**, 40-49.
80. Drake, J.W. (1970) *The Molecular Basis of Mutation* (Holden Day, San Francisco), pp82-185.
81. Drake, J.W. & Baltz, R.H. (1976) *Ann.Rev.Biochem.* **45**, 11-38.
82. Duffel, M.W., Graham, J.M. & Ziegler, D.M. (1981) *Mol.Pharmacol.* **19**, 134-139.
83. Dunkel, V.C. (1979) *J.Assoc.Off.Anal.Chem.* **62**, 874-882.
84. Durston, W.E. & Ames, B.N. (1974) *Proc.Nat.Acad.Sci.USA* **71(3)**, 737-741.

85. Einarsson, K., Ericsson, J.L., Gustafsson, J-A., Sjövall, J. & Zietz, E. (1974) *Biochim.Biophys.Acta* **369**, 278-288.
86. Estabrook, R.W, Martinez-Zadillo, G., Young, S., Peterson, J.A. & McCarthy, J. (1975) *J.Steroid Biochem.* **6**, 419-425.
87. Feuer, G. & Kardish, R. (1975) *Int.J.Clin.Pharmacol.Biopharm.* **11(4)**, 366-374.
88. Feuer, G., Kardish, R. & Farkas, R. (1977) *Biochem.Pharmacol.* **26**, 1495-1499.
89. Feuer, G. & Liscio, A. (1969) *Nature* **223**, 68-70.
90. Feuer, G., Sosa-Lucero, J.C. & De La Iglesia, F.A. (1977) *Toxicology* **7**, 107-114.
91. Fishman, J., Hellman, L., Zumoff, B. & Gallagher, T.F. (1965) *J.Clin. Endocr.Metab.* **25**, 365-368.
92. Förlin, L. & Hansson, T. (1982) *J.Endocrinol.* **95(2)**, 245-252.
93. Fouts, J.R. & Rogers, L.A. (1965) *J.Pharmacol.Exp.Ther.* **147**, 112-117.
94. Franklin, T.J. & Snow, G.A. (1981) *Biochemistry of Antimicrobial Action* (Chapman & Hall, London), 95-98.
95. Freese, E. (1971) in *Chemical Mutagens - Principles and Methods for Their Detection*, ed. Hollaender, A. (Plenum Press, N.Y.), Vol.1, pp1-56.
96. Freudenthal, R.I. & Amerson, E. (1974) *Biochem.Pharmacol.* **23**, 2651-2656.
97. Friederich, U. & Würgler, F.E. (1983) *Teratogenesis Carcinog.Mutagen.* **3(2)**, 177-182.
98. Gallagher, T.F.(Jr.), Mueller, M.N. & Kappas, A. (1966) *Medicine* **45**, 471-479.
99. Garner, R.C. (1979) *Proc.R.Soc.Fond.(Biol.)* **205(1158)**, 121-134.
100. Garner, R.C., Miller, E.C. & Miller, J.A. (1972) *Cancer Res.* **32**, 2058-2066.
101. Gielen, J.E., Van Cantfort, J. & Kremers, P. (1976) *Arch.Toxicol.* **36(3-4)**, 255-266.
102. Gillette, J.R. (1963) *Prog.Drug Res.* **6**, 11-73.
103. Gillette, J.R. (1979) *Ann.N.Y.Acad.Sci.* **179**, 43-66.
104. Gletten, E., Weekes, U. & Brusick, D. (1975) *Mutat.Res.* **28**, 113-122.
105. Göggelmann, W., Grafe, A., Vollmar, J., Baumeister, M., Kramer, P.J. & Poole, B.L. (1983) *Teratogenesis Carcinog.Mutagen.* **3(2)**, 205-213.

106. Goodman, L.S. & Gillman, A. (eds.) (1975) *The Pharmacological Basis of Therapeutics*, 5th edit. (Macmillan,N.Y.), pp1423-1450.
107. Gower, D.B. (1974) *J.Steroid Biochem.* **5**, 501-523.
108. Gower, D.B. (1979) *Steroid Hormones*, (Croom Helm, London), pp1-40.
109. Gram, T.E. & Gillette, J.R. (1969) in *Metabolic Effects of Gonadal Hormones and Contraceptive Steroids*, eds. Salhanick, H.A., Kipnis, D.M. & Van de Wiele, R.L. (Plenum Press, N.Y.), pp86-94.
110. Gram, T.E., Guarino, A.M., Schroeder, D.H., Davis, D.C., Reagan, R.L. & Gillette, J.R. (1970) *J.Pharmacol.Exp.Ther.* **175(1)**, 12-20.
111. Green, M.H.L. (1981) in *Progress in Mutation Research*, ed. Kappas, A. (Elsevier/North-Holland, Amsterdam), Vol.2, pp159-165.
112. Greim , H.A. (1981) in *Concepts in Drug Metabolism : Part B*, eds. Jenner, P. & Testa, B. (Marcel Dekker, N.Y.), pp219-263.
113. Grover, P.L. (ed.) (1979) *Chemical Carcinogens and DNA* (CRC Press, Florida), Vols. 1 & 2.
114. Guengerich, F.P. (1979) *Clin.Pharmacol.Ther.* **6**, 99-121.
115. Guenthner, T., Jernström, B. & Orrenius, S. (1979)*Biochem.Biophys. Res.Commun.* **91(3)**, 842-848.
116. Guenthner, T., Jernström, B. & Orrenius, S. (1980) *Carcinogenesis* **1**, 407-418.
117. Guenthner, T. & Mannering, G. (1977) *Biochem.Pharmacol.* **26**, 567-575.
118. Guenthner, T. & Mannering, G. (1977) *Biochem.Pharmacol.* **26**, 577-584.
119. Guenthner, T. & Oesch, F. (1983) in *Drug Metabolism and Distribution*, ed. Lamble, J.W. (Elsevier Biomedical Press, Amsterdam), pp30-35.
120. Gumbmann, M. & Williams, S. (1970) *Biochem.Pharmacol.* **19**, 2861-2866.
121. Gustafsson, J-A., Carlstedt-Duke, J., Mode, A. & Rafter, J. (eds.) (1980) *Biochemistry, Biophysics and Regulation of Cytochrome P-450* (Elsevier/North-Holland Biomedical Press, Amsterdam), pp405-441.
122. Gustafsson, J-A. & Skett, P. (1979) *Acta Biol.Med.Ger.* **38(2-3)**, 307-320.
123. Gustafsson, J-A. & Stenberg, A. (1974) *Endocrinology* **95(3)**, 891-896.
124. Hamrick, M.E., Zampaglione, N.G., Stripp, B. & Gillette, J.R. (1973) *Biochem.Pharmacol.* **22**, 293-310.
125. Haroun, L. & Ames, B.N. (1981) in *Short Term Tests for Chemical Carcinogens*, eds. Stich, H.F. & San, R.H.C. (Springer-Verlag,Berlin), pp108-119.

126. Heath, E.C. & Dingell, J.V. (1974) *Drug Metab.Dispos.* **2(6)**, 556-565.

127. Heddle, J.A. & Bruce, W.R. (1977) in *Origins of Human Cancer*, eds. Hiatt, H.H., Watson, J.D. & Winsten, J.A. (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.), Bk.C, pp1549-1557.

128. Herbold, B.A. (1982) *Mutat.Res.* **97**, 191-192.

129. Herbold, B.A., Arni, P., Driesel, A., Engelhardt, G., Jager, J., Joosten, H.F., King, M.T., Klemp, H., Lang, R., Miltenburger, H.G., de Raat, W.K., Trager, H., Waalkens-Berendsen, D.H., Wallat, S. & Willems, M. (1983) *Teratogenesis Carcinog.Mutagen.* **3(2)**, 187-193.

130. Herbold, B. & Buselmaier, W. (1976) *Mutat.Res.* **40**, 73-84.

131. Hesse, S., Jernström, B., Martinez, M., Guenthner, T. & Orrenius, S. (1980) *Biochem.Biophys.Res.Commun.* **94(2)**, 612-617.

132. Holder, G., Yagi, H., Dansette, P., Jerina, D.M., Levin, W., Lu, A.Y.H. & Conney, A.H. (1974) *Proc.Nat.Acad.Sci.USA* **71(11)**,4356-4360.

133. Hollstein, M., McCann, J., Angelosanto, F.A. & Nichols, W.W. (1979) *Mutat.Res.* **65**, 133-226.

134. Horn, L., Kaldor, J. & McCann, J. (1983) *Mutat.Res.* **109(2)**, 131-141.

135. Ho Yuen, E. (1977) *Secondary Effects of Oral Contraceptives* (M.Sc. thesis, School of Pharmaceutical Sciences, Rhodes University).

136. Hsieh, D.P.H., Wong, J.J., Wong, Z.A., Michas, C. & Ruebner, B.H. (1977) in *Origins of Human Cancer*, eds. Hiatt, H.H., Watson, J.D. & Winsten, J.A. (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.), Bk.B, pp697-707.

137. Huberman, E., Sachs, L., Yang, S.K. & Gelboin, H.V. (1976) *Proc.Nat. Acad.Sci.USA* **73(2)**, 607-611.

138. *IARC Monograph on the Evaluation of Carcinogenic Risk of Chemicals to Humans* (1973) (IARC, Lyons) **Vol.3**, 91-131.

139. *IARC Monograph on the Evaluation of Carcinogenic Risk of Chemicals to Humans* (1974) (IARC, Lyons) **Vol.6**, 19-51, 99-115, 135-145.

140. *IARC Monograph on the Evaluation of Carcinogenic Risk of Chemicals to Humans* (1976) (IARC, Lyons) **Vol.10**, 51-71, 171-179.

141. *IARC Monograph on the Evaluation of Carcinogenic Risk of Chemicals to Humans* (1978) (IARC, Lyons) **Vol.17**, 125-135.

142. *IARC Monograph on the Evaluation of Carcinogenic Risk of Chemicals to Humans* (1979) (IARC, Lyons) **Vol.21**, 83-134, 279-326, 491-515.

143. *IARC Monograph on the Evaluation of Carcinogenic Risk of Chemicals to Humans* (1980) (IARC, Lyons), Supplement No.2.

144. Ingelman-Sunberg, M. (1983) in *Drug Metabolism and Distribution*, ed. Lamble, J.W. (Elsevier Biomedical Press, Amsterdam), pp22-29.
145. Ingerowski, G.H., Scheutwinkel-Reich, M. & Stan, H-J (1981) *Mutat. Res.* **91**, 93-98.
146. Isono, K. & Yourno, J. (1974) *Proc.Nat.Acad.Sci.USA* **71(5)**, 1612-1617.
147. Iyer, V.N. & Szybalski, W. (1964) *Science* **145**, 55-58.
148. Jacob, F. & Monod, J. (1961) *J.Molec.Biol.* **3**, 318-356.
149. Jerina, D.M. & Daly, J.W. (1974) *Science* **185(4151)**, 573-582.
150. Jernström, B., Guenthner, T., Hesse, S. & Orrenius, S. (1980) in *Carcinogenesis : Fundamental Mechanisms and Environmental Effects*, eds. Pullman, B., Ts'o, P.O.P. & Gelboin, H. (D.Reidel, Dordrecht), pp157-165.
151. Jernström, B., Orrenius, S., Undeman, O., Gräshund, A. & Ehrenberg, A. (1978) *Cancer Res.* **38**, 2600-2607.
152. Jori, A., Bianchetti, A. & Prestini, P.E. (1969) *Eur.J.Pharmacol.* **7**, 196-200.
153. Jori, A., Guiso, G. & Ravazzani, C. (1976) *J.Pharm.Pharmacol.* **28**, 714-716.
154. Juchau, M.R. & Fouts, J.R. (1966) *Biochem.Pharmacol.* **15**, 891-898.
155. Juchau, M.R., Namkung, M.J. & Chao, S.T. (1982) *Drug Metab.Dispos.* **10(3)**, 220-224.
156. Kado, N.Y. (1983) *Mutat.Res.* **121(1)**, 25-32.
157. Kahl, R. (1983) in *Drug Metabolism and Distribution*, ed. Lamble, J.W. (Elsevier Biomedical Press, Amsterdam), pp70-75.
158. Kamataki, T., Ando, M., Yamazoe, Y., Ishii, K. & Kato, R. (1979) *Biochem.Pharmacol.* **29**, 1015-1022.
159. Kapitulnik, J., Levin, W., Yagi, H., Jerina, D.M. & Conney, A.H. (1976) *Cancer Res.* **36**, 3625-3628.
160. Kappas, A., Anderson, K.E., Conney, A.H., Pantuck, E.J., Fishman, J. & Bradlow, H.L. (1983) *Proc.Nat.Acad.Sci.USA* **80(12)**, 7646-7649.
161. Kastenbaum, M.A. & Bowman, K.O. (1970) *Mutat.Res.* **9**, 527-549.
162. Kato, R. (1974) *Drug Metab.Rev.* **3(1)**, 1-32.
163. Kato, R., Chiesara, E. & Fromtino, G. (1962) *Biochem.Pharmacol.* **11**, 221-224.
164. Kato, R. & Onoda, K. (1970) *Biochem.Pharmacol.* **19(5)**, 1649-1660.
165. Kawajiri, K., Yonekawa, H., Gotoh, O., Watanabe, J., Igarashi, S. & Tagashira, Y. (1983) *Cancer Res.* **43**, 819-823.

166. Kier, L.D., Yamasaki, E. & Ames, B.N. (1974) *Proc.Nat.Acad.Sci.USA* **71(10)**, 4159-4163.

167. King, H., Thompson, M. & Brookes, P. (1976) *Int.J.Cancer* **18**, 339-344.

168. Kirk, R.E. (1968) *Experimental Design : Procedures for the Behavioural Sciences* (Brookes/Cole, Belmont, California).

169. Krahn, D.F. & Heidelburger, C. (1977) *Mutat.Res.* **46**, 27-44.

170. Kramer, R.E., Greiner, J.W., Rumbaugh, R.C., Sweeney, T.D. & Colby, H.D. (1979) *J.Pharmacol.Exp.Ther.* **208**, 19-23.

171. La Du, B.N., Mandel, H.G. & Way, E.L. (eds.) (1971) *Fundamentals of Drug Metabolism and Drug Disposition* (Williams & Wilkins, Baltimore).

172. Lake, B.G. & Gangolli, S.D. (1981) in *Concepts in Drug Metabolism*, eds. Jenner, P. & Testa, B. (Marcel Dekker, N.Y.), Part B, pp167-218.

173. Lake, L.M. & Gerschenson, L.E. (1983) *Cancer Lett.* **19(2)**, 173-179.

174. Lang, R. & Redmann, U. (1979) *Mutat.Res.* **67**, 361-365.

175. Lawley, P.D. (1979) *Nature* **282**, 134-135.

176. Lax, E.R., Baumann, P. & Schriefers, H. (1983) *Exp.Clin.Endocrinol.* **82(2)**, 145-152.

177. Lax, E.R., Ghraf, R. & Schriefers, H. (1978) *Acta Endocrinol.* **89(2)**, 352-358.

178. Lax, E.R., Kreuzfelder, E. & Schriefers, H. (1979) *Hoppe-Seylers Z. Physiol.Chem.* **360**, 1807-1812.

179. Lax, E.R., Kreuzfelder, E. & Schriefers, H. (1980) *Steroids* **36(5)**, 521-529.

180. Lax, E.R., Rumstadt, F., Plasczyk, H., Peetz, A. & Schriefers, H. (1983) *Endocrinology* **113(3)**, 1043-1055.

181. Lee, S. & Chen, C. (1971) *Steroids* **18(5)**, 565-575.

182. Legator, M.S. (1972) in *Mutagenic Effects of Environmental Contaminants*, eds. Sutton, H.E. & Harris, M.I. (Academic Press, N.Y.), 67-79.

183. Lemon, H.M. (1977) *Cancer* **40**, 1825-1832.

184. Levin, D.E., Hollstein, M., Christman, M.F. & Ames, B.N. (1984) *Methods Enzymol.* **105**, 249-254.

185. Levin, D.E., Hollstein, M., Christman, M.F., Schwiers, E.A. & Ames, B.N. (1982) *Proc.Nat.Acad.Sci.USA* **79(12)**, 7445-7449, including two addenda on storage conditions.

186. Levin, D.E., Yamasaki, E. & Ames, B.N. (1982) *Mutat.Res.* **94**, 315-330.

187. Levin, W., Welch, R.M. & Conney, A.H. (1968) *Endocrinol.* **83**, 149-156.

188. Levin, W., Wood, A.W., Yagi, H., Dansette, P., Jerina, D.M. & Conney, A.H. (1976) *Proc.Nat.Acad.Sci.USA* **73(1)**, 243-247.

189. Li, S.A., Lam, L.K.T. & Li, J.J. (1983) *Biochem.Pharmacol.* **32(19)**, 2847-2850.

190. Lijinsky, W., Loo, J. & Ross, A.E. (1968) *Nature* **218**, 1174-1175.

191. Lipsett, M.B. (1971) *Ann.N.Y.Acad.Sci.* **179**, 442-449.

192. Lown, J. (1979) in *Mitomycin C - Current Status and New Developments* eds. Carter, S.K., Crooke, S.T. & Alder, N.A. (Academic Press, N.Y.), pp5-26.

193. Lown, J.W. (1982) *Acc.Chem.Res.* **15**, 381-387.

194. Lu, A.Y., Somogyi, A., West, S., Kuntzman, R. & Conney, A.H. (1972) *Arch.Biochem.Biophys.* **152**, 457-462.

195. Magee, P.N. (1974) *Essays Biochem.* **10**, 105-136.

196. Malaveille, C., Planche, G. & Bartsch, H. (1977) *Chem.-Biol.Interact.* **17**, 129-136.

197. Malling, H.V. (1971) *Mutat.Res.* **13**, 425-429.

198. Malling, H.V. (1974) *Mutat.Res.* **26**, 465-472.

199. Margolin, B.H., Kaplan, N. & Zeiger, E. (1981) *Proc.Nat.Acad.Sci.USA* **78(6)**, 3779-3783.

200. Margolin, B.H., Risko, K.J., Shelby, M.D. & Zeiger, E. (1984) *Mutat. Res.* **130(1)**, 11-25.

201. Maron, D.M., personal communication.

202. Maron, D.M. & Ames, B.N. (1983) *Mutat.Res.* **113(3-4)**, 173-215.

203. Maron, D.M., Katzenellenbogen, J. & Ames, B.N. (1981) *Mutat.Res.* **88**, 343-350.

204. Marr, W., White, J.O., Elder, M.G. & Lim, L. (1980) *Biochem.J.* **190**, 17-23.

205. Marshall, W.J. & McLean, A.E.M (1969) *Biochem.Pharmacol.* **18**, 153-157.

206. *Martindale : The Extra Pharmacopoeia* (1977), 27th edition (The Pharmaceutical Press, London), pp1415-1424.

207. Matsushima, T., Sugimura, T., Nagao, M., Yahagi, T., Shirai, A. & Sawamura, M. (1980) in *Short-Term Test Systems for Detecting Carcinogens*, eds. Norpoth, K.H. & Garner, R.C. (Springer-Verlag, Berlin), pp273-285.

208. Maugh II, T.H. (1978) *Science* **201**, 1200-1205.

209. Mayne, R., Forsyth, I.A. & Barry, J.M. (1968) *J.Endocrinol.* **41**, 247-251.

210. Mazel, P. in *Fundamentals of Drug Metabolism and Drug Distribution*

eds. La Du, B.N., Mandel, H.G. & Way, E.L. (Williams & Wilkins, Baltimore), pp546-582.

211. McCann, J. (1976) *Chemtech.US* **6**, 682-687.
212. McCann, J. (1983) *Hospital Practice* **Sept**, 73-85.
213. McCann, J. & Ames, B.N. (1976) *Proc.Nat.Acad.Sci.USA* **73(3)**, 950-954.
214. McCann, J. & Ames, B.N. (1977) in *Origins of Human Cancer*, eds. Hiatt, H.H., Watson, J.D. & Winsten, J.A. (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.), Bk.C, pp1431-1450.
215. McCann, E., Choi, E., Yamasaki, E. & Ames, B.N. (1975) *Proc.Nat.Acad.Sci.USA* **72(12)**, 5135-5139.
216. McCann, J., Spingarn, N.E., Kobori, J. & Ames, B.N. (1975) *Proc.Nat.Acad.Sci.USA* **72(3)**, 979-983.
217. McLean, A.E.M. & Marshall, A. (1971) *Br.J.Exp.Pathol.* **52**, 322-329.
218. McCulloch, B., personal communication.
219. Meselson, M. & Russell, K. (1977) in *Origins of Human Cancer*, eds. Hiatt, H.H., Watson, J.D. & Winsten, J.A. (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.), Bk.C, pp1473-1482.
220. Miller, E.C. & Miller, J.A. (1966) *Pharmacol.Rev.* **18**, 805-838.
221. Miller, E.C. & Miller, J.A. (1971) in *Chemical Mutagens - Principles and Methods for Their Detection*, ed. Hollaender, A. (Plenum Press, N.Y.), Vol.1, pp 83-119.
222. Miller, J.A. (1970) *Cancer Res.* **30**, 559-576.
223. Miller, J.A. & Miller, E.C. (1977) in *Origins of Human Cancer*, eds. Hiatt, H.H., Watson, J.D. & Winsten, J.A. (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.), Bk.B, pp605-627.
224. Mitchell, I de G. (1978) *Mutat.Res.* **54**, 1-16.
225. Moore, D. & Felton, J.S. (1983) *Mutat.Res.* **119**, 95-102.
226. Neale, M.G. & Parke, D.V. (1973) *Biochem.Pharmacol.* **22**, 1451-1461.
227. Nebert, D.W., Bausserman, L.L. & Bates, R. (1970) *Int.J.Cancer* **6**, 470-480.
228. O'Malley, B.W. & Means, A.R. (1974) *Science* **183**, 610-620.
229. O'Malley, B.W. & Schrader, T.W. (1976) *Sci.Am.* **234(2)**, 32-43.
230. O'Malley, K., Stevenson, I. & Crooks, J. (1972) *Clin.Pharmacol.Ther.* **13**, 552-557.
231. Orrenius, S., Das, M. & Gnosspelius, Y. (1969) in *Microsomes and Drug Oxidations*, eds. Gillette, J.R., Conney, A.H., Cosmides, G.J., Estabrook, R.W., Fouts, J.R. & Mannering, G.J. (Academic Press,N.Y.), pp251-277.

232. Paine, A.J. (1981) *Essays Biochem.* **17**, 85-126.
233. Pasleau, F., Kremers, P. & Gielen, J.E. (1981) *Chem.-Biol.Interact.* **34(3)**, 279-286.
234. Pence, D.H. & Schnell, R.C. (1979) *Pharmacology* **18(1)**, 52-56.
235. Peraino, C., Fry, P.J.M. & Staffeldt, E. (1971) *Cancer Res.* **31**, 1506-1512.
236. Peto, R. (1977) in *Origins of Human Cancer*, eds. Hiatt, H.H., Watson, J.D. & Winsten, J.A. (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.), Bk.C, pp1403-1428.
237. Prival, M.J. (1983) *Ann.N.Y.Acad.Sci.* **406**, 154-163.
238. Prival, M.J., King, V.D. & Sheldon, A.T. (1979) *Environ.Mutagen.* **1**, 95-104.
239. Purchase, I.F.H. (1982) *Mutat.Res.* **99**, 53-71.
240. Purchase, I.F.H., Longstaff, E., Ashby, J., Styles, J.A., Anderson, D., Lefevre, P.A. & Westwood, F.R. (1976) *Nature* **264**, 624-627.
241. Purchase, I.F.H., Longstaff, E., Ashby, J., Styles, J.A., Anderson, D., Lefevre, P.A. & Westwood, F.R. (1978) *Br.J.Cancer* **37**, 873-959.
242. Quinn, G.P, Axelrod, J. & Brodie, B.B. (1958) *Biochem.Pharmacol.* **1**, 152-159.
243. Ramel, C. (1983) *Environ.Health Perspect.* **47**, 153-159.
244. Rao, G.S., Haueter, G., Rao, M.L. & Breuer, H. (1977) *Biochem.J.* **162(3)**, 545-556.
245. Rasmussen, R.E. & Wang, I.Y. (1974) *Cancer Res.* **34**, 2290-2295.
246. Reich, S.D. (1979) in *Mitomycin C - Current Status and New Developments*, eds. Carter, S.K., Crooke, S.T. & Alder, N.A. (Academic Press, N.Y.), pp243-250.
247. Remers, W.A. (1979) in *Mitomycin C - Current Status and New Developments*, eds. Carter, S.K., Crooke, S.T. & Alder, N.A. (Academic Press, N.Y.), pp27-32.
248. Remmer, H. (1969) *J.Mond.Pharm.* **3(12)**, 169-185.
249. Rinkus, S.J. & Legator, M.S. (1979) *Cancer Res.* **39**, 3289-3318.
250. Rinkus, S.J. & Legator, M.S. (1981) *Cancer Res.* **41**, 4196-4203.
251. Rubin, H. (1976) *Science* **191**, 241.
252. Rumbaugh, R.C. & Colby, H.D. (1980) *Endocrinology* **107(3)**, 719-724.
253. Rümke, C.L. & Noordhoek, J. (1969) *Eur.J.Pharmacol.* **6**, 163-168.
254. Russell, J.F.(Jr.) & Krahn, D.F. (1981) *Environ.Mutagen.* **3**, 352.

255. Sachan, D.S. (1976) *J.Nutr.* **106**, 1569-1576.

256. Sailer, D. (1978) *Fortschr.Med.* **96(9)**, 446-449.

257. Salmeen, I. & Durisin, A.M. (1981) *Mutat.Res.* **85**, 109-118.

258. Schenkman, J.B. & Gibson, G.G. (1983) in *Drug Metabolism and Distribution*, ed. Lamble, J.W. (Elsevier Biomedical Press, Amsterdam), pp7-11.

259. Scheutwinkel-Reich, M., Ingerowski, G.H. & Stan, H.-J. (1981) *Mutat. Res.* **85**, 265-266.

260. Seiler, J.P. (1983) *Teratogenesis Carcinog.Mutagen.* **3(2)**, 183-185.

261. Seiler, J.P. (1983) *Teratogenesis Carcinog.Mutagen.* **3(2)**, 215-217.

262. Selkirk,J.K. (1977) *Nature* **270**, 604-607.

263. Shelby, M.D. & Purchase, I.F.H. (1981) in *Evaluation of Short-Term Tests for Carcinogens*, eds. De Serres, F.J. & Ashby, J. (Elsevier /North-Holland, Amsterdam), Vol.1, pp16-20.

264. Sims, P. & Grover, P.L. (1974) *Adv.Cancer Res.* **20**, 165-274.

265. Slater, E.E., Anderson, M.D. & Rosenkranz, H.S. (1971) *Cancer Res.* **31**, 970-973.

266. Snee, R.D. & Irr, J.D. (1984) *Mutat.Res.* **128**, 115-125.

267. Söderkvist, P., Busk, L., Toftgard, R. & Gustafsson, J-A. (1983) *Chem.-Biol.Interact.* **46**, 151-163.

268. Solymoss, B., Varga, S. & Classen, H.G. (1970) *Eur.J.Pharmacol.* **10**, 127-130.

269. Solymoss, B., Werringloer, J. & Toth, S. (1971) *Steroids* **17**, 427-433.

270. Soyka, L.F. & Deckert, F.W. (1974) *Biochem.Pharmacol.* **23**, 1629-1639.

271. Soyka, L.F. & Long, R. (1972) *J.Pharmacol.Exp.Ther.* **182(2)**, 320-327.

272. Stanton, C.A., Riley, S. & Garner, R.C. (1984) *Mutat.Res.* **141**, 7-9.

273. Stark, A.A., Essigmann, J.M., Demain, A.L., Skopek, T.R. & Wogan, G.N. (1979) *Proc.Nat.Acad.Sci.USA* **76(3)**, 1343-1347.

274. Stead, A.G., Hasselblad, V., Creason, J.P. & Claxton, L. (1981) *Mutat.Res.* **85**, 13-27.

275. Stegeman, J.J., Pajor, A.M. & Thomas, P. (1982) *Biochem.Pharmacol.* **31(24)**, 3979-3989.

276. Stohs, S.J., Al-Turk, W.A. & Roche, E.B. (1980) in *Microsomes, Drug Oxidations and Chemical Carcinogenesis*, eds. Coon, M.J., Conney, A.H., Estabrook, R.W., Gelboin, H.V., Gillette, J.R. & O'Brien, P.J. (Academic Press, N.Y.), Vol.2, pp1239-1242.

277. Sugimura, T., Kawachi, T., Matsushima, T., Nagao, M., Sato, S. &

Yahagi, T. (1977) in *Progress in Genetic Toxicology*, eds. Scott, D., Bridges, B.A. & Sobels, F.H. (Elsevier/North-Holland, Amsterdam), pp125-140.

278. Sugimura, T., Nagao, M., Kawachi, T., Honda, M., Yahagi, T., Seino, Y., Sato, S., Matsukura, N., Matsushima, T., Shirai, A., Sawamura, M. & Matsumoto, H. (1977) in *Origins of Human Cancer*, eds. Hiatt, H.H., Watson, J.D. & Winsten, J.A. (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.), Bk.C, 1561-1577.
279. Sugimura, T., Yahagi, T., Nagao, M., Takeuchi, M., Kawachi, T., Hara, K., Yamasaki, E., Matsushima, T., Hashimoto, Y. & Okada, M. (1976) in *Screening Tests in Chemical Carcinogenesis*, eds. Montesano, R., Bartsch, H., Tomatis, L. & Davis, W. (IARC Scientific Publication no.12), pp81-101.
280. Tan, L., Rao, A.J. & Yu, P. (1982) *J.Steroid Biochem.* **17(1)**, 89-94.
281. Tauc, M., Hermann, M., Dansette, P.M. & Vandersteele, J.P. (1984) *Mutat.Res.* **125(2)**, 123-133.
282. Tephly, T.R. & Mannering, G.J. (1968) *Mol.Pharmacol.* **4**, 10-14.
283. Teschke, R. & Wiese, B. (1982) *J.Endocrinol.Invest.* **5(4)**, 243-250.
284. Tomatis, L., Agthe, C., Bartsch, H., Huff, J., Montesano, R., Saracci, R., Walker, E. & Wilborn, J. (1978) *Cancer Res.* **38**, 877-885.
285. Trosko, J.E. & Chu, E.H.Y. (1975) *Adv.Cancer Res.* **21**, 391-392.
286. Tüttenberg, K.H., Hüthwohl, B., Kahl, R. & Kahl, G.F. (1974) *Biochem. Pharmacol.* **23**, 2037-2043.
287. Vadi, H., Jernström, B. & Orrenius, S. (1976) in *Polynuclear Aromatic Hydrocarbons : Chemistry, Metabolism, and Carcinogenesis*, eds. Freudenthal, R.I. & Jones, P.W. (Raven Press, N.Y.), pp45-61.
288. Van den Berg, A., Noordhoek, J., Savenije-Chapel, E.M. & Koopman-Kool, E. (1978) *Biochem.Pharmacol.* **27**, 627-633.
289. Vesell, E.S., Lang, C.M., White, W.J., Passananti, G.T. & Tripp, S.L. (1973) *Science* **179**, 896-897.
290. Vodicnik, M.J., Franklin, R.B., Elcombe, C.R. & Lech, J.J. (1981) *Biochem.Pharmacol.* **30(10)**, 1091-1097.
291. Vodicnik, M.J. & Lech, J. (1983) *J.Steroid Biochem.* **18(3)**, 323-328.
292. Vogel, H.J. & Bonner, D.M. (1956) *J.Biol.Chem.* **218**, 97-106.
293. Vollmar, J. (1981) in *Progress in Mutation Research*, ed. Kappas, A. (Elsevier/North-Holland, Amsterdam), Vol.2, pp179-186.
294. Vore, M. & Soliven, E. (1979) *Drug Metab.Dispos.* **7(5)**, 247-251.

295. Watanabe, M., Tamura, Y. & Abe, T. (1980) *Toxicol.Lett.* **5(1)**, 55-60.
296. Watanabe, K. & Yoshizawa, I. (1983) *J.Pharmacobiodyn.* **6(6)**, 438-440.
297. Weinberger, M.J., Aten, R.F. & Eisenfeld, A.J. (1978) *Biochem.Pharmacol.* **27**, 2469-2474.
298. Weinstein, D. & Lewinson, T.M. (1978) *Mutat.Res.* **51**, 433-434.
299. Weisburger, J.H. (1980) *Ann.N.Y.Acad.Sci.* **349**, 228-231.
300. Welch, R.M., Levin, W. & Conney, A.H. (1968) *J.Pharmacol.Exp.Ther.* **160**, 171-178.
301. Williams, K., Inmon, J. & Lewtas, J. (1983) *Teratogenesis Carcinog. Mutagen.* **3(4)**, 367-376.
302. Williams, M.T. & Hendley, R. (1982) *Biochem.Biophys.Res.Commun.* **104(4)**, 1349-1355.
303. Wislocki, P.G., Wood, A.W., Chang, R.L., Levin, W., Yagi, H., Hernandez, O., Dansette, P.M., Jerina, D.M. & Conney, A.H. (1976) *Cancer Res.* **36**, 3350-3357.
304. Wong, J.J. & Hsieh, D. (1976) *Proc.Nat.Acad.Sci.USA* **73(7)**, 2241-2244.
305. Wood, A.W., Goode, R.L., Chang, R.L., Levin, W., Conney, A.H., Yagi, H., Dansette, P.M. & Jerina, D.M. (1975) *Proc.Nat.Acad.Sci.USA* **72(8)**, 3176-3180.
306. Wood, A.W., Wislocki, P.G., Chang, R.L., Levin, W., Lu, A.Y.H., Yagi, H., Hernandez, O., Jerina, D.M. & Conney, A.H. (1976) *Cancer Res.* **36**, 3358-3366.
307. Yahagi, T., Nagao, M., Seino, Y., Matsushima, T., Sugimura, T. & Okada, M. (1977) *Mutat.Res.* **48**, 121-130.
308. Yamasaki, E. & Ames, B.N. (1977) *Proc.Nat.Acad.Sci.USA* **74(8)**, 3555-3559.
309. Zeiger, E., Pagano, D.A. & Robertson, I.G.C. (1981) *Environ.Mutagen.* **3**, 205-209.
310. Zeigler, D.M., Duffel, M.W. & Poulsen, L.L. (1979) *Ciba Found.Symp.* **72**, 191-204.