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The antimutagenic and antioxidant potential of stercobilin and urobilin in the Ames Salmonella test

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3 LIST OF ABBREVIATIONS

1-NP	1-nitropyrene
2-AF	2-aminofluorene
2-NF	2-nitrofluorene
3-NFA	3-nitrofluoranthene
AFB1	aflatoxin B1
AMVN	2,2-azobis (2,4-dimethylvaleronitrile)
B[α]P	benzo[α]pyrene
BV	biliverdin
BRT	bilirubin ditaurate
CAT	catalase
DMSO	dimethylsulfoxide
DPPH	1,1-diphenyl-2-picrylhydradyl
GPx	glutathione-peroxidase
his^+	histidine independence
ID_{50}	bile pigment dose [µmol/plate] that inhibit 50% of the positive control mutagenicity
LDL	low density lipoprotein
NADP	nicotineamideadeninedinocleotide phosphate
NaN_3	sodium azide
OVNC	overnight culture

PETN	pentaerithrityl tetranitrate
Phip	2-amino-1-methyl-6-phenylimidazo [4, 5,-b] pyridine
(U) BR	(unconjugated) bilirubin
RONS	reactive oxygen and nitrogen species
ROS	reactive oxygen species
S9	S9-rat liver homogenate
SOD	superoxide dismutase
TA102	histidine auxotrophe *salmonella typhimurium* tester strain 102
TA98	histidine auxotrophe *salmonella typhimurium* tester strain 98
***t*-BuOOH**	*teriary*-butylhydroxyperoxide
TNFone	2, 4, 7- Trinitro-9-Fluorenone
UDP glucuronosyl transferase	uridinediphosphoglucuronate glucuronosyl transferase
UGT1A1	hepatic uridine diphosphate glucuronosyl transferase

4 INTRODUCTION

Bile pigments including bilirubin and biliverdin belong to the class of 'porphyrin' molecules. These coloured compounds are tetrapyrrolic, dicarboxylic acids that are produced in the body. An adult human produces approximately 300mg bilirubin per day which is the principal mammalian bile pigment and a catabolic end product of the degradation of heme [PETRYKA and HOWE, 1979]. Urobilinoids including urobilin and stercobilin are degradation products formed by bacterial reduction of bilirubin in the gastrointestinal tract. These compounds occur primarily in the stools of human beings and provide the colouration of faeces [VITEK et al., 2006].

For a long time, bilirubin has been associated with toxicity or illness. Furthermore, bile pigments have been considered as waste products of heme-catabolism leading to adverse effects if they accumulate in the body [BULMER et al., 2008].

Over the past 20 years, researchers have expressed their interest to the variety of endogenous antioxidant compounds that may prevent oxidative damage [TEMME, 2001]. A series of studies has been performed to explain the physiological relevance of bile pigments. In the mid 1900s, the beneficial effects of bilirubin were described for the first time in an *in vivo* study. It was found that bilirubin can provide antioxidant potential by protecting vitamin A from oxidation in the lumen [BERNHARD, 1954]. As mentioned in a recent publication, unconjugated bilirubin, biliverdin and bilirubin ditaurate possess antimutagenic and antioxidant effects. It was shown that these compounds inhibit the genotoxicity of different mutagens in *Salmonella* bacteria [BULMER et al., 2007]. Furthermore, numerous studies have demonstrated that the impact of heme oxygenase and slightly increased serum bilirubin concentrations may protect against oxidative stress induced diseases, such as atherosclerosis and cancer [VITEK and OSTROW, 2009].

So far, there are only a few published studies dealing with the beneficial effects of stercobilin and urobilin. From 1868 to 1871, researchers examined these two bile pigments in urine and feces which are formed by bacterial reduction of bilirubin [PETRYKA and HOWE, 1979].

In continuation of formerly published data on a variety of bile pigments it was the aim of the present study to investigate the potential antimutagenic and antioxidant properties of stercobilin and urobilin in the Ames *Salmonella* test. To my knowledge, these compounds have never been used before in this test model. The bile pigments were tested in the presence of two bacterial strains and different mutagens (TNFone, Phip, AFB1 and *t*-BuOOH) were used to provide the formation of mutant revertants. Mutagenicity assays were performed to ensure the non-mutagenic potential of stercobilin and urobilin.

The present study was part of the project: 'The physiological relevance of bile pigments - *In vitro* to *in vivo* evidence of antioxidant, anti- mutagenic and anti-carcinogenic potential and their mechanisms of action'. Principal investigator is A.o. Univ.-Prof. Dr. Mag. Karl-Heinz Wagner and his experimental working group 'Oxidative stress and DNA damage'.

The entire research included eight bile pigments. My workmate Gesa Ziesel and me investigated the modulatory effects of bilirubin dimethyl ester, biliverdin dimethyl ester, stercobilin and urobilin and split these up between the two of us. Mag. Christine Mölzer and Hedwig Huber studied the antimutagenic and antioxidant effects of bilirubin, biliverdin, bilirubin ditaurate and protoporphyrin under the same test conditions. The research activities were funded by the Austrian Science Fund (FWF, Vienna).

5 LITERATURE SURVEY

5.1 The Ames test

5.1.1 ***Main features of the test procedure***

The Ames *Salmonella* test is defined as a short-term bacterial reverse mutation assay. This assay is used all around the world to determine the mutagenic potential of new chemicals and drugs that can produce genetic damage that leads to gene mutations. Furthermore, the test is an important method for registration or acceptance of various chemicals, including drugs and biocides. It is very important to comply with formalities and international guidelines (e.g., Organisation for Economic Co-operation and Development (OECD); International Commission on Harmonization (ICH)) to ensure uniformity of the testing procedures [MORTELMANS and ZEIGER, 2000].

5.1.1.1 Historical aspects

The Ames *Salmonella*/microsome mutagenicity assay is named after its developer, Dr. Bruce Ames.

In 1966, Ames and Whitfield used a spot test procedure with several histidine mutant strains for screening chemically induced mutagenesis. In order to perform the spot test it is necessary to apply a small amount of the test chemical directly to the center of an agar plate. A concentration gradient is formed after diffusion of the chemical into the agar. Mutagenic chemicals can be detected by a ring of revertant colonies surrounding the area where the chemical was applied. Toxic effects of a chemical are characterized by a zone of growth inhibition [MORTELMANS and ZEIGER, 2000].

In 1973, Ames et al. established the plate incorporation assay. This assay procedure was performed in the present study to investigate the potential

antimutagenic and antioxidant effects of stercobilin and urobilin. The development of the plate incorporation assay was a major contributing factor to the success of the Ames test and replaced spot test or liquid suspension procedures. The plate incorporation assay is more sensitive and quantitative than the spot test. A further advantage is the easier and cheaper test performance [MORTELMANS and ZEIGER, 2000].

5.1.2 ***The Salmonella typhimurium tester strains***

The Ames test uses a number of different *Salmonella typhimurium* strains which are histidine dependent and contain some type of mutation in the histidine operon. The bacterial strains are not able to grow on a minimal glucose agar plate unless the cultures are supplemented with a trace amount of histidine. That implies that only those bacteria that revert to histidine independence (his^+) are able to form colonies [BARILE, 1994].

5.1.2.1 Genotypes of the bacterial strains

In the present study bile pigments were tested in the presence of two bacterial strains, *TA98* and *TA102*. As mentioned above, all tester strains are histidine dependent by virtue of a mutation in the histidine operon.

The mutations are of several types (*e.g.*, base substitutions, frameshifts) and each tester strain carries a known type of mutation.

TA98 carries the *his*D3052 mutation and *TA102* contains the *his*G428 mutation. The *his*D3052 mutation is defined as a -1 frameshift mutation and influences the reading frame of a nearby repetitive –C–G–C–G–C–G –C-G- sequence. Frameshift mutagens, for example 2-nitrofluorenone, are necessary for the reversion of the *his*D3052 allele [ISONO and YOURNO, 1974].

Strain *TA102* which carries the *his*G428 mutation contains A·T base pairs at the site of mutation in contrast to the other *Salmonella* strains that detect mutagens damaging G·C base pairs. Furthermore, *TA102* detects a number of oxidative mutagens, for example hydroperoxides. *His*G428 mutation is an ochre mutation that can be reverted by all six possible base-pair changes [LEVIN et al., 1982].

The plasmid pKM101 is present in both bacterial tester strains and carries an ampicillin resistance gene. The R factor plasmid improves the error-prone DNA repair. Additional introduction of the multi-copy plasmid pAQ1 in strain *TA102* contains a tetracycline resistance gene, which is useful for detecting the presence of the plasmid [MORTELMANS and ZEIGER, 2000].

Moreover, strain *TA98* and *TA102* contain the rfa-mutation which affects the characteristics of the bacterial cell wall by inducing a partial loss of the lipopolysaccharide barrier. The result is an increased permeability of the cells [MORTELMANS and ZEIGER, 2000].

Strain *TA98* includes the *uvrB* deletion mutation which appears as a repair pathway for DNA damage and eliminates the exact excision repair. As a result, more DNA lesions can be repaired by the error-prone DNA repair mechanism. This type of shift is not performed in *TA102* [AMES et al., 1973].

Summing up, it can be said that *TA98* is a kind of a frameshift sensitive strain that supplies information on frameshift mutations. The tester strain *TA102* can be described as a base-pair substitution-strain which detects DNA cross-linking damage and is sensitive to mutagens causing oxidative damage [MARON and AMES, 1983]. Before starting the test series the strains were checked for genetic integrity.

5.1.3 **Metabolic activation systems**

Some classes of chemicals are not self-acting and therefore require metabolic activation to be biologically active. A distinction is made between oxidative- and reductive enzyme systems [MORTELMANS and ZEIGER, 2000].

5.1.3.1 Oxidative metabolism

Several carcinogenic chemicals, such aromatic amines or polycyclic aromatic amines have to be activated by a metabolic activation system; otherwise they would be biologically ineffective. Humans and lower animals possess the cytochrome-based P450 system which is present primarily in the liver. This metabolic oxidation system is able to metabolize various chemicals into DNA-reactive, electrophilic forms. A relevant disadvantage of the *Salmonella typhimurium* strains and bacterial test systems in general, is their lack of metabolic abilities, including the cytochrome-based P450 metabolic oxidation system. This fact has led to the use of an exogenous mammalian organ activation system which consists of a 9000xg supernatal fraction of a rat liver homogenate [MARON and AMES, 1983].

5.1.3.2 Reductive metabolism

A reductive enzyme system can be used for the metabolic activation of chemicals containing azo and diazo bonds. A reduction of such chemicals can be obtained in the anaerobic intestinal microflora, in the intestinal wall and in the liver of mammals, including man. Two different types of reductive *in vitro* systems are commonly in use. Type 1 is based on a liver homogenate supplemented with FMN [PRIVAL et al., 1984]. Type 2 is based on rat intestinal microflora preparations [REID et al., 1984].

5.1.4 ***Positive and negative controls***

Each experiment in the present study included solvent controls and diagnostic positive controls specific for each strain and for the metabolic activation system [MORTELMANS and ZEIGER, 2000].

5.1.4.1 Positive control chemicals

Diagnostic mutagens are used in the Ames test to ensure the efficacy of the S9 rat liver homogenate and the reversion properties of the tester strains. The concentration of the positive control should be calculated for each new S9-charge, because each batch of metabolic activation is subject to fluctuations [MORTELSMANS and ZEIGER, 2000].

5.1.4.2 Negative control chemicals

Each tester strain has a relatively constant number of spontaneous revertant colonies. Day-to-day and laboratory-to-laboratory variations usually arise. The spontaneous mutant frequency can also be influenced by the utilization of S9. For instance, the spontaneous revertant values of strain *TA102* will increase in the presence of metabolic activation because the strain is highly sensitive to the S9 addition [MORTELMANS and ZEIGER, 2000].

Strain	Number of revertants	
	without S9	with S9
TA97	**75-200**	**100-200**
TA98	**20-50**	**20-50**
TA100	**75-200**	**75-200**
TA102	**100-300**	**200-400**
TA104	**200-300**	**300-400**
TA1535	**5-20**	**5-20**
TA1537	**5-20**	**5-20**
TA1538	**5-20**	**5-20**

Table 1: Ranges of spontaneous revertant colonies in the Ames *Salmonella* test [modified: MORTELMANS and ZEIGER, 2000]

5.2 Bile pigments – History, Chemistry, Metabolism

5.2.1 ***Historical background***

Over the years the opinion about the physiological importance of bile pigments has considerably changed. These coloured compounds were used in the traditional Chinese medicine for thousands of years to prevent and heal different diseases like epilepsy, convulsions and tetany. The treatment particularly involved the oral supplementation of gall stones and the salts of bilirubin. Furthermore, a regular administration should imply life prolonging properties [READ, 1976]. In the modern medicine the beneficial properties of bile pigments were ignored for a long time. Especially bilirubin was described as a useless by-product of heme catabolism that can be toxic [BULMER et al., 2008].

The origin of scientific studies dates back to the 19th century. In that time, the term ‘bilirubin’ was established [STADELER, 1864]. Fischer et al. classified all potential isomers of protoporphyrin and the mesoporphyrins in fifteen groups [FISCHER and ORTH, 1934].

The history of urobilinoids began in 1868. At this time, Jaffe discovered the bile pigment urobilin in urine and feces. Urobilin combined with zinc salts showed a strong green fluorescence [JAFFE, 1868]. In 1871, stercobilin was identified in feces [LAIR and MASIUS, 1871]. At this time, the formation of urobilinoids by heme degradation was still unknown.

More than forty years later, the identity of these two pigments was proven. Further research was done in this field resulting in very important discoveries. It was established that stercobilin and urobilin were formed by bacterial reduction of bilirubin. The formation of both pigments via bacterial reduction was introduced by Maly and later confirmed by other researchers [WATSON et al., 1969].

5.2.2 ***General chemical aspects***

The term ‘bile pigment’ describes all linear open-chain tetrapyrroles [MC DONAGH, 1979]. Tetrapyrroles possess many different functions, such as light harvesting pigments in higher plants, energy generation in bacterial and eukaryotic cells, antioxidant effects, generation of ROS and cell signaling in eukaryotic cells [VITEK and OSTROW, 2009].

The endogenous pigments, unconjugated and conjugated bilirubin, belong to this group. Additionally, bile pigments include biliverdin and the urobilinoids, stercobilin and urobilin. These pigments are tetrapyrrolic, dicarboxylic acids that belong to the porphyrin family of molecules. These coloured compounds possess two propionic acid side chains and are formed in the human body

[BULMER et al., 2008]. The unique chemical structure of the porphyrins is responsible for their biochemical and genetic features.

Chlorophylls, hemoglobins and heme-containing enzymes are classified as porphyrin derivatives that include metalloporphyrins as the active part [ODIN, 1997]. All bile pigments contain two free or conjugated propionate groups and several double bonds which are susceptible to oxidation. Therefore, these pigments can serve as antioxidants by transferring their free electrons to other unstable chemicals. The solubility of bilirubin in water and in most lipid solvents is poor. Bilirubin dissolves best in hydrogen bond-breaking solvents, for example DMSO [OSTROW and CELIC, 1984].

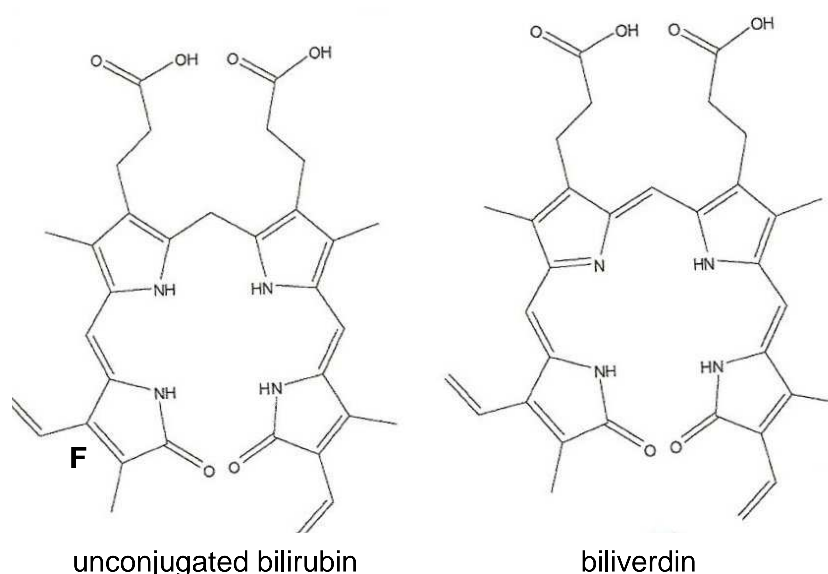
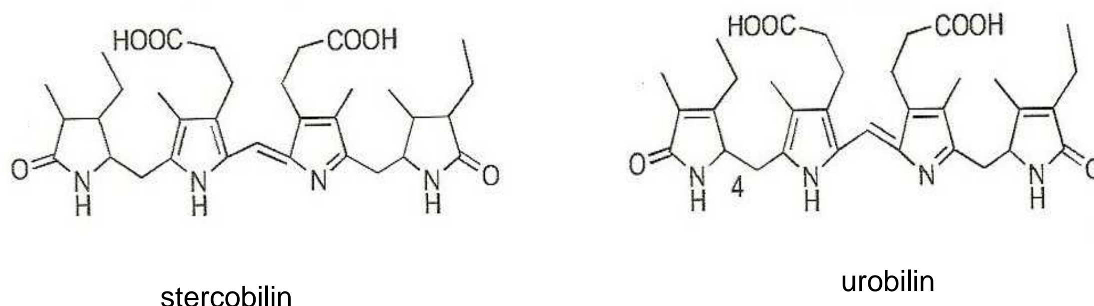


Figure 1: Two dimensional structure of bilirubin and biliverdin [BULMER et al., 2008]



stercobilin

urobilin

Figure 2: Chemical structures of stercobilin and urobilin [VITEK et al., 2006]

5.2.3 Formation of bile pigments

Bile pigments are formed in humans via the degradation of heme which acts as prosthetic group for several hemoproteins (*e.g.* hemoglobin, myoglobin, cytochrome P-450 and peroxidase) [STOCKER, 2004].

All red blood cells have a limited life span of approximately 120 days. After that time, they are degraded by the reticuloendothelial system. The phagocytosis takes place in spleen, liver and bone marrow and provides approximately 80% of heme. The remaining 20% originate from the catabolism of other heme proteins. This pathway is the initial step in the formation of the pigments [SCHMID and MCDONAGH, 1975].

The first step of heme catabolism is initiated by heme oxygenase-1 which requires NADPH and molecular oxygen. This enzyme converts heme to biliverdin, carbon monoxide and iron by cleaving the heme ring. Heme oxygenase-1 also includes biliverdin reductase which reduces the blue-green pigment biliverdin to the yellow pigment bilirubin [STOCKER, 2004].

As mentioned above, bilirubin is insoluble in water at physiological pH. Therefore, bilirubin is present as a complex with albumin in the blood circulation. In this form bilirubin is called unconjugated or indirect bilirubin [STOCKER et al., 1987].

Bilirubin dissociates from its carrier protein and is taken up from blood into hepatocytes. In the liver, unconjugated bilirubin is conjugated with glucuronic acid by the enzyme UDP glucuronosyl transferase (UGT1A1). In this form the bilirubin is called conjugated or direct bilirubin. Bilirubin diglucuronide is then transported into the bile caniculi and thence into the bile. Finally, the conjugated bilirubin reaches the duodenum via the bile duct. There it is hydrolyzed to hydrogenated derivatives of bilirubin and finally to urobilinoids by bacterial enzymes including β-glucuronidase [STOCKER, 2004].

The major portions of urobilinoids are excreted into the stool and provide the colouration of feces [STOCKER, 2004]. It is established that unconjugated bilirubin is reduced to colorless urobilinoids in the gastrointestinal tract by intestinal microflora. The most important compounds are urobilinogen and stercobilinogen as well as their oxidation products, urobilin and stercobilin [VITEK et al., 2006].

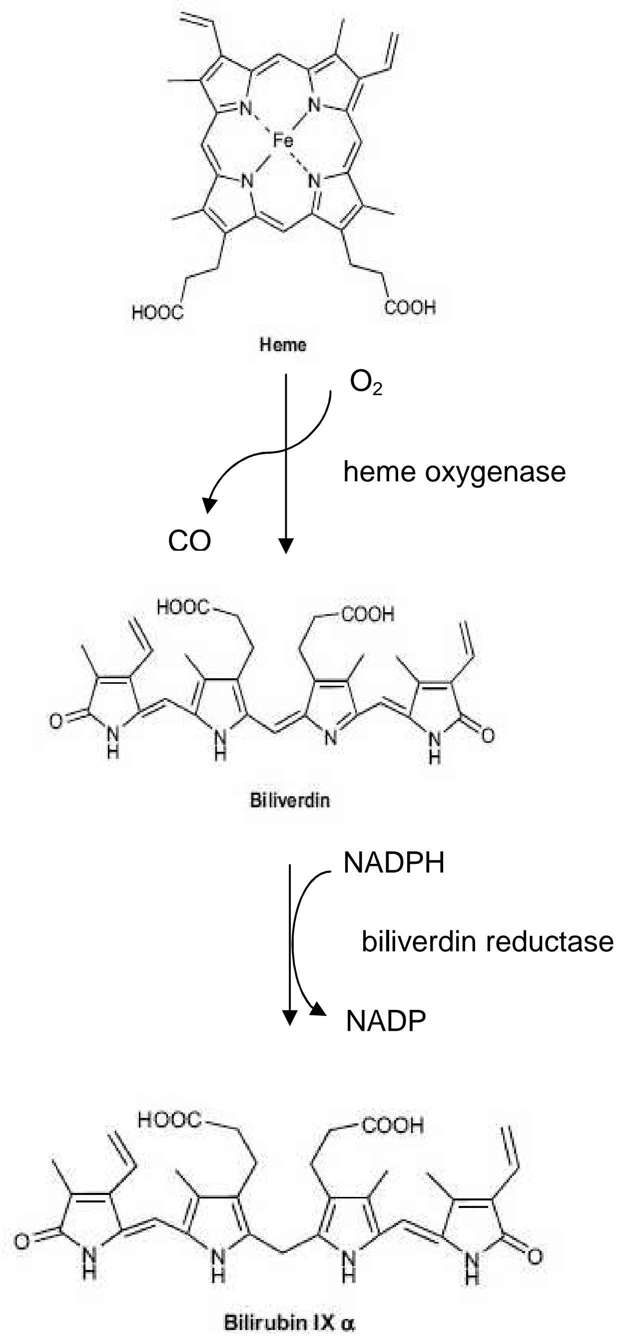


Figure 3: Heme degradation from biliverdin to bilirubin [modified: VITEK and OSTROW, 2009]

Approximately 10-15% of the urobilinoids reach the enterohepatic circulation which is characterized by reabsorption by hepatocytes, transport through the portal vein back to the liver and re-excretion via the bile. A minor portion (1.0-3.5mg urobilinoids per day) is eliminated from the body in the urine as water-soluble compounds via the kidneys [KUNTZ and KUNTZ, 2008]. The reduction of unconjugated bilirubin to urobilinoids is very efficient; therefore only 5-20mg bilirubin per day can be found in stools of humans while the excretion of urobilinoids range from 50-250mg per day [WITH, 1968, VITEK et al., 2006].

The involvement of specific bacteria in the reduction of unconjugated bilirubin to urobilinoids is poorly described in the literature. *Clostridium perfringens*, *Clostridium ramosum*, *Clostridium difficile* and *Bacteroides fragilis* are a few microbial species which are uncontroversially involved in the reduction [VITEK et al., 2000, GUSTAFSSON and LANKE, 1960, FAHMY et al., 1972].

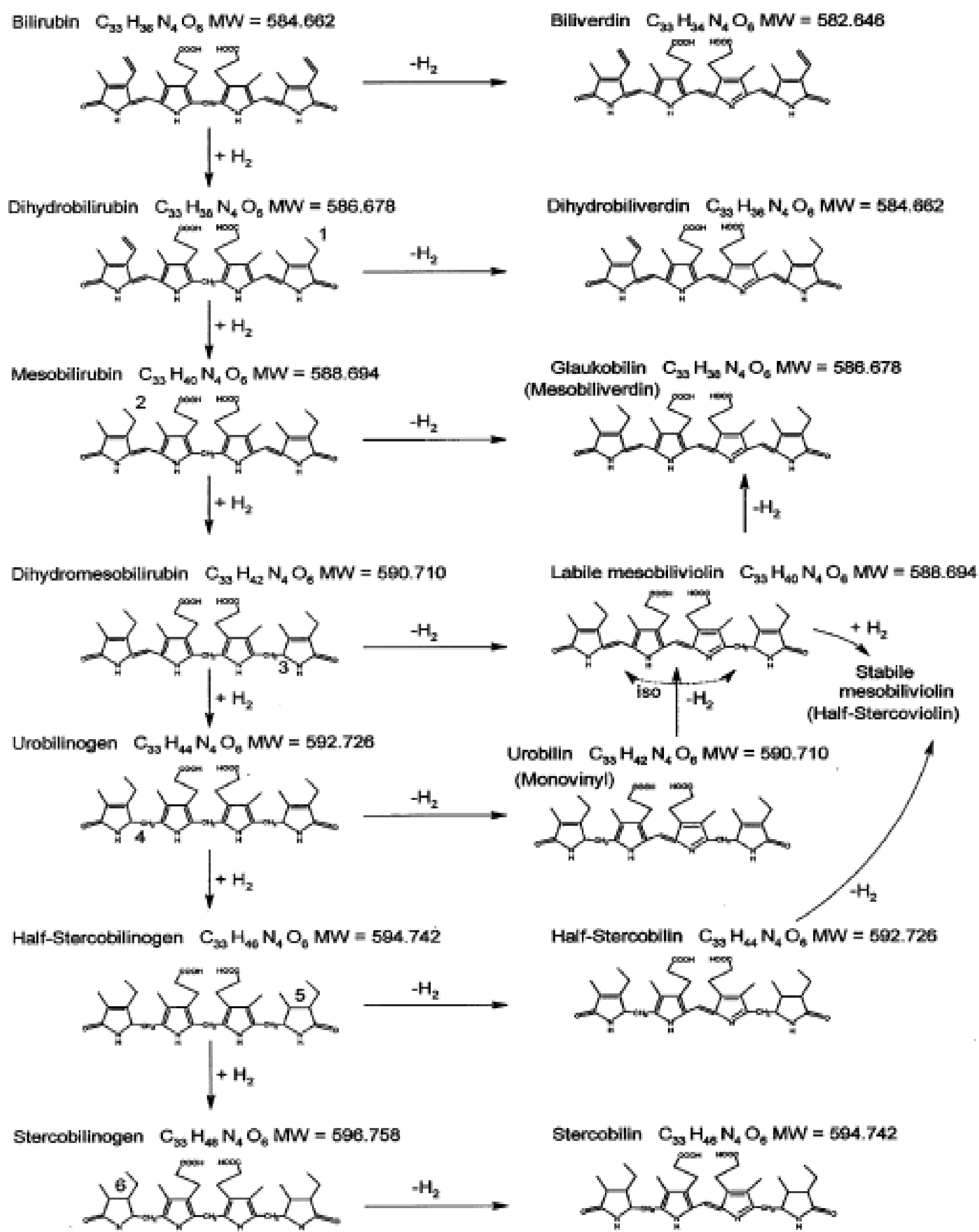


Figure 4: Multiple steps in the reduction of unconjugated bilirubin by intestinal microflora showing the chemical structure of the products formed [VITEK et al., 2006]

5.2.4 ***Bile pigments in mammals and other animals***

The physiological plasma bilirubin concentration in the human body ranges from 5 to 17µ*M*/l. A concentration over 300µ*M*/l is related to the occurrence of neurologic diseases [STOCKER, 2004]. An elevated circulating unconjugated bilirubin concentration of more than 17.1µmol/L is defined as Gilbert syndrome. The Crigler-Najjar syndrome is characterized by a concentration of 335µmol/L [IHARA et al., 1999].

The organism produces approximately 300mg bilirubin per day [SCHMID and MCDONAGH, 1975]. As mentioned above, in mammals, blue-green biliverdin is reduced to yellow bilirubin. Thus, biliverdin acts as a transient intermediate of heme catabolism. In some vertebrates (*e.g.* birds, amphibians, reptiles) the heme degradation is interrupted after the formation of biliverdin. In that case, biliverdin acts as the predominant endproduct [O´CARRA et al., 1975].

However, the formation of bilirubin appears to occur also in fish. Therefore, the sequence of bile pigment formation (heme → biliverdin → bilirubin) is not restricted to human beings and mammals [SCHMID and MCDONAGH, 1979].

5.3 Bile pigments – Harmful and protective aspects

5.3.1 ***Harmful aspects and bile pigment related diseases***

5.3.1.1 Toxicity of bile pigments

Only a few studies have been carried out in the past exploring the potential toxic effects of bile pigments. The main focus was dedicated to the adverse properties of unconjugated bilirubin. These studies reported that bilirubin possesses prooxidant and toxic properties referring to its incorrect association with neonatal jaundice and brain damage [TOMARO et al., 2002]. Bilirubin has

cytoprotective properties at normal to slightly increased concentrations, whereas high levels of plasma bilirubin are often cytotoxic [VITEK and OSTROW, 2009].

5.3.1.2 Neonatal jaundice and bilirubin encephalopathy

Neonatal jaundice appears in almost all newborn babies. During the first two weeks of life, unconjugated bilirubin concentrations are increased because of the degradation of fetal red blood cells and the temporary inability of bilirubin glucuronide formation. At the age of one month, the enzyme UDP glucuronosyl transferase reaches its fully active and reduces the unconjugated bilirubin concentration to the adult level [GOURLEY, 1997]. Therefore, the link between unconjugated bilirubin and the development of neonatal jaundice or brain damage is not exclusionary, but only relevant at high bilirubin concentrations.

Excessively elevated unconjugated bilirubin is not able to bind albumin and therefore it is defined as free unconjugated bilirubin which is highly lipophilic. This fraction can diffuse into cells by passive diffusion and causes toxic effects. Unconjugated bilirubin at high concentration can cause various types of neurological dysfunctions (*e.g.* kernicterus) which are known as bilirubin encephalopathy. Newborn infants are particularly at risk of brain damage. The increased susceptibility is caused by the delayed development of the hepatic conjugation system (*e.g.* in premature infants), the reduced binding capacity of albumin and possible genetic lesions (*e.g.* Crigler-Najjar syndrome patients) [SHAPIRO, 2003].

5.3.1.3 Crigler-Najjar-syndrome

A further example for an unconjugated hyperbilirubinemia is the Crigler-Najjar syndrome. This potentially lethal disorder is characterized by an increased

unconjugated bilirubin concentration (15-50mg/dl) in the plasma resulting from a deficiency of the uridinediphosphoglucuronate glucuronosyl transferase. This enzyme is predominately responsible for the detoxification and excretion of bilirubin [GANTLA et al., 1998]. In 1952, the rare syndrome was described by Crigler and Najjar and was formerly called “a severe congenital familial non-hemolytic jaundice with kernicterus”. Possible treatments are orthotopic liver transplantation and phototherapy [CRIGLER and NAJJAR, 1952].

5.3.1.4 Gilbert Syndrome

Gilbert Syndrome is a relatively common disorder (3-17% of the general population) which is characterized by a mildly increased serum bilirubin concentration of approximately 17µmol/L. Individuals with Gilbert syndrome have a reduced activity of the enzyme UDP glucuronosyl transferase. This enzyme is needed for the synthesis of hepatic uridine diphosphate glucuronosyl transferase (UGT1A1). In healthy people, UGT1A1 conjugates bilirubin and eliminates it from the body. The UGT1A1 polymorphism (UGT1A1*28) is characterized by decreased bilirubin excretion and increased unconjugated bilirubin levels [IHARA et al., 1999, BULMER et al., 2008].

Numerous studies have been published during the past years demonstrating the positive effects of mildly elevated serum bilirubin concentrations in the prevention of oxidative stress-mediated disorders, especially atherosclerotic diseases [NOVOTNY and VITEK, 2003]. Furthermore, individuals with Gilbert syndrome suffer lower rates of cardiovascular diseases contrary to individuals with reduced or normal serum bilirubin levels [BULMER et al., 2008]. Therefore, investigating bile pigments’ mechanism of action constitutes an exciting new field of research.

In 2008, Bulmer et al. examined the reasons for decreased risk of cardiovascular disease in Gilbert syndrome patients compared to healthy individuals. The authors investigated several markers (plasma antioxidant

status, erythrocytes antioxidant enzyme activities, plasma malondialdehyde, blood lipid profile, resistance to serum oxidation) in 21 subjects. Nine individuals represented the Gilbert syndrome group, whereas the remaining twelve were controls. It was established that the Gilbert syndrome group was characterized by a significantly higher trolox equivalent antioxidant capacity and significantly increased concentrations of unconjugated bilirubin. The antioxidant activity was measured by FRAP-test (ferric reducing ability of plasma) which showed significantly better results in the Gilbert syndrome individuals. Additionally, the Gilbert syndrome patients were characterized by a 13.6% improved resistance to serum oxidation compared to controls.
This study represented the first attempt to investigate the mechanism how bilirubin could protect from cardiovascular disease. In order to realize the important beneficial effects of bile pigments, this still unknown mechanism has to be explained. After their mechanism of action has been clarified, researchers could use the physiological effect of bile pigments to prevent cardiovascular diseases and cancer [BULMER et al., 2008].

5.3.1.5 Cell proliferation

Only a few studies have been designed in the past to explain the potential cell stimulating effects of biliverdin. A significant result in this field was yielded by Lafarge-Frayssinet et al. who studied the promoting effects of biliverdin for hepatic cells mediated by AFB1. The authors demonstrated that, in the presence of AFB1, biliverdin enhanced the impact of neoplastic liver cell transformation. In the absence of AFB1, this observation was not confirmed [LAFARGE-FRAYSSINET et al., 1983]. Although some studies suggest biliverdin can stimulate cell growth, it is important to review the findings critically due to the high and hence non physiological biliverdin concentrations that have been assayed [BREINHOLT et al., 1995].

5.3.2 ***Protective aspects***

In the past 20 years, the physiological relevance of bile pigments has been growing. Numerous studies have been carried out during the last years to investigate the beneficial effects of bile pigments. As already mentioned, the majority of research is dealing with the positive properties of bilirubin and biliverdin. These compounds are said to possess antioxidant, antimutagenic, anti-complement, anti-viral and anti-apoptotic effects [DUDNIK et al., 2001, ROMERT et al., 1994, NAKAGAMI et al., 1993]. Only a few studies have been published dealing with the beneficial properties of stercobilin and urobilin. To my knowledge, these compounds have never been used before to investigate their antimutagenic and antioxidant potency in the Ames *Salmonella* test.

Furthermore, numerous papers have been published exploring the positive effects of hemin, chlorophyllin and related pyrrole pigments [ARIMOTO et al., 1995].

Nowadays, the proof of antimutagenic and anticarcinogenic compounds is of great importance due to the negative effects of an increased mutation rate and the related cancer risks [ODIN, 1997].

5.3.2.1 Antioxidant potential of bile pigments

Antioxidants are a group of compounds that delay or inhibit the oxidation of an oxidizable substrate. There are two groups of antioxidants, enzymatic and non-enzymatic. The enzymatic antioxidant system includes superoxide dismutase (SOD), catalase (CAT) and glutathione-peroxidase (GPx). Non-enzymatic antioxidants are vitamin A, vitamin C, vitamin E, flavonoids, uric acid and bilirubin [HALLIWELL and GUTTERIDGE, 1985]. Non enzymatic antioxidants are essential in cellular defense, whereas enzymatic antioxidants are more important in the extracellular space [STOCKER, 2004].

Many studies have been published in the last years to elucidate the possible antioxidant properties of bile pigments, particularly of unconjugated bilirubin and biliverdin. To my knowledge, only one study has been carried out to investigate the antioxidant activity of urobilinoids. In this section the postulated antioxidant effects of bile pigments will be presented and discussed.

In 1953, Bernhard et al. studied the antioxidant activity of bile pigments for the first time. It was shown that unconjugated bilirubin and biliverdin protect vitamin A from oxidation [BERNHARD et al., 1954]. More than 60 years later, Stocker et al. reawaked the interest in bilirubin and its antioxidant potential. The authors showed that bilirubin was a more efficient peroxyl radical scavenger than α-tocopherol and vitamin C [STOCKER et al., 1987].

In a subsequent experiment, Stocker et al. studied the antioxidant potential of albumin-bound bilirubin at concentrations found in plasma of healthy men. The results indicated that albumin-bound bilirubin inhibited the oxidation of albumin-bound fatty acids *in vitro*, particularly of albumin-bound linoleic acid. 1mol of albumin-bound bilirubin was able to scavenge 2mol of peroxyl radicals. Furthermore, the antioxidant potential of albumin-bound bilirubin was compared to ascorbate and urate. It was demonstrated that albumin-bound bilirubin was more or less equally effective in scavenging peroxyl radicals like uric acid, but less effective than vitamin C. In conclusion, the authors established that albumin-bound bilirubin has antioxidant properties and acts as an endogenous antioxidant [STOCKER et al., 1987].

A growing body of evidence supports the view that albumin-bound bilirubin is also able to prevent proteins of being oxidatively damaged by different types of reactive oxygen and nitrogen species (RONS) [STOCKER, 2004]. 40% of the human albumin is located in the blood circulation, whereas the remaining 60% is present in the extracellular space. Thus, the antioxidant potential of albumin-bound bilirubin is not restricted to the plasma [BRODERSEN and STERN, 1980]. Further research revealed that albumin was present in inflammatory

exudates after leaving the blood circulation. This implies a higher possibility for antioxidant activity at sites of elevated occurrence of RONS by phagocytic cells [STOCKER, 2004].

Further research suggests that bile pigments possess antioxidant properties towards lipid oxidation. In 1995, Hulea et al. studied the antioxidant effects of free and albumin-bound bilirubin at concentrations found in blood plasma. These two compounds were tested for their potency to inhibit the metal-catalyzed oxidation of low density lipoprotein (LDL). Several markers were used for determining LDL peroxidation (decreased reactivity of thiobarbituric acid, electrophoretic mobility changes, fewer apo B fragmentations, reduced quantity of cholesterol oxidation products). It was shown that the albumin-bilirubin complex suppressed the metal-catalyzed oxidation of LDL in a dose depent manner. In conclusion, bilirubin protected against oxidation of lipid membranes *in vitro*. Interestingly, the inhibition in the LDL-iron-BR system (54% inhibition) was more effective than in the copper-catalyzed reaction (30% inhibition). This could be due to the fact that bilirubin and copper can form an unstable complex [HULEA et al., 1995].

The antioxidant activity of urobilinoids was first described in 1996 by Nakamura et al. who showed that these reductive bilirubin metabolites have an antioxidant function. Urobilinoids had not been used previously to investigate their antioxidant potential and their specific function.

Nakamura et al. prepared a synthetic urobilinogen for the experiment which was defined as i-urobilinogen. The synthesis of the metabolite was based on the reduction of commercial bilirubin. NMR analysis and mass spectroscopy were used for structural conformation [NAKAMURA et al., 2006].

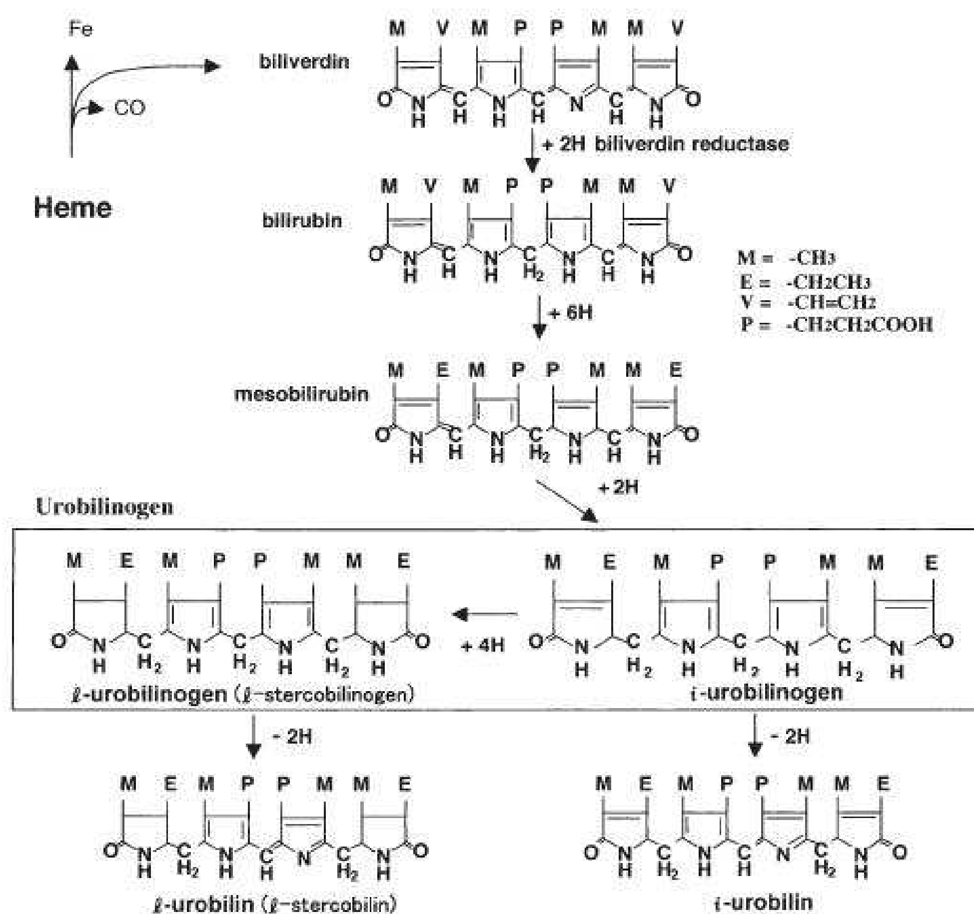


Figure 5: Metabolism and structures of fecal pigments in mammals [NAKAMURA et al., 2006]

The aim of the previously mentioned study was to explore the radical scavenging potential of urobilinogen compared with other antioxidants (bilirubin, α-tocopherol and β-carotene). The radical DPPH (1,1-diphenyl-2-picrylhydradyl) was used to evaluate antioxidant potential. Furthermore a "challenge test" was incorporated into the study to investigate the effects of urobilinogen on the formation of linolate hydroperoxide by AMVN (2,2-azobis (2,4-dimethylvaleronitrile)). Generally speaking, antioxidant potential is essential to inhibit the formation of linolate hydroperoxide. In order to explore the effects on the formation, HPLC was used. The DPPH radical trapping effects of the antioxidants were established by a UV/VIS spectrophotometer at

517nm absorption. In order to determine the radical scavenging activity, each antioxidant sample was compared to the absorbance of a blank test. It was shown that urobilinogen was the most potent among the other antioxidants. The bilirubin metabolite decreased the absorption by 79% of the control, whereas α-tocopherol and β-carotene showed a 35 and 29.1% reduction in absorption, respectively. The DPPH radical scavenging activity of bilirubin amounted 72% of the control. A similarly positive effect of urobilinogen was obtained in the “challenge test”. In the presence of urobilinogen, the formation of linolate hydroperoxide was inhibited by 50%. The authors concluded that urobilinogen was able to inhibit lipid oxidation induced by free radicals [NAKAMURA, 2006].

5.3.2.2 Antimutagenic activity of bile pigments

A number of studies have been published to elucidate the antimutagenic potential of bile pigments. Most of the studies have used the Ames *Salmonella* test for their experiments which is a suitable test system for exploring the antimutagenic activity of different compounds. This section includes important findings for bilirubin, biliverdin and related pyrrole pigments in this field and the major differences are summarized in Table 2.

In 1980, researchers studied the antimutagenic effects of bilirubin, biliverdin, hemin and chlorophyllin against benzo[α]pyrene (B[α]P) in the Ames *Salmonella* test. B[α]P is characterized as a polycyclic aromatic hydrocarbon which is often used in the Ames test. The assays were performed in the presence of metabolic activation with the *Salmonella typhimurium* strain *TA98*. It was established that all compounds inhibited the B[α]P induced mutagenicity. Hemin showed the greatest antimutagenic effect, followed by chlorophyllin, bilirubin and biliverdin [ARIMOTO et al., 1980].

This fact was confirmed in 1995 by Arimoto et al. who reported the antimutagenic properties of hemin, chlorophyllin, bilirubin, biliverdin, protoporphyrin, Fe- and Cu-chlorins in the Ames *Salmonella* test. These compounds were investigated for their potential to inhibit the mutagenicity of B[α]P and the metabolites benzo[α]pyrene-7,8-diol, benzo[α]pyrene-4,5-epoxide and benzo[α]pyrene-7,8-diol-9,10-epoxide. The tests were carried out with and without metabolic activation in the presence of the *Salmonella typhimurium* strain *TA100*. All assays were performed with a preincubation time of 30min. The authors reported that bilirubin and biliverdin inhibited the mutagenicity of B[α]P with and without metabolic activation. In contrast, the bile pigments provided only slightly antimutagenic properties against B[α]P4,5-epoxide and B[α]P-7,8-diol-9,10-epoxide and benzo[α]pyrene-7,8-diol. Hemin was most effective against the mutagenicity of all mutagens. It was also shown that hemin and Fe-chlorin inhibited the enzymatic formation of 3-OH-B[α]P. A similar effect was not observed for the bile pigments. In order to investigate the mechanism of the effective inhibition of the 3-OH-B[α]P formation the compounds were incubated with benzo[α]pyrene-7,8-diol-9,10-epoxide. The different stabilities on the B[α]P metabolites were monitored with a spectrophotometer. A degradation of benzo[α]pyrene-7,8-diol-9,10-epoxide was observed with hemin and Cu-chlorin, whereas bilirubin and biliverdin showed no effects.

To conclude, these two studies have shown that bile pigments inhibited the B[α]P induced mutagenicity in the Ames *Salmonella* test. Bilirubin and biliverdin were not effective in speeding up the degradation of B[α]P metabolism [ARIMOTO et al., 1980, ARIMOTO et al., 1995].

Tang and Edenharder investigated whether porphyrins including hemin, chlorophyllin, chlorophyll, biliverdin and bilirubin could inhibit the mutagenic effects of 2-nitrofluorene (2-NF), 3-nitrofluoranthene (3-NFA) and 1-nitropyrene (1-NP). These mutagens are tetracyclic (3-NFA, 1-NP) and tricyclic (2-NF) nitroarenes. Additionally, vitamins and fruit juices were tested for their

antimutagenic potential. The antimutagenic effects of these compounds have been tested in the *Salmonella typhimurium* strain *TA98*. All Ames assays were performed without metabolic activation. It was shown that all tested compounds had antimutagenic effects against the three tested mutagens. Hemin and bilirubin were the most potent antimutagens, whereas biliverdin and chlorophyllin showed less antimutagenic potential [TANG and EDENHARDER, 1997].

In 2007, Bulmer and colleagues studied the antimutagenic and antioxidant effects of bile pigments in the Ames *Salmonella* test. Unconjugated bilirubin, biliverdin and bilirubin ditaurate (a synthetic, water soluble conjugate) were tested in six different concentrations with three *Salmonella typhimurium* tester strains (*TA98*, *TA100* and *TA102*). The bile pigments were studied for their potential to inhibit the mutagenicity of various mutagens including benzo[α]pyrene (B[α]P), trinitrofluorenone (TNFone), 2-aminofluorene (2-AF), sodium azide (NaN_3) and tertiary-butyl hydroxyperoxide (*t*-BuOOH). The assays were performed with (B[α]P, 2-AF and *t*-BuOOH) and without metabolic activation. The assays with TNFone, NaN_3 and *t*-BuOOH were conducted without S9. It was shown that unconjugated bilirubin, biliverdin and bilirubin ditaurate were antimutagenic and antioxidative against all mutagens, except for NaN_3. In this study, the *Salmonella* tester strain *TA102* was used for the first time to investigate the antimutagenic and antioxidant effects of bile pigments. It was shown that bilirubin, biliverdin and bilirubin ditaurate were able to inhibit the genotoxic effects of *t*-BuOOH induced oxidative stress [BULMER et al., 2007].

Author	Salmonella strain	Compounds	Mutagen	Metabolic activation	Results, Discussion
Arimoto et al. 1980	*TA98*	hemin, chlorophyllin BR [0-1,6µmol/plate] BV [0-0,75µmol/plate]	B[α]P	with metabolic activation	Effects of compounds against B[α]P: hemin>BR>chlorophyllin>BV ID_{50} (BR/BV/hemin): 0.05/0.5/0.01µmol
Arimoto et al. 1995	*TA100*	hemin, chlorophyllin, protoporphyrin, BR, BV, Cu-chlorin, Fe-chlorin	B[α]P, B[α]P(7,8)D, B[α]P(4,5)E, B[α]P(7,8)D(9,10)E	B[α]P, 2-AF, t-BuOOH (with S9) TNFone, NaN_3, t-BuOOH (without S9) with and without S9	Effects of compounds against B[α]P: hemin=protoporphyrin>hematoporphyrin= Cu-chlorin> Fe-chlorin≥chlorophyllin>BR>BV Effects of compounds against B[α]P(4,5)E: hemin>chlorophyllin>protoporphyrin> hematoporphyrin>BR=BV BR and BV inhibited the mutagenic effects of B[α]P with/without S9. They do not speed up the reduction of B[α]P metabolites.

Tang and Edenharder 1997	*TA98*	hemin, chlorophyllin, chlorophyll BR [7µmol/plate] BV[1µmol/plate]	2-NF, 3-NFA, 1-NP	without S9	Effects of compounds against all three mutagens: BR>BV ID_{50} for 2-NF (BR/BV/hemin): 0.05/0.39/0.04µmol ID_{50} for 1-NP (BR/BV/hemin): 0.04/0.7/0.02µmol ID_{50} for 3-NF (BR/BV/hemin): 0.04/0.11/0.05µmol
Bulmer et al. 2007	*TA98, TA100, TA102*	BR, BV, BRT [0-2µmol]	B[α]P, TNFone, 2-AF, NaN_3, t-BuOOH	B[α]P, 2-AF, *t*-BuOOH (with S9) TNFone, NaN_3, *t*-BuOOH (without S9)	Antimutagenic effects of bile pigments against all mutagens, except NaN_3. BR≥BRT>BV (TNFone) BV≥BRT≥BR (2-AF) BV>BRT>BR (B[α]P) BR≥BV>BRT (*t*-BuOOH) ID_{50} (BR/BV/BRT): 0,557/1/2,756µmol

Table 2: The antimutagenic potential of bile pigments and related pyrrole pigments in the Ames test [modified: BULMER et al., 2008]

5.3.2.3 Other postulated effects of bile pigments

As previously mentioned, the enzyme heme oxygenase-1 catalyzes the degradation of heme to biliverdin, carbon monoxide and iron and subsequently to bilirubin by biliverdin reductase. A number of studies have shown that this enzyme is able to prevent oxidative stress by decreasing the amount of ROS (reactive oxygen species). These studies established, amongst other findings, that heme oxygenase-1 is highly overproduced in atherosclerotic lesions in humans [WANG et al., 1998]. Further research revealed that heme oxygenase-1 is an important protective factor which is lowly expressed in vascular diseases and cancer [EXNER et al., 2004].

As mentioned above, bilirubin has strong antioxidant and antimutagenic properties *in vitro*. This fact was confirmed and supported by several clinical observations. An increased body of evidence confirms that bilirubin acts as protective factor in several diseases including atherosclerosis, cancer, inflammatory and autoimmune disorders [VITEK and SCHWERTNER, 2007].

In 2001, the link between serum bilirubin concentrations and the mortality of cardiovascular diseases and cancer was a study background by Temme et al. The risk of cancer mortality was divided into three groups, total cancer, lung and non-lung cancer. Additionally, total mortality was examined. The 10-year follow-up mortality data of approximately 10.000 subjects were provided from the Belgium Inter-university Research on Nutrition and Health study. The representative sample of the Belgian population included 5450 men and 4843 women. Serum bilirubin concentrations of men and women were classified into five groups (≤ 0.2, 0.3, 0.4, 0.5, ≥ 0.6 mg/dl) and four groups (≤ 0.2, 0.3, 0.4, ≥ 0.5 mg/dl), respectively. In order to reduce the number of confounders, some variables were adjusted (age, smoking status, body mass index, total cholesterol concentrations). Temme et al. reported that men in the highest range (≥ 0.6mg/dl) of serum bilirubin concentrations had a 27% lower total mortality risk compared with men in the lowest range (≤ 0.2mg/dl). Furthermore, men in the fifth group showed a significantly lower risk (58%) for total cancer

compared with men in the first group. Data observed for non-lung cancer were significant. The association between serum bilirubin concentration and mortality was not shown for cardiovascular diseases. Women in the highest range (≥ 0.5mg/dl) of serum bilirubin concentrations showed a 24% lower risk for total cancer compared with women in the lowest range (≤ 0.2mg/dl). However, this observation was not significant. Furthermore, inverse associations for total mortality and cardiovascular diseases were not established in women. A *p*-value of 0.05 was considered as significant [TIMME et al., 2001].

	Serum bilirubin [mg/dl]		
	≤ 0.2	≥ 0.6	*p*-value
No. of men	740	1190	
Total mortality			
Deaths/1000 person-years	19.2	10.6	
No adjustment	1.00	0.55 (0.43-0.70)	0.0001
adjusted	1.00	0.73 (0.57-0.94)	0.0960
Cardiovascular diseases			
Deaths/1000 person-years	6.0	4.6	
No adjustment	1.00	0.78 (0.52-1.2)	0.4306
adjusted	1.00	1.02 (0.68-1.5)	0.3667
Total cancer			
Deaths/1000 person-years	7.6	2.4	
No adjustment	1.00	0.31 (0.20-0.49)	0.0001
adjusted	1.00	0.42 (0.26-0.68)	0.0040

Table 3: Association between mortality risk and highest and lowest serum bilirubin concentration in men (relative risk and confidence interval) [modified: TEMME et al., 2001]

	Serum bilirubin [mg/dl]		
	≤ 0.2	≥ 0.5	*p*-value
No. of women	1343	950	
Total mortality			
Deaths/1000 person-years	7.0	5.6	
No adjustment	1.00	0.78 (0.55-1.1)	0.8414
adjusted	1.00	0.87 (0.62-1.2)	0.5771
Cardiovascular diseases			
Deaths/1000 person-years	2.7	2.6	
No adjustment	1.00	0.96 (0.57-1.6)	0.9274
adjusted	1.00	1.04 (0.62-1.8)	0.7392
Total cancer			
Deaths/1000 person-years	2.0	1.3	
No adjustment	1.00	0.70 (0.36-1.4)	0.3014
adjusted	1.00	0.76 (0.39-1.5)	0.1562

Table 4: Association between mortality risk and highest and lowest serum bilirubin concentration in women (relative risk and confidence interval) [modified: TEMME et al., 2001]

This prospective study showed that the determination of serum bilirubin concentrations could be a helpful tool to evaluate the relative cancer risk [TIMME et al., 2001].

Furthermore, bile pigments may possess cell apoptotic and regulatory effects on carcinogenesis. For example, bilirubin and carbon monoxide provide endothelial protection by an active PETN metabolite (Pentaerithrityl tetranitrate) [OBERLE et al., 2003].

Further research has demonstrated that bile pigments might possess antiviral properties. 15 different compounds were tested for their potential to inhibit HIV-1 protease activity. Additionally, their effects in HIV-1 viral maturation were examined. Of these 15 compounds, only bilirubin, biliverdin, secalonic acid D and alphazurin suppressed human and simian immunodeficiency virus protease activity by blocking viral entry into HeLaT4 target cells. Furthermore it was shown that stercobilin, urobilin, biliverdin dimethylester and xanthobilirubic acid have similar antiviral properties under the same conditions. However, results of cell culture experiments provided no inhibition of HIV-1 viral maturation by these compounds. The results of this study suggest that increased bile pigment concentrations may affect the course of HIV infections [McPHEE et al., 1996].

6 MATERIALS AND METHODS

6.1 General principles of the Ames test

The Ames *Salmonella* test is defined as a short-term bacterial reverse mutation assay which is a popular and frequently used test system for the detection of chemicals and drugs that can induce mutations [MORTELMANS and ZEIGER, 2000]. This assay was used to investigate the possible antimutagenic and antioxidant effects of stercobilin and urobilin. Furthermore, mutagenicity assays were performed with both bile pigments. In principle the test procedure followed the standard plate incorporation assay which had been established by Bruce Ames in 1973 [MARON and AMES, 1973].

6.2 Solutions used for the assay procedure

6.2.1 ***Ampicillin solution***

0.2mg Ampicillin was solubilised in 25ml of 0.2N sodiumhydroxide. After mixing the solution was strained through an aseptic filter. This antibiotic solution could be stored at 4℃ for four weeks.

6.2.2 ***Tetracycline solution***

80mg of tetracycline was solubilised in 10ml of 0.2N hydrochloric acid. After mixing the solution was aseptically filtered. This antibiotic solution could be stored at 4℃ for two weeks.

6.2.3 ***Glucose solution (40%)***

For a 40% solution 400g of glucose was dissolved in 1000ml of boiling deionised water. The prepared solution was autoclaved each time before use.

6.2.4 ***Glucose-6-phosphate solution (304mg/ml)***

1g of glucose-6-phosphate was dissolved in 3.289ml of deionised water. Finally the solution was sterilised through an aseptic filter. Portions of 1ml each were stored at -20℃.

6.2.5 ***Histidine-biotin solution***

30.9mg D-biotin and 24mg L-histidine were weighed into two separate weighing boats. After transferring the amino acids into a 250ml Pyrex flask, 250ml of deionised water were added. The solution was sterilised each time before use and was stored at 4℃.

6.2.6 ***Histidine solution***

0.25g of histidine was dissolved in 50ml of deionised water. Finally the solution was aseptically filtered and stored at 4℃.

6.2.7 ***Biotin solution***

6.1mg of D-biotin was mixed with 50ml of deionised water. The prepared solution was strained through an aseptic filter and stored at 4℃.

6.2.8 *Overnight culture*

5g of Nutrient Broth No.2 are weighed into a Pyrex flask and filled up to 200ml with deionised water. After shaking the solution was autoclaved and as soon as the nutrient broth was cooled down strain specific antibiotics were added. For strain *TA98* 625µl ampicillin solution are used, whereas *TA102* additionally needed 156µl tetracycline solution. Thereof, 12ml each were transferred into two sterile 250ml Erlenmeyer flasks. One bacterial colony (either *TA98* or *TA102*) was taken with an inoculation loop from a master plate and was added to the flask. After whirling the flasks were recapped with a dabber and aluminium film and were shaken in a 37℃ incubator for 12 hours at 55rpm.

Figure 6: OVNC incubated for 12 hours at 37℃

6.2.9 *Master plates*

For the preparation of two master plates, 3.2g of Oxoid agar were made up with 200ml of deionised water in Pyrex flasks. Afterwards the prepared solution was autoclaved. Next 4.3ml of vogel-bonner- and 10.8ml of glucose solution were added. After shaking the flasks 2.17ml of histidine- and 1.3ml of biotin solution were added. After the agar was cooled down strain specific antibiotics were added:

Salmonella strain	Antibiotics
TA98	625µl of ampicillin
TA102	625µl of ampicillin 156µl of tetracycline

Table 5: Strain specific antibiotics for preparation of the master plates

After the antibiotics were added the agar was poured into sterile Petri dishes, the thickness of the agar amounted to approximately 1cm. The prepared master plates were left under sterile conditions to dehumidify for 24 hours. After drying the requested bacteria strain was taken with an inoculation loop from the overnight culture and was applied on the plates lattice-like. The master plates were incubated for 48 hours at 37℃ and sealed with a tape after that. Once the master plates had been inoculated they could be kept for 2 weeks (*TA102*) or for two months (*TA98*), respectively at 4℃.

6.2.10 ***$MgCl_2$/KCl solution***

61.5g of KCl and 40.7g of $MgCl_2$ were dissolved in 500ml of deionised water. The solution was sterilised trough an aseptic filter and stored at 4℃.

6.2.11 ***Minimal glucose agar plates***

For preparation of approximately 320 minimal glucose agar plates eight 1000ml Erlenmeyer flasks were needed. Into each flask 11.25g of Oxoid agar are weighed and filled up to 700ml with deionised water. The flasks were recapped with a dabber and aluminium film, and sterilised in the autoclave together with glucose- and vogel-bonner solutions. After about 2 hours the flasks were ready to be transferred under the laminar flow. To each of the flasks 15ml of vogel-bonner solution and 37.5ml of glucose-solution were added under sterile conditions. After shaking the warm agar was poured into Petri dishes. The poured plates were dried for about 24 hours. The next day they were packed in plastic bags and stored at 4℃. The storage time wa s 14 days.

6.2.12 ***NADP solution***

A 0.135g/2ml solution was produced. 1g of NADP was dissolved in 14.814ml of deionised water. The solution was sterilised through an aseptic filter and frozen in portions of 1ml at -20℃.

6.2.13 ***S9-Mix***

The use of a rat-liver S9 metabolic activation system was necessary because bacteria do not have a cytochrome-based P450 metabolic oxidation system.

The solution was always prepared fresh and kept on ice throughout the test. For a maximum of 100 plates 19.75ml of deionised water, 25ml of PBS buffer (without Ca and Mg), 1ml of $MgCl_2$, 2ml of NADP solution and 250µl of glucose-6-phosphate-solution were pipetted into a sterile 50ml centrifuge tube. At last, 2 ml of the rat liver homogenate were added. The S9-Mix had to be cooled on ice constantly during the test, because the solution contains highly susceptible enzymes which are highly susceptible to oxidation. The cooled solution had to be used within one hour.

6.2.14 ***Top agar***

1.2g of Oxoid agar and 1g of sodium chloride were dissolved in 200ml of deionised water and autoclaved just before each test. Before use, 20ml of the histidine-biotin solution are added. The top agar had to be kept in a water bath at 55℃ throughout the test.

6.2.15 ***Vogel-Bonner solution***

10g of magnesium sulphate heptahydrate, 100g of citric acid monohydrate, 500g of dibasic potassium phosphate and 175g of sodium ammonium

phosphate tetrahydrate were dissolved in 670ml of deionised water (45℃). The solution was autoclaved each time before use.

Substance	Abbreviation/ empirical formula	Source	Order No.	Utilisation
2-Amino-1-methyl-6-phenylimidazo[4,5,-b] pyridine	PhiP	Toronto Research Chemicals	A617000	Positive control
Aflatoxin B1	AFB1	Sigma	A6636	Positive control
Agar No I		Oxoid/	LP011P	Plates, Top agar
Ampicillin Trihydrate		Sigma	A6140	MP, OVNC
D-Biotin	$C_{10}H_{16}N_2O_3S$	Sigma	B4639	MP
Dimethylsulfoxide	DMSO	Sigma	D5879	solvent, negative control
D-Glucose	$C_6H_{12}O_6$	Sigma	G8270	GM agar plates,
Glucose-6-Phosphat	Glu-6-P	Sigma	G7250	S9-Mix
Potassium chloride	KCl	Sigma	P5405	S9-Mix
Potassium phosphate dibasic	K_2HPO_4	Sigma	P3786	Vogel-Bonner-solution
L-Histidine	$C_6H_9N_3O_2$	Sigma	H8125	Top Agar, MP

Magnesium chloride	$MgCl_2 . 6H_2O$	Sigma	M9272	S9-Mix
Magnesium sulfate	$MgSO_4 . H_2O$	Sigma	M9397	Vogel-Bonner-solution
Nutrient Broth		Oxoid/Bertoni	CM067B	OVNC
Sodium ammonium phosphate	$Na_2NH_2PO_4 . 4H_2O$	Sigma	S9638	Vogel-Bonner-solution
Sodium chloride	NaCl	Sigma	S5886	Top Agar
Sodium hydroxide	NaOH	Sigma	O6203	Ampicillin-solution
Nicotinamide adenine dinucleotide	NADP	Sigma	N0505	S9-Mix
Dulbecco´s phosphate buffered saline	PBS	PAA Laboratories	H15002	Preincubation, S9-Mix
Rat liver homogenate	S9	Biomedica/ICN	50412	Plate incorporation test with S9-Mix
Tertiary-Butyl-hydroxyperoxide	*t*-BuOOH	Sigma	B2633	Oxidative mutagen
Tetracycline hydrochloride		Sigma	T3383	MP, OVNC
2,4,7-Trinitro-9-fluorenone	TNFone			Positive control
Citric acid monohydrate	$C_6H_8O_7 . H_2O$	Sigma	C1909	Vogel-Bonner-solution

Table 6: Chemicals and reagents used within the test procedure

6.3 Experimental design

Experiments in the bacterial system were designed to explore the potential antimutagenic and antioxidant effects of stercobilin (0.01-2µmol/plate) and urobilin (0.01-2µmol/plate and 0.0001-2µmol/plate) in the Ames *Salmonella* test.

6.3.1 ***Chemicals and reagents***

The bile pigments, stercobilin hydrochloride (S) [34217-90-8] and urobilin hydrochloride (U) [28925-89-5], were purchased from Frontier Scientific. In order to protect bile pigments from light and avoid possible oxidative events, the highly sensitive compounds were sealed with some adhesive tape (see Figure 7). The S9 liver homogenate was obtained from MP Biomedicals (Illkirch, France).

All chemicals and reagents were stored at -80°C if necessary. All solutions, glass ware, tubes, pipettes, etc. had to be sterile to ensure a contamination free test system. The whole equipment was sterilised in an autoclave.

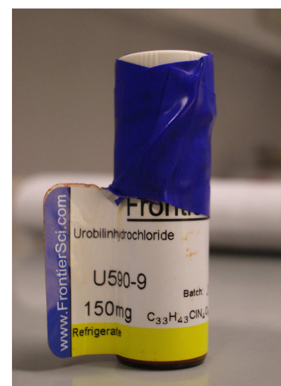


Figure 7: Urobilin hydrochloride

6.3.2 ***Bacterial strains***

The antimutagenic and antioxidant effects of stercobilin and urobilin were tested in the presence of two bacterial strains, *TA98* and *TA102*. It is very important to use different *Salmonella typhimurium* tester strains. Since specific mutations within the strains make them more sensitive to respond on different mutagens. Mutations of the *TA102* can be reverted by mutagens that cause oxidative damage. The *TA102* strain detects cross-linking agents and the *TA98* strain gives information on frame-shift mutations. Because of that various tester strains are essential for the experimental procedure [MARON and AMES 1983].

6.3.3 *Positive control chemicals*

In order to ensure the reversion properties, the specificity of each tester and the activity of the S9-mix, diagnostic mutagens (positive control chemicals) were used in each Ames assay [MORTELMANS and ZEIGER, 2000].

All tests included three positive control tubes (mutagen only). Therefore a variety of mutagens including 2, 4, 7-trinitrofluorenone (TNFone), 2-amino-1-methyl-6-phenylimidazo [4, 5,-b] pyridine (PhiP) and aflatoxin B1 (AFB1) were used to confirm the formation of mutant revertants. Furthermore the prooxidans *tertiary*-butyl hydroxyperoxide (*t*-BuOOH) was used.

Tests were conducted with and without metabolic activation incorporating the addition of the microsomal liver homogenate. The solutions of TNFone, PhiP and AFB1 were dissolved in DMSO. The *t*-BuOOH solution was dissolved in deionised water.

TNFone is a non-planar polycyclic nitro-amine and was previously found in certain photocopy processes. In the Ames *Salmonella* test TNFone turned out to be mutagenic with and without metabolic activation. A number of oral feeding studies in animals have been carried out in the past showing that TNFone caused different lesions including black discoloration of the skin and hair, dark brain and gallbladder, lymphoid depletion and seminal vesicle atrophy in rats and mice [KARI, 1992].

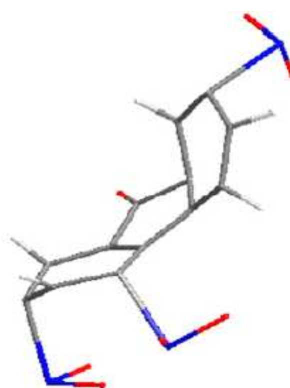


Figure 8: Three-dimensional structure of TNFone [BULMER et al., 2007]

t-BuOOH is an organic hydroperoxide and oxidant which is known as an initiator of the lipid peroxidation. Furthermore, *t*-BuOOH has the potential to increase oxidative stress *in vivo* and therefore this organic peroxide is used as a marker for oxidative stress in various cell culture experiments [HWANG et al., 2005].

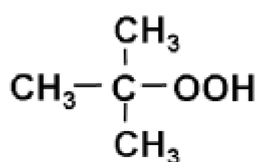


Figure 9: Chemical structure of *t*-BuOOH [INAMI et al., 2009]

PhiP is a hazardous heterocyclic aromatic amine which may increase the risk of cancer in human, in particular colon, prostate and mammary cancer. Heterocyclic aromatic amines are generally formed in cooked meats, fish and poultry at high temperatures. Furthermore different types of heterocyclic aromatic amines are present in tobacco smoke condensate and diesel exhaust [TURESKY, 2007].

Generally speaking, PhiP is a food related mutagen arising when meat and fish is grilled or fried and therefore it should be explained whether stercobilin and urobilin are able to inhibit the genotoxic effects of this mutagen [TURESKY, 2007].

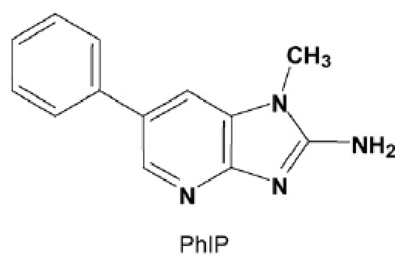


Figure 10: Chemical structure of PhiP [TURESKY, 2007]

Aflatoxins belong to the substance class of mycotoxins which are mainly produced as secondary metabolites by Aspergillus flavus and Aspergillus parasiticus. These compounds are toxic, mutagenic and carcinogenic. Different types of aflatoxins with various potency and carcinogenicity are known, important members are aflatoxin B1, B2, G1 and G2. Of these, AFB1 turned out to be the most common and toxic aflatoxin in animals and humans. The major metabolite of AFB1 is M1 which is primarily present in milk [DIEKMAN and GREEN, 1992]. Aflatoxins are food-related mutagens which occur predominantly in peanuts, maize and corns [STROKA, 2000].

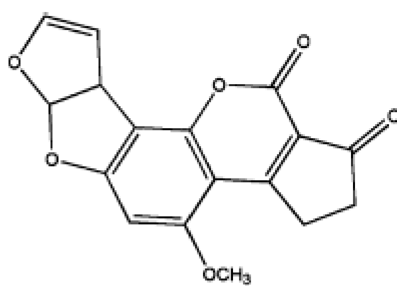


Figure 11: Chemical structure of AFB1 [HAN et al., 2010]

TNFone, PhiP and AFB1 induced genotoxicity was used to test the possible antimutagenic effects of stercobilin and urobilin. *t*-BuOOH induced pro-oxidative effects were used to investigate the possible antioxidant potential of these two bile pigments.

	without metabolic activation	**with metabolic activation**
TA98	TNFone [0,0001mg/ml]	Phip [0,1µmol/ml]
		AFB1 [5µg/ml]
TA102	TNFone [0,07mg/ml]	AFB1 [120µg/ml]
	t-BuOOH [0,75*10^{-6}mol/plate]	*t*-BuOOH [0,75*10^{-6}mol/plate]

Table 7: General overview of mutagens used in the Ames test

6.3.4 *Negative control chemicals*

All tests included six negative control plates which contained DMSO. These plates were necessary to determine the spontaneous mutant frequency of the *Salmonella typhimurium* strains *TA98* and *TA102.*

6.4 Assay procedure

Antimutagenic testing was performed to explain the possible antimutagenic effects of stercobilin and urobilin against TNFone, PhiP and AFB1. Antioxidant testing was carried out to investigate the possible antioxidant effects of the two bile pigments against *t*-BuOOH. In order to assess the mutagenic potential of stercobilin and urobilin, mutagenicity assays were performed for each mutagen and each tester strain.

6.4.1 *Preparation of the bile pigment samples*

For each test a fresh bile pigment stock solution was prepared. Therefore 9mg aliquots of the bile pigments were weighed out into eppendorf cups and dissolved in 618µl of DMSO. After mixing, the cups were placed in an ultrasonic bath for 3min to dispense the bile pigments homogenously. To elucidate the efficacy of the inhibitory action of stercobilin and urobilin against the mutagenicity of various mutagens, the Salmonella assays were performed in the presence of varying doses of the pigments.

Six different concentrations (2, 1, 0.5, 0.1, 0.05 and 0.01µmol/plate) of stercobilin and eight different

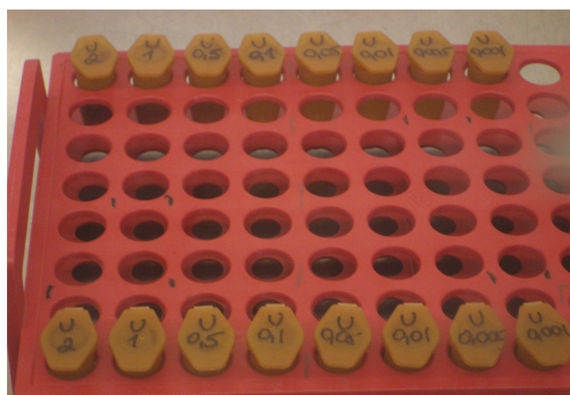


Figure 12: Preparation of urobilin in eight different concentrations

concentrations (2, 1, 0.5, 0.1, 0.05, 0.01, 0.005 and 0.001µmol/plate) of urobilin were investigated. Only one test for urobilin was conducted with six concentrations as it applied for stercobilin (TNFone, *TA102*).

The following dilution series was established:

	stock solution [µl]	**DMSO [µl]**
2µmol/plate	320	480
1µmol/plate	160	640
0,5µmol/plate	80	720
0,1µmol/plate	100 [1µM]	900
0,05µmol/plate	100 [0,5µM]	900
0,01µmol/plate	100 [0,1µM]	900
0,005µmol/plate	100 [0,05µM]	900
0,001µmol/plate	100 [0,01µM]	900

Table 8: Preparation of the bile pigment samples

*6.4.2 **Antimutagenic/Antioxidant assays***

Antimutagenic and antioxidant activities of stercobilin and urobilin were tested in a broad ranging spectrum (0.001 to 2µmol/ plate) in *TA98* and *TA102* whereas bile pigments were dissolved in DMSO. Three plates were counted for every concentration of each condition. Every assay included three positive control tubes (mutagen only) and six negative control (no mutagen, no bile pigment) tubes.

The test procedure consisted of adding 500µl PBS buffer or S9-mix, 100µl of histidine dependent overnight culture bacteria, 100µl mutagen solution and

200µl PBS buffer in positive and negative control. Finally, 200µl bile pigment solution were added to the test tubes.

All assays were performed with a preincubation time of 25 min at 37°C. After that time, 2 ml of top agar containing biotin and histidin were added. Finally, the contents of the tubes were vortexed and poured onto the surface of minimal glucose agar plates.

As soon as the top agar had hardened (after 20-25 min) the plates were placed in a 37°C incubator for 48hours.

After expiration of this time the histidine revertant colonies were counted.

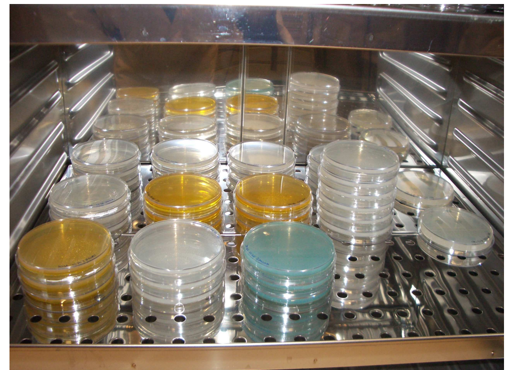


Figure 13: Poured plates drying in the incubator for 48h

The white spots were placed onto a flat lamp and counted manually. The background lawns of the plates were examined by eye for thinning and for the presence of precipitate [MORTELMANS and ZEIGER, 2000].

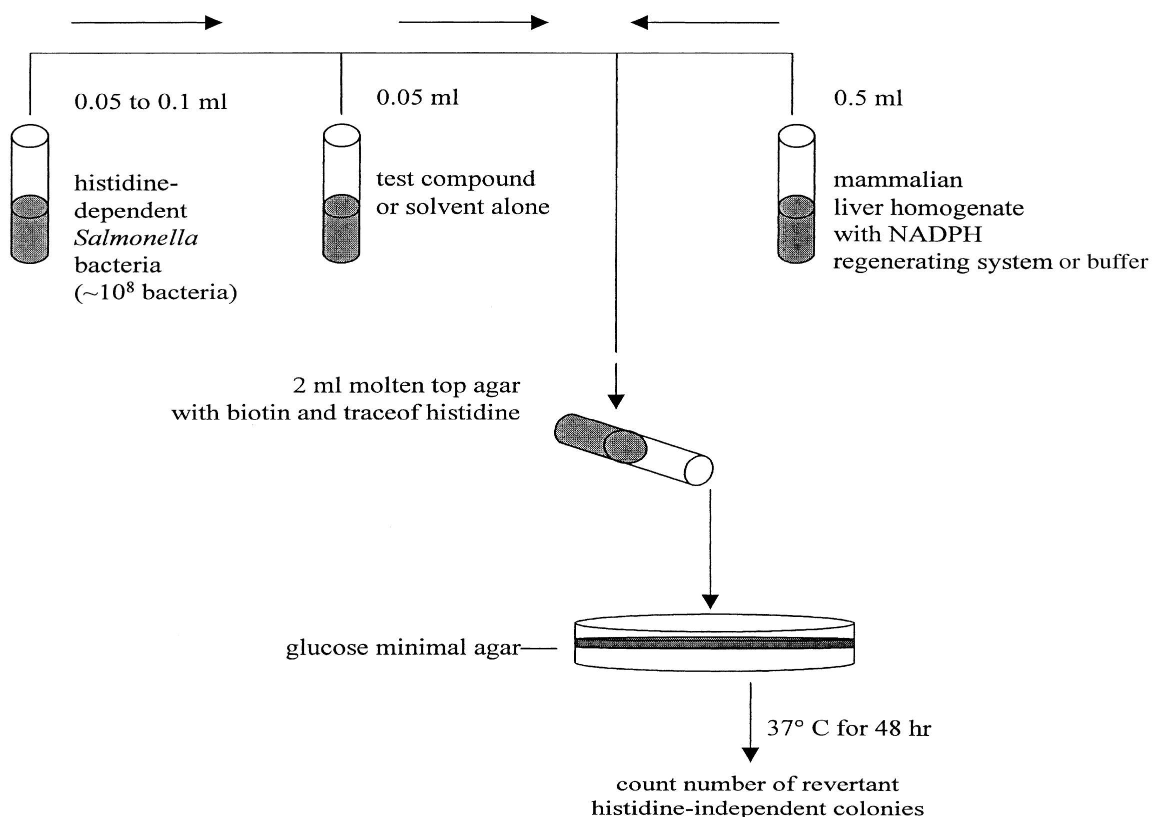


Figure 14: Diagram depicting the steps involved in the plates incorporation assay [MORTELMANS and ZEIGER, 2000]

*6.4.3 **Mutagenicity assays***

Mutagenicity assays were performed to demonstrate that stercobilin and urobilin are not mutagenic. Therefore, the highest and the lowest concentration of the bile pigments were screened (0.01 and 2µmol/plate). Every assay included three positive control plates and six negative control plates. These tests were conducted with and without metabolic activation.

In a typical mutagenicity assay the following substances were mixed in the **positive control tubes**:

- 500µl of PBS buffer or S9-mix
- 100µl of histidine dependent overnight culture bacteria
- 100µl of the respective mutagenic substance
- 200µl of PBS buffer

In a typical mutagenicity assay the following substances were mixed in the **negative control tubes**:

- 500µl of PBS buffer or S9-mix
- 100µl of histidine dependent overnight culture bacteria
- 100µl of DMSO
- 200µl of PBS buffer

In a typical mutagenicity assay the following substances were mixed in the **sample tubes**:

- 500µl of PBS buffer or S9-mix
- 200µl of bile pigment solution

6.5 Statistical analysis

6.5.1 ***Non statistical evaluation***

According to Mortelmans and Zeiger [MORTELMANS and ZEIGER, 2000] a non-statistical procedure was used to evaluate the results of Salmonella experiments:

- *Positive:*

  A *mutagenic* compound forms a reproducible, dose dependent elevation in the number of revertant colonies in one or more strains.

  A *weak mutagen* compound forms a reproducible, dose dependent elevation in the number of revertant colonies in one or more strains. However, the revertant number is not double the background colonies number.

- *Negative:*

  A *non-mutagenic* compound forms no dose dependent elevation in the number of revertant colonies in two independent assays.

- *Inconclusive:*

  An *inconclusive* compound cannot be considered as a mutagen or a nonmutagen (*e.g.*, if there is a spread of slightly revertant counts or one increased count).

In order to classify compounds as antimutagenic or promutagenic the following equation was used:

$$\frac{\text{No. of revertants (test compound)} - \text{No. of revertants (negative control)} \times 100}{\text{No. of revertants (positive control)} - \text{No. of revertants (negative control)}}$$

According to this equation, a compound was defined as '*antimutagenic*' if the relative mutagenic response fell below 50%. A compound was assessed to be '*promutagenic*' if the relative mutagenic response elevated beyond 200% [BULMER et al., 2007].

6.5.2 ***Statistical evaluation***

In order to illustrate the collected test data graphically Microsoft Excel 2007 and SPSS 17.0 were used. First of all, a Microsoft Excel spreadsheet was created including all the counted revertant data to illustrate the modulatory effects of stercobilin and urobilin in diagrams and curves (see Figures in chapter 7). In order to determine antimutagenic/antioxidant effects of bile pigments, the percent inhibition of revertant colonies compared to the positive control values was evaluated. In mutagenicity assays, results were put in relation to negative control values.

Appropriate statistical algorithms were used to illustrate each bile pigment in each condition optimally. Therefore the R^2 co-efficient was used to quantify the strength of the correlation. This coefficient of determination as well as the linear equation was superimposed onto the results figures.

The software SPSS 17.0 was used to analyse statistical significance of the test data. First of all, the data were tested for normal distribution using the K-S test of goodness of fit (Kolmogorov-Smirnov). This test showed that all data were

normally distributed. In order to investigate the revertant growth inhibition differences between the bile pigment concentrations related to positive and negative control values, the data were analysed with the one-way analysis of variance (ANOVA) and the post hoc tests Bonferroni and Scheffe. A p-value < 0.05 was considered significant.

7 RESULTS AND DISCUSSION

The statistical analysis revealed that stercobilin and urobilin show modulatory effects towards genotoxicity of different mutagens. As mentioned before, stercobilin and urobilin have not previously been investigated in the Ames *Salmonella* test. Therefore, findings might be of high interest and importance. The following section gives an overview on all the achieved results. Table 9 summarizes the collected data for both bile pigments.

Strain	Mutagen [mol/plate]	S9	Compound	ID_{50} [µmol/plate]	ID_{75} [µmol/plate]	$I_{0.5}$ [%]	I_{max} [%]	His^+
TA98	TNFone [0.3*10^{-6}]	-	S	2.98*	5.77*	15.8	32.3	453
			U	4.02*	17.8*	19.3	50.6	362
	PhiP [0.1*10^{-8}]	+	S	0.29*	3.14	56.8	68.9	442
			U	0.35	2.75	48.3	71.9	488
	AFB1 [1.2*10^{-8}]	+	S	1.73*	-	43.9	13.1	274.5
			U	-	0.18	76.9	78	316
TA102	TNFone [0.2*10^{-7}]	-	S	3.35*	13.4*	45.8	43.6	1218
			U	1.4	6.08*	54.1	48	1218
	AFB1 [2.9*10^{-7}]	+	S	0.46	-	40.8	-	1008
			U	1.35	7.18*	45	55.7	764
	t-BuOOH [0.75*10^{-6}]	-	S	-	-	2.1	-	1168
			U	7.15	-	25.9	22.8	1482
	t-BuOOH [0.75*10^{-6}]	+	S	4.68	-	34.4	48.8	2312
			U	1.97	-	50.5	42.9	824

Table 9: The modulatory effects of stercobilin and urobilin on genotoxicity in the *TA98* and *TA102 Salmonella typhimurium* strains [according to BULMER et al., 2007]

- **ID_{50}**: bile pigment dose [µmol/plate] required for 50% inhibition of the genotoxicity (positive control)
- **ID_{75}**: bile pigment dose [µmol/plate] required for 75% inhibition of the genotoxicity (positive control)
- **$I_{0.5}$:** inhibition of genotoxic effects [%] at 0.5µmol bile pigment per plate
- **I_{max}:** inhibition of genotoxic effects [%] at 2µmol bile pigment per plate
- **His^+:** mean number in the positive control plates of one and two experiments, respectively
- * Result was extrapolated beyond the concentration range tested [BULMER et al., 2007]

7.1 Antimutagenic testing with *TA98*

In the presence of *Salmonella typhimurium* tester strain *TA98* stercobilin and urobilin were tested for their potency to inhibit the mutagenicity of TNFone, PhiP and AFB1. Assays with TNFone were conducted without metabolic activation whereas tests with PhiP and AFB1 were performed with S9. Almost all data were significantly different from positive control values ($p<0.05$) and most of the assays were repeated under the same conditions.

7.1.1 ***Mutagenicity induced by TNFone***

Under this condition the tests on antimutagenic effects of stercobilin and urobilin were performed without metabolic activation. Figure 15 illustrates that both bile pigments inhibited the TNFone (0.0001 mg/ml) induced mutagenic response in the strain *TA98* in a dose dependent manner. Table 12-16 in the appendix provide the single revertant numbers for positive/negative control and different bile pigment concentrations. According to the extrapolated ID_{50} values, stercobilin (2.98µmol) was more effective than urobilin (4.02µmol). The latter compound showed a significant effect at all tested concentrations (with an

exception of 0.1µmol/plate), whereas stercobilin was at or above 1 µmol/ plate significantly different from positive control values (p<0.05). In general, the two bile pigments showed a common trend at the concentrations 2, 1, 0.5 and 0.1µmol/plate. Interestingly, lower concentrations of urobilin (0.05, 0.01, 0.005 and 0.001µmol/plate) increased the number of revertants above the positive control level (about 150%). Lower concentrations of stercobilin have leveled off at 100%. That implies that high concentrations of these two bile pigments effectively inhibited the TNFone induced genotoxic effects, whereas low concentrations did not. The coefficient of determination R^2 for stercobilin and urobilin was 0.86 and 0.94, respectively which expressed a strong correlation between single values.

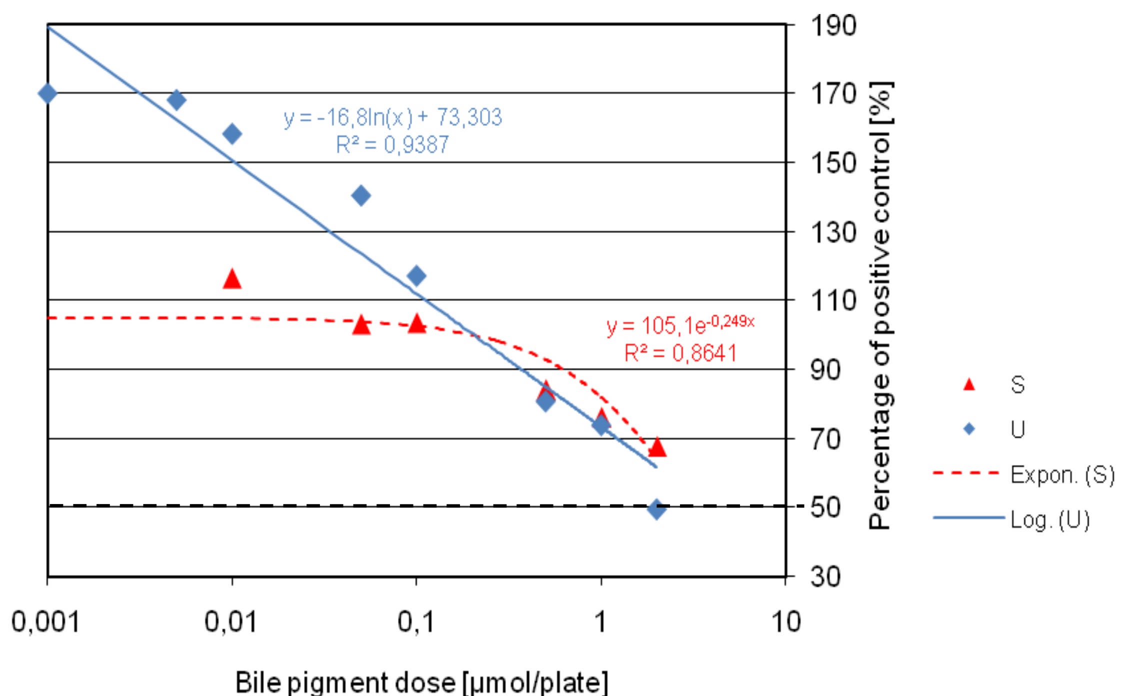


Figure 15: The modulatory effects of bile pigments, stercobilin ▲- - - ▲ (S) and urobilin ♦——— ♦ (U) towards TNFone induced genotoxicity in *TA98 Salmonella typhimurium* (S≥1µmol/plate, U≥0.5 and U≤0.05, p<0.05)

7.1.2 Mutagenicity induced by PhiP

PhiP (0.1µmol/ml) induced mutagenicity was used to investigate the possible antimutagenic effects of stercobilin and urobilin in the presence of metabolic activation. Single revertant numbers, mean values and standard deviations can be found in chapter 12, table 12-16. Figure 16 shows that the two bile pigments suppressed the PhiP induced genotoxic effects in a dose dependent manner. The remarkable significant antimutagenic properties of stercobilin and urobilin against PhiP followed the same trend. Both compounds were significantly effective at any tested concentration ($p<0.05$). The low ID_{50} of stercobilin (0.29µmol) was similar to that of urobilin (0.35µmol). Interestingly, even low concentrations of both bile pigments crossed the 50% line of inhibition. To sum up, stercobilin and urobilin showed strong antimutagenic effects towards PhiP induced genotoxicity in *TA98* at any tested concentration. As mentioned above, high R^2 values (0.83 and 0.95) confirmed a strong dose dependent correlation.

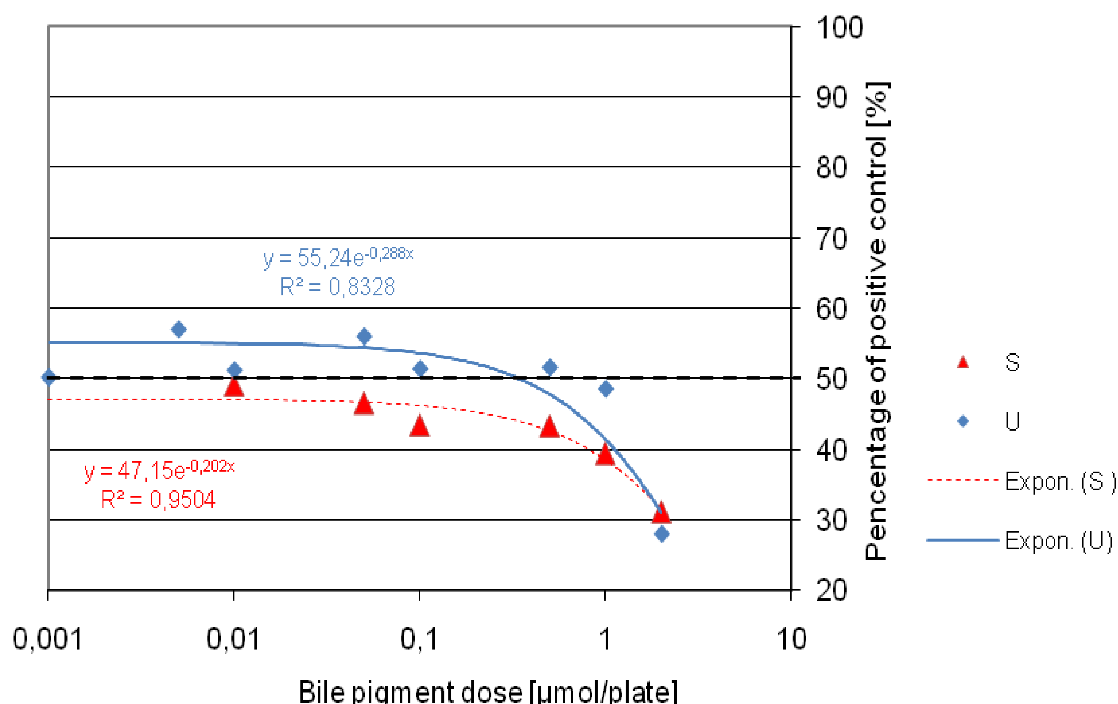


Figure 16: The modulatory effects of bile pigments, stercobilin ▲- - - ▲ (S) and urobilin ♦——♦ (U) towards PhiP induced genotoxicity in *TA98 Salmonella typhimurium* (*p*<0.05 at all concentrations tested)

7.1.3 ***Mutagenicity induced by AFB1***

In this condition, stercobilin and urobilin were tested for their antimutagenic effects in the presence of metabolic activation. Figure 17 clearly shows that urobilin was more effective than stercobilin. More specifically, urobilin showed strong significantly antimutagenic effects against AFB1 (5µg/ml), even at lower concentrations (p<0.05). A decrease in revertants was observed at higher concentrations and vice versa. Furthermore, R^2 of 0.864 pointed out a strong correlation which ensured the results concerning antimutagenicity.

Generally speaking, stercobilin showed weak antimutagenic effects against AFB1 characterized by an extrapolated ID_{50} value of 1.73µmol. Interestingly, stercobilin showed neither a dose-relation nor a twofold reduction of revertants. However, significant effects at any tested concentration (p<0.05), except for 2 µmol/plate (p=0.852), could be observed.

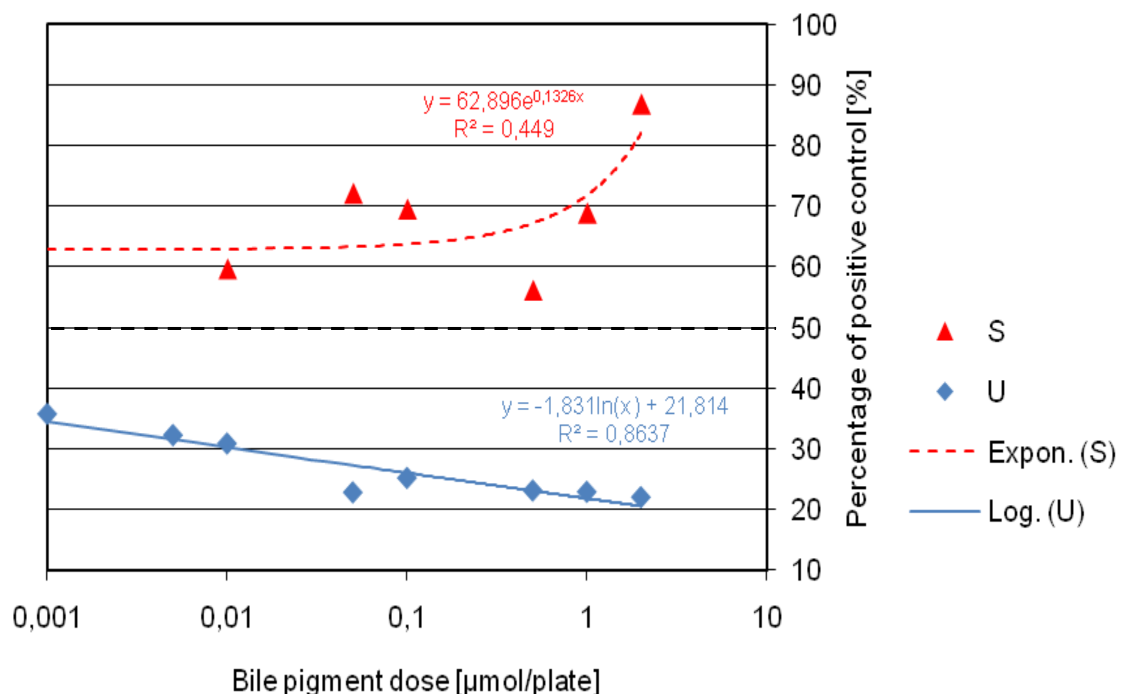


Figure 17: The modulatory effects of bile pigments, stercobilin ▲- - - ▲ (S) and urobilin ♦——♦ (U) towards AFB1 induced genotoxicity in *TA98 Salmonella typhimurium* (S≤1µmol/plate, U at all concentrations tested, *p*<0.05)

7.2 Antimutagenic and antioxidant testing with *TA102*

Salmonella typhimurium indicator strain *TA102* was used to study the antimutagenic and antioxidant properties of stercobilin and urobilin against the mutagenicity of TNFone, AFB1 and *t*-BuOOH.

TNFone as well as *t*-BuOOH were tested without a metabolic activation system. Furthermore, *t*-BuOOH and AFB1 were tested in the presence of S9.

Almost all data were significantly different from positive control values (p<0.05) and most of the assays were repeated under the same test conditions.

In 2007, Bulmer et al. used the tester strain *TA102* for the first time to investigate the antimutagenic and antioxidant effects of bile pigments (bilirubin, biliverdin, bilirubin ditaurate) in the Ames *Salmonella* test [BULMER et al., 2007].

7.2.1 ***Mutagenicity induced by TNFone***

To elucidate the efficacy of inhibitory action of stercobilin and urobilin against the mutagenicity of TNFone (0.07mg/ml), the *Salmonella* assays were performed without metabolic activation.

Both bile pigments showed a significant and dose-dependent inhibition of TNFone induced genotoxic effects (p<0.05). The ID_{50} for stercobilin and urobilin was approximately 3.35 and 1.4µmol, respectively. According to this value urobilin was more effective than stercobilin.

In general, both compounds shared a common trend in suppressing the number of His^+ revertants compared to the positive control values (see Figure 18). Furthermore, high concentrations of stercobilin and urobilin reduced the number of revertants approximately by 50%.

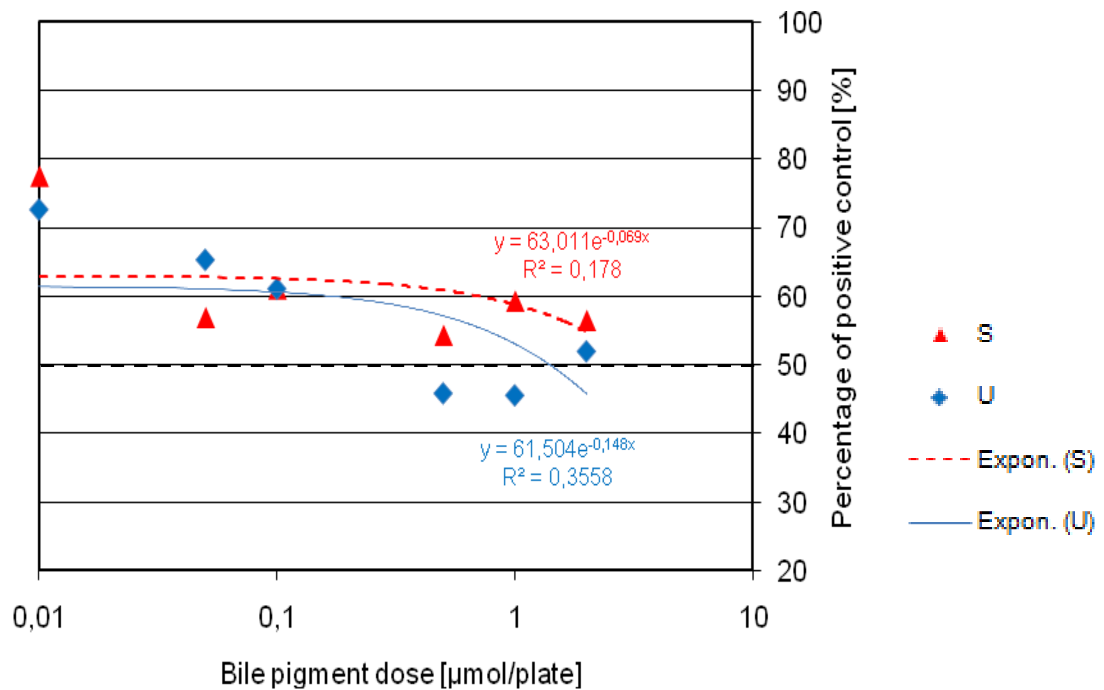


Figure 18: The modulatory effects of bile pigments, stercobilin ▲- - - ▲ (S) and urobilin ♦———♦ (U) towards TNFone induced genotoxicity in *TA102 Salmonella typhimurium* (*p*<0.05 at all concentrations tested)

7.2.2 ***Mutagenicity induced by AFB1***

In this condition the antimutagenic effects of stercobilin and urobilin were tested in the presence of metabolic activation. 120mg/ml AFB1 was used as mutagen.

Urobilin inhibited the AFB1 induced mutagenic response in a dose dependent manner. All used concentrations provided strong significant antimutagenic effects (*p*<0.05). In order to inhibit 50% of positive control, 1.35µmol urobilin per plate were needed. Furthermore, a twofold reduction was achieved at the highest concentration tested (see Figure 19).

Under these conditions, only an antimutagenic tendency could be observed for stercobilin. More precisely, no dose-related inhibitory response could be

observed. However, 0.01, 0.05, 0.1, 0.5 and 1µmol/plate of stercobilin were able to reduce the number of revertants significantly (*p*<0.05) from positive control values. Surprisingly, the tested concentration 2µmol/plate provoked a number of revertants that was slightly above the positive control level (about 105%). Therefore, statistical analysis did not show significant results for this concentration (*p*=1.000).

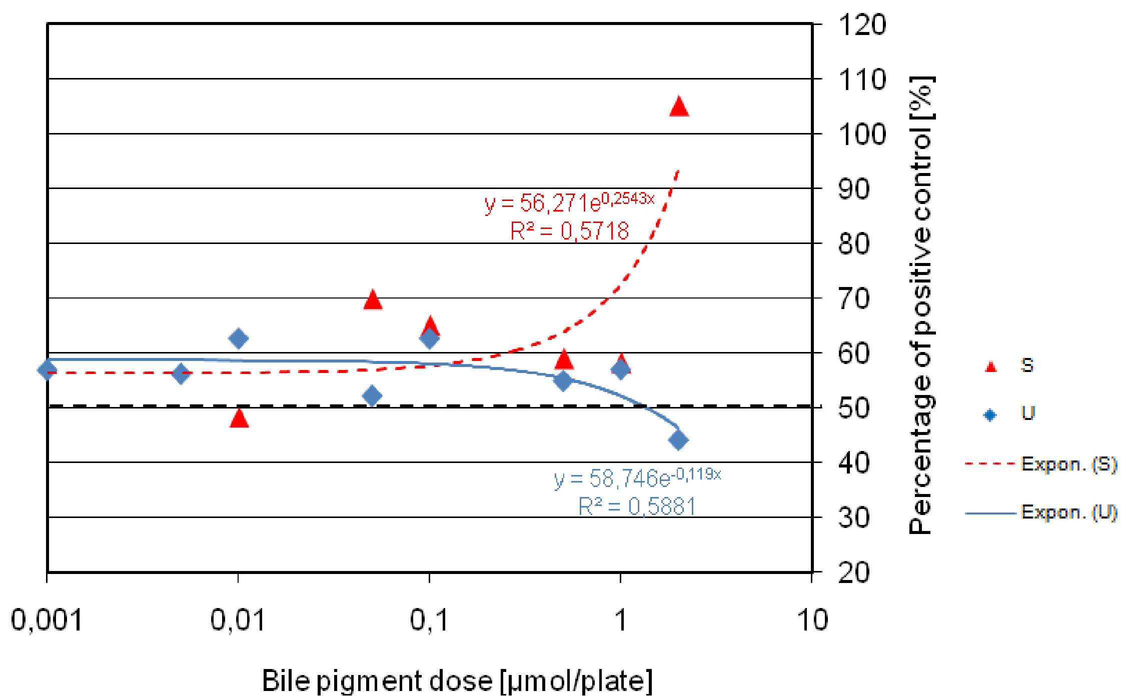


Figure 19: The modulatory effects of bile pigments, stercobilin ▲- - - ▲ (S) and urobilin ♦——— ♦ (U) towards AFB1 induced genotoxicity in *TA102 Salmonella typhimurium* (S≤1µmol/plate, U at all concentrations tested, *p*<0.05)

*7.2.3 **Mutagenicity induced by t-BuOOH without S9***

t-BuOOH (0.75*10^{-6} mol/plate) induced genotoxicity was used to investigate the possible antioxidant effects of stercobilin and urobilin. When metabolic activation was absent, the bile pigments provoked an inconclusive effect that cannot be observed as antioxidant. Stercobilin and urobilin were not able to reduce the number of revertants in a dose dependent manner. It can be seen from Figure 20 that urobilin was more effective than stercobilin. However, none of the tested concentrations afforded a significant protection against *t*-BuOOH induced pro-oxidative effects (p>0.05). Stercobilin caused a significant effect at the concentrations 0.05, 0.1 and 1µmol/plate. Nevertheless, this bile pigment did not reveal any antioxidant potential under the chosen test conditions. It is crucial to note that these results do not imply promutagenicity of bile pigments. In summary, these findings suggest that stercobilin and urobilin were not able to attenuate *t*-BuOOH induced mutation.

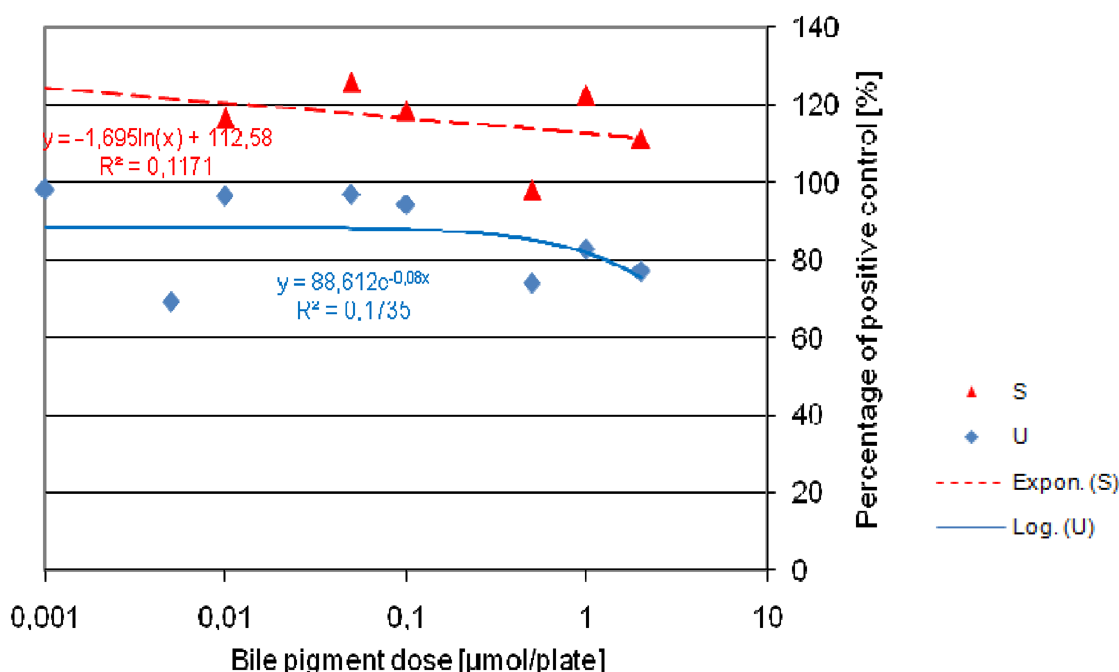


Figure20: The modulatory effects of bile pigments, stercobilin ▲- - - ▲ (S) and urobilin ♦——— ♦ (U) towards *t*-BuOOH induced genotoxicity in *TA102 Salmonella typhimurium* (S at 1, 0.1 and 0.05µmol/plate, *p*<0.05)

*7.2.4 **Mutagenicity induced by t-BuOOH with S9***

As mentioned above, this mutagen was used to elucidate the potential antioxidant properties of stercobilin and urobilin. When metabolic activation was utilized in this test model, weak antioxidant effects of both compounds could be observed.

The bile pigments inhibited the *t*-BuOOH induced mutagenic response not in a dose dependent manner. Urobilin reduced the number of revertants significantly different from positive control values at or above 0.01µmol/plate (p<0.05).

Although some stercobilin concentrations were able to decrease the number of revertants to half the positive control level, none of the tested concentrations showed significant differences (p>0.05).

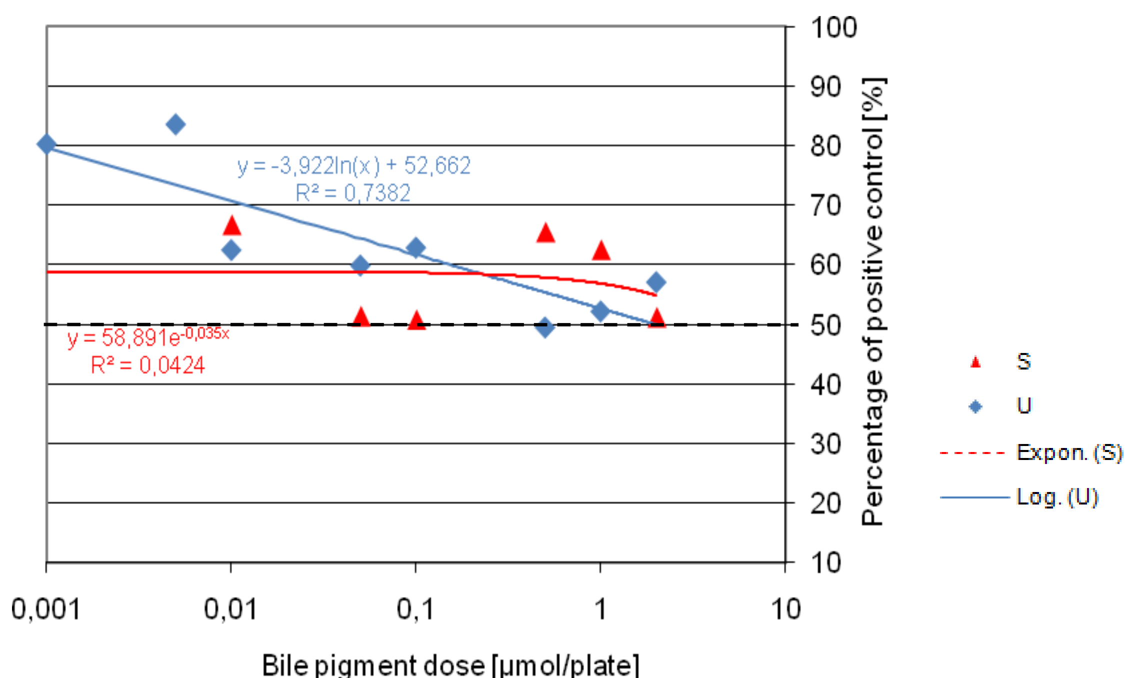


Figure 21: The modulatory effects of bile pigments, stercobilin ▲- - - ▲ (S) and urobilin ♦—— ♦ (U) towards *t*-BuOOH with S9 induced genotoxicity in *TA102 Salmonella typhimurium* (U≥0.01µmol/plate, S at all concentrations tested, *p*<0.05)

Numerous studies have been carried out during the past investigating the beneficial effects of bile pigments. These studies focused predominantly on the antimutagenic and antioxidant effects of bilirubin, biliverdin and related pyrrole pigments including hemin and chlorophyllin. However, available data on the beneficial effects of stercobilin and urobilin are sparse and therefore, this project attempted to fill this gap in current research. The present study aimed to test whether stercobilin and urobilin could selectively suppress the genotoxic effects of specific mutagens with different conformations.

In *Salmonella typhimurium* strain *TA98*, both bile pigments were able to inhibit the mutagenicity of TNFone, PhiP and AFB1. In the presence of this tester strain, stercobilin was the most effective antimutagen for PhiP. Urobilin was most active for AFB1. As mentioned earlier, tester strain *TA98* is suitable for detecting frameshift mutations [MARON and AMES, 1983].

More precisely, in the presence of $0.3*10^{-6}$ mol TNFone per plate, stercobilin was more effective than urobilin. The bile pigment dose that inhibited mutagenicity to 50% of the positive control was found to be approximately 3µmol for stercobilin and 4µmol for urobilin. Therefore, the inhibition potency was in the order S>U (see Figure 15).

In a recently published study, bilirubin, biliverdin and bilirubin ditaurate were tested for their potency to inhibit the mutagenicity of TNFone in *Salmonella typhimurium* strain *TA98*. All three compounds suppressed this genotoxic effect in a dose dependent manner (BR≥BRT>BV) [BULMER et al., 2007].

Taken together, these findings as well as our achieved results suggest that stercobilin, urobilin, bilirubin, biliverdin and bilirubin ditaurate influenced the impact of TNFone to destroy DNA structure [BULMER et al., 2008].

Concerning the PhiP results, stercobilin and urobilin showed strong antimutagenic effects in the presence of metabolic activation. $0.1*10^{-8}$ mol of PhiP were added to each plate. Both compounds were potent antimutagens which reduced the number of histidine revertants dose-related and significantly

different from positive control (p<0.05). In *Salmonella typhimurium* strain *TA98*, stercobilin was the most effective antimutagen for PhiP. Since there are no published studies available dealing with the modulatory effects of bile pigments towards PhiP induced genotoxicity, comparative results are lacking.

As previously mentioned, urobilin was the most potent antimutagen for AFB1 in the presence of *Salmonella typhimurium* tester strain *TA98.* A remarkable ID_{75} of 0.18µmol as well as an I_{max} of approximately 80% could be observed. As shown in Figure17, AFB1 induced genotoxicity ($1.2*10^{-8}$ mol/plate) was less strongly inhibited by stercobilin. Therefore, the order of effectiveness was U>S. In the present study, antimutagenicity of stercobilin and urobilin towards AFB1 induced genotoxicity was tested for the first time, therefore comparative data are missing.

In 1995, Breinholt et al. studied the antimutagenic properties of chlorophyllin against the mutagenicity of AFB1. It was shown that this compound of porphyrin-like structure suppressed AFB1 induced cancer in fish [BREINHOLT et al., 1995].

	TNFone	PhiP	AFB1
Stercobilin	-	↓↓↓	-
Urobilin	↓	↓↓	↓↓↓

Table 10: Modulatory effects of stercobilin and urobilin in *Salmonella typhimurium* strain *TA98*

-	ID_{50} not calculable
↓	50% inhibition of positive control at 2µmol/plate
↓↓	50% inhibition of positive control between 1 and 0.1µmol/plate and at 0.01 and 0.001 µmol/plate
↓↓↓	50% inhibition of positive control at each concentration

In *Salmonella typhimurium* strain *TA102*, stercobilin and urobilin showed different modulatory effects against the induced mutagenicity of TNFone, AFB1 and *t*-BuOOH. In the presence of strain *TA102,* stercobilin and urobilin were the most effective antimutagens for TNFone and AFB1, respectively.

As mentioned above, this tester strain was used for the first time by Bulmer et al. in 2007 who studied the antimutagenic and antioxidant properties of bilirubin, biliverdin and bilirubin ditaurate [BULMER et al., 2007]. This tester strain is sensitive to mutagens causing oxidative damage [MARON and AMES, 1983].

In the presence of $0.2*10^{-7}$mol TNFone per plate, urobilin was more effective than stercobilin. The ID_{50} of urobilin was approximately half of that for stercobilin (3.35, 1.4µmol, respectively) and therefore the order of effectiveness was U>S. Interestingly, this trend was not consistent in both tester strains. As previously described, in *TA98* the reverse order could be observed.

These positive findings agreed with the results gained by Bulmer et al. in 2007. In this study, bilirubin, biliverdin and bilirubin ditaurate inhibited the TNFone induced mutagenic response dose-related [BULMER et al., 2007].

Mutagenicity caused by AFB1 in the presence of *Salmonella typhimurium* strain *TA102* was prevented most effectively by urobilin, whereas weaker antimutagenic effects were observed for stercobilin. The AFB1 concentration chosen was 120µg/ml, which means that $2.9*10^{-7}$mol of AFB1 were added to each plate. Urobilin was found to inhibit the mutagenicity of AFB1 dose-related and significantly different from positive control values at all concentrations tested ($p<0.05$). This trend was consistent in both tester strains and therefore suggests that urobilin is a very strong antimutagen for AFB1. As mentioned before, unfortunately there are no comparative data available.

In order to investigate the antioxidant potential of stercobilin and urobilin, *t*-BuOOH induced pro-oxidative effects were used. The modulatory effects of both compounds on *t*-BuOOH induced mutation were similar with and without metabolic activation. $0.75*10^{-6}$ mol of the mutagen were added to each plate.

When metabolic activation was missing, stercobilin and urobilin were not effective in inhibiting the mutagenicity of *t*-BuOOH in *Salmonella typhimurium TA102* (see Fig.20). More precisely, urobilin was less active and stercobilin had no effect.

In the presence of metabolic activation, both compounds were not able to reduce number of revertants to half of the positive control (see Fig.21). Taken together, stercobilin and urobilin did not reveal antioxidant effects towards *t*-BuOOH induced genotoxicity.

The results obtained differ from a recently published study where bilirubin and biliverdin were demonstrated to inhibit *t*-BuOOH induced mutation [BULMER et al., 2007]. Under these conditions, bilirubin was more effective than biliverdin.

A growing body of evidence supports the view that bilirubin possesses strong antioxidant properties and acts as a chain-breaking compound [STOCKER et al., 1987a].

Furthermore, there is increasing evidence that the antioxidant potential of bilirubin could be explained by bilirubin´s capacity to donate two electrons and form a non-radical complex. Stocker et al. demonstrated that biliverdin can only accept one electron [STOCKER et al., 1987]. This fact could be a possible explanation why bilirubin was more effective than biliverdin in inhibiting *t*-BuOOH induced mutagenic response.

As mentioned above, in the presence of *t*-BuOOH, stercobilin and urobilin showed inconclusive effects that cannot be observed as being antioxidant. Unfortunately there are no comparative data available on the antioxidant properties of stercobilin and urobilin towards mutagenicity induced by *t*-BuOOH. Therefore further research is needed to confirm or refute our findings in the here presented study.

	TNFone	AFB1	*t*-BuOOH (-S9)	*t*-BuOOH (+S9)
Stercobilin	-	↓	-	↓↓↓
Urobilin	↓↓↓	↓↓	-	↓↓

Table 11: Modulatory effects of stercobilin and urobilin in *Salmonella typhimurium* strain *TA102*

- ID$_{50}$ not calculable

↓ 50% inhibition of positive control at 0.01µmol/plate

↓↓ 50% inhibition of positive control at 1 and 0.05µmol/plate (AFB1) and at 1 and 0.5µmol/plate (*t*-BuOOH +S9)

↓↓↓ 50% inhibition of positive control at 2, 0.1 and 0.05µmol/plate (stercobilin) and at 2, 1 and 0.5µmol/plate (urobilin)

7.3 Mutagenicity assays

A recent study revealed that bilirubin, biliverdin and bilirubin ditaurate had no mutagenicity against *Salmonella typhimurium* strains *TA98*, *TA100* and *TA102* [BULMER et al., 2007]. In order to ensure the non-mutagenic potential of stercobilin and urobilin in the present study, mutagenicity assays were conducted in concentrations of 0.01 and 2µmol/plate. Ames assays were carried out with and without metabolic activation using *TA98* and *TA102* strains. Altogether, four tests (*TA98* TNFone, *TA98* PhiP and AFB1 with S9, *TA102* TNFone and *t*-BuOOH and *TA102* AFB1 and *t*-BuOOH with S9) were performed to confirm non-mutagenic potential of the bile pigments.

Obviously, stercobilin and urobilin did not show any mutagenic effects in *TA98* and *TA102* (see Figure 23-26). This was true for every chosen test condition. All tested concentrations were significantly different from doubled negative control values ($p<0.001$) and significantly different from positive control values ($p>0.05$).

The following figures 23-26 show that none of the mutagenicity assays provided a dose-related increase in the number of revertant colonies. Furthermore, a two-fold increase of revertants could not be noticed in any test. Single revertant numbers as well as positive/negative control values for each test can be found in chapter 12 (Table 22-36). Generally it can be said that all data were situated in the negative control range. Interestingly, the majority of tested bile pigment concentrations reduced the number of histidine revertants compared to the negative control (DMSO).

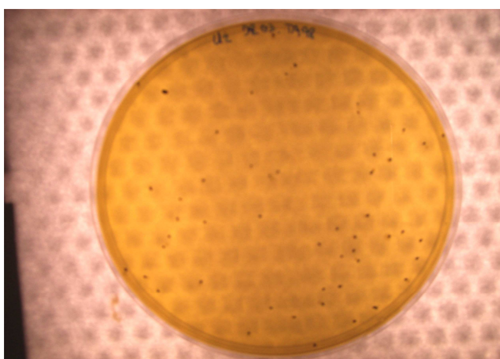


Figure 22: *TA98* colonies in a mutagenicity assay without metabolic activation (urobilin)

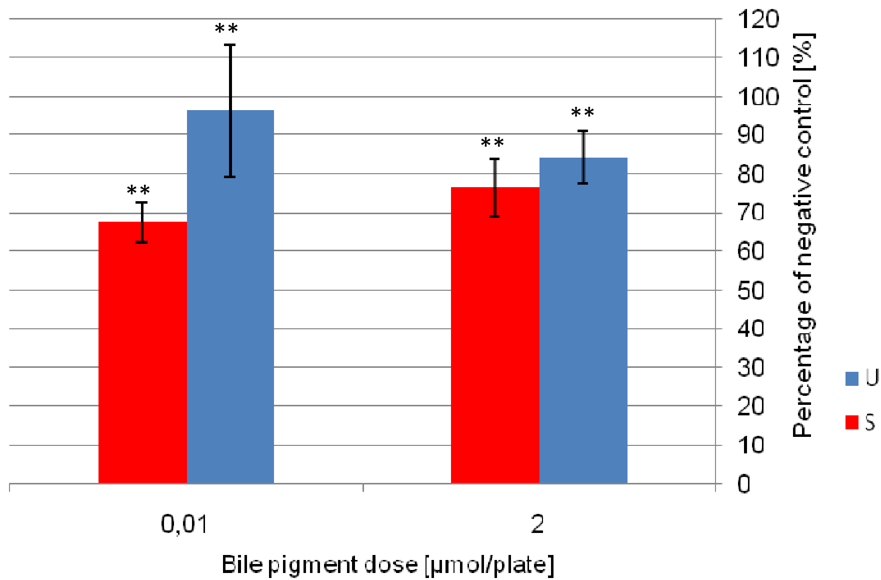


Figure 23: Non-mutagenic potential of stercobilin (S) and urobilin (U) towards TNFone induced genotoxicity in *TA98 Salmonella typhimurium* (* *, *p*<0.001 significantly different to the negative control)

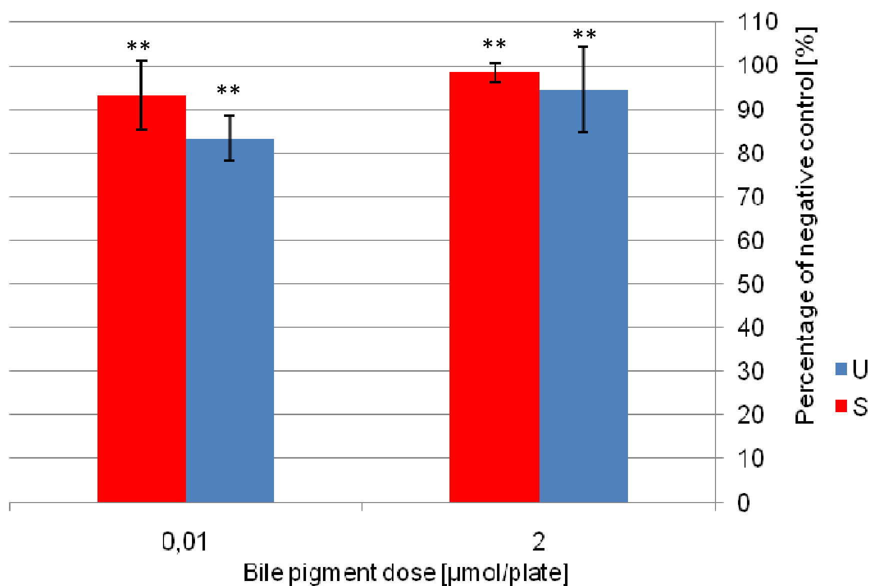


Figure 24: Non-mutagenic potential of stercobilin (S) and urobilin (U) towards PhiP and AFB1 induced genotoxicity in *TA98 Salmonella typhimurium* (* *, *p*<0.001 significantly different to the negative control)

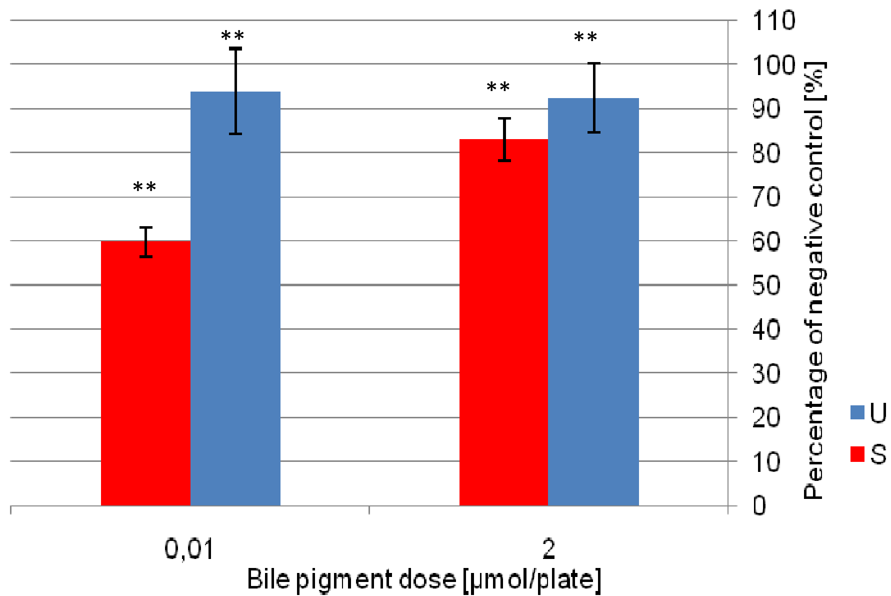


Figure 25: Non-mutagenic potential of stercobilin (S) and urobilin (U) towards TNFone and *t*-BuOOH induced genotoxicity in *TA102 Salmonella typhimurium* (* *, *p*<0.001 significantly different to the negative control)

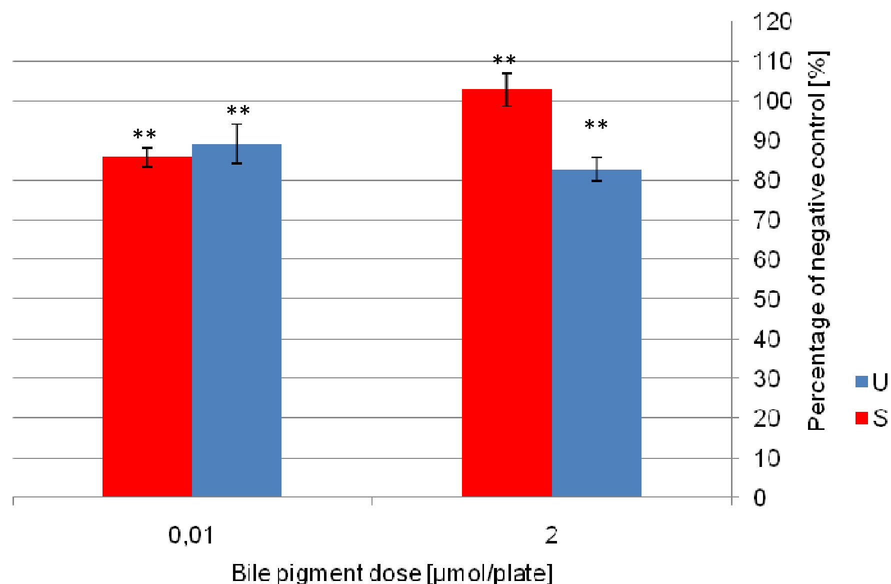


Figure 26: Non-mutagenic potential of stercobilin (S) and urobilin (U) towards AFB1 and *t*-BuOOH induced genotoxicity in *TA102 Salmonella typhimurium* (* *, *p*<0.001 significantly different to the negative control)

8 CONCLUSION

The aim of the present study was to investigate the antimutagenic and antioxidant potential of stercobilin and urobilin in the Ames *Salmonella* test. This is the first study to report that degradation products of bilirubin possess modulatory effects towards the genotoxicity of different mutagens. In continuation of formerly published data on a variety of bile pigments including bilirubin and biliverdin, novel findings of the present study might be of great physiological relevance.

As mentioned at the beginning, this study was part of the project: 'The physiological relevance of bile pigments *in vitro* to *in vivo* evidence of antioxidant, antimutagenic and anti-carcinogenic potential and their mechanisms of action'. In the course of this project the protective effects of eight bile pigments (bilirubin, biliverdin, bilirubin ditaurate, protoporphyrin, bilirubin dimethyl ester, biliverdin dimethyl ester, stercobilin and urobilin) were investigated in bacterial system, cell culture experiments and a human trial.

Experiments in the bacterial system revealed that stercobilin and urobilin alone did not induce mutagenicity in *TA98* and *TA102.* All presented data were significantly different from doubled negative control values ($p<0.001$) and significantly different from positive control values ($p>0.05$). This fact supports antimutagenicity of bile pigments and their physiological importance.

Antimutagenic testing in the presence of *Salmonella typhimurium TA98* and *TA102* indicated that stercobilin and urobilin possessed antimutagenic properties against the mutagenicity induced by TNFone, PhiP and AFB1.

Almost all experimental data were significantly different from positive control values ($p<0.05$). Chapter 12 includes all single revertant numbers, positive/negative control values, mean values and standard deviations. An

Antimutagenic potential could be observed independent of tester strain and metabolic activation.

TNFone induced mutagenic response was used to investigate the antimutagenic potential of stercobilin and urobilin in the absence of metabolic activation. The results clearly show that both compounds were able to suppress the TNFone induced genotoxic effect dose-related in *TA98* and *TA102*. The order of effectiveness according to ID_{50} values was S>U for *TA98* and U>S for *TA102*. However, *TA98* provided weaker antimutagenic effects, especially at lower concentrations.

PhiP induced mutagenicity was used to test the possible antimutagenic effects of stercobilin and urobilin in the presence of metabolic activation. Assays were conducted with *Salmonella typhimurium* strain *TA98*. Both compounds showed strong antimutagenic effects against the genotoxicity of this food related mutagen. The order of effectiveness was S>U.

Another food related mutagen, aflatoxin (AFB1), was used to investigate the antimutagenic potential of stercobilin and urobilin in the presence of metabolic activation. Antimutagenic testing with this mutagen was carried out with *Salmonella typhimurium* strain *TA98* and *TA102.* Mutagenicity caused by AFB1 was prevented most effectively by urobilin, whereas weaker antimutagenic effects were observed for stercobilin. This trend was consistent in both tester strains (U>S).

In order to determine the antioxidant potential of stercobilin and urobilin, pro-oxidative effects of an organic hydroperoxide were used. Regarding *Salmonella typhimurium* strain *TA102* in the antioxidant assays, stercobilin and urobilin could not show protection against oxidative stress induced by *t*-BuOOH. However, in the presence of metabolic activation, both compounds showed a tendency to act as antioxidant.

Summarizing the results of the antimutagenic assays in *TA98* and *TA102*, strong antimutagenic potential (PhiP, AFB1 TA98 and TNFone) as well as antimutagenic tendencies (AFB1 TA102) of stercobilin and urobilin were observed in the presence of all mutagens. Most significant results were obtained against the mutagenicity induced PhiP (both compounds) and the mutagenicity of AFB1 (urobilin).

Summarizing the findings of the antioxidant assays in *TA102*, no protective effect was observed towards the genotoxicity of *t*-BuOOH without metabolic activation. However, stercobilin and urobilin showed weak antioxidant effects in the presence of metabolic activation.

The total findings of the present study suggest that both degradation products of bilirubin, stercobilin and urobilin, possess broad ranging antimutagenic effects against a variety of mutagens in the Ames *Salmonella* test. Nevertheless, further confirmation of our findings is required to clarify the mechanisms of antimutagenic action. Furthermore, more research is needed to determine the antioxidant potential of stercobilin and urobilin.

9 SUMMARY

The aim of the present study was to investigate the antimutagenic and antioxidant potential of the bile pigments, stercobilin and urobilin, in the Ames *Salmonella* test. The experiments in the bacterial system were designed with two *Salmonella typhimurium* tester strains, *TA98* and *TA102*. Different mutagens including 2, 4, 7- trinitro-9-fluorenone (TNFone), aflatoxin B1 (AFB1), 2-amino-1-methyl-6-phenylimidazo [4, 5,-b] pyridine (PhiP) und *tert*-butylhydroxyperoxide (*t*-BuOOH) were used to confirm the formation of mutant revertants. Six doses of stercobilin (0.01, 0.05, 0.1, 0.5, 1 and 2 μmol/plate) and eight doses of urobilin (0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1 and 2 μmol/plate) were screened. In order to ensure non-mutagenic potential of bile pigments, mutagenicity assays were performed. Tests were conducted without (TNFone, *t*-BuOOH) and with (PhiP, AFB1 and *t*-BuOOH) metabolic activation.

Summarizing the results of the present study, stercobilin and urobilin could afford significant protection against TNFone, PhiP and AFB1 induced mutagenicity. Antimutagenicity was observed in the presence of these three mutagens independent of tester strain and metabolic activation. Antioxidant testing in the *TA102* strain revealed that stercobilin and urobilin could not effectively inhibit the genotoxic effects of *t*-BuOOH induced oxidative mutations. Therefore, stercobilin and urobilin showed weak antioxidant effects in this test system.

To conclude, these findings suggest apparent beneficial properties of stercobilin and urobilin. During the course of this project, further research will be done in this field exploring the mechanisms of antimutagenic and antioxidant action.

Studies in literature so far have primarily focused on bilirubin and biliverdin, so there are no comparative data for stercobilin and urobilin in the Ames *Salmonella* test available. Therefore, results of the present study might be of great interest and importance for further research in this field.

10 ZUSAMMENFASSUNG

Das Ziel der vorliegenden Studie war die Abschätzung des antimutagenen bzw. antioxidativen Potentials von den beiden Gallenpigmenten Stercobilin und Urobilin im *in vitro* Ames Test. Für die Untersuchung wurden die Bakterienstämme *Salmonella typhimurium TA98* und *TA102* verwendet. Als mutagene Substanzen wurden 2, 4, 7- Trinitro-9-Fluorenon (TNFone), Aflatoxin B1 (AFB1), 2-Amino-1-methyl-6-phenylimidazo [4, 5,-b] Pyridin (PhiP) und *tert*-Butylhydroxyperoxide (*t*-BuOOH) herangezogen. Die Testkonzentrationen der Gallenfarbstoffe reichten von 0.001 bis 2 µmol/Platte (Urobilin) bzw. von 0.01 bis 2 µmol/Platte (Stercobilin). Um sicherzustellen, dass die beiden Gallenpigmente kein mutagenes Potential aufweisen, wurde dies in Mutagenitätstests überprüft. Die Tests wurden sowohl ohne (TNFone, *t*-BuOOH) als auch mit (PhiP, AFB1, *t*-BuOOH) metabolischer Aktivierung durchgeführt.

Die Ergebnisse der vorliegenden Studie haben gezeigt, dass die beiden Gallenpigmente antimutagenes Potential gegenüber TNFone, PhiP und AFB1 aufweisen. Die entsprechende induzierte Mutagenität konnte, unabhängig von Bakterienstamm und metabolischer Aktivierung, signifikant inhibiert werden. Die durch *t*-BuOOH induzierten pro-oxidativen Effekte wurden von Stercobilin und Urobilin nicht durchgehend signifikant gehemmt. Dies lässt bei diesem Test auf eine tendenzielle antioxidative Schutzwirkung der Gallenpigmente schließen.

Zusammenfassend lässt sich sagen, dass Stercobilin und Urobilin antimutagene Eigenschaften aufweisen, die im Zuge des Gesamtprojektes noch näher erforscht und beleuchtet werden.

Da es sich hierbei um Pigmente handelt, deren antimutagenes bzw. antioxidatives Potential im Ames Test noch nicht erforscht worden ist, sind diese Untersuchungsergebnisse möglicherweise von erheblicher physiologischer Relevanz und großem Interesse für weitere Forschung.

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12 APPENDIX

12.1 Antimutagenic assays with *TA98*

12.1.1 *Single revertant numbers for TA98*

Mutagen	**Bile pigment dose [µmol/plate]**							
	2	**1**	**0.5**	**0.1**	**0.05**	**0.01**	**0.005**	**0.001**
TNF*one*								
Stercobilin	303	347	379	500	460	554	-	-
	312	343	384	427	472	567	-	-
	306	345	381	480	470	461	-	-
mean	307	345	381	469	467	527	-	-
sd	4.6	2.0	2.5	37.7	6.4	57.8	-	-
% of Pos	67.8	76.2	84.2	103.6	103.2	116.5	-	-
sd % of Pos	1.0	0.4	0.6	8.3	1.4	12.8	-	-
Urobilin	181	264	284	402	532	572	621	634
	192	269	294	445	494	613	594	582
	163	268	298	425	499	534	611	630
mean	179	267	292	424	508	573	609	615
sd	14.6	2.6	7.2	21.5	20.6	39.5	13.7	28.9
% of Pos	49.4	73.8	80.7	117.1	140.4	158.3	168.1	170.0
sd % of Pos	4.0	0.7	2.0	5.9	5.7	10.9	3.8	8.0
PhiP +S9								
Stercobilin	143	196	191	199	226	257	-	-
	151	202	214	221	219	242	-	-
	164	182	196	215	230	241	-	-
mean	153	193	200	212	225	247	-	-
sd	10.6	10.3	12.1	11.4	5.6	9.0	-	-
% of Pos	31.1	39.4	40.9	43.2	45.9	50.3	-	-
sd % of Pos	2.2	2.1	2.5	2.3	1.1	1.8	-	-
Urobilin	123	226	272	268	261	266	292	236
	147	238	255	237	302	246	290	269

	142	250	230	250	259	239	254	232
mean	137	238	252	252	274	250	279	246
sd	12.7	12.0	21.1	15.6	24.3	14.0	21.4	20.3
% of Pos	28.1	48.7	51.7	51.5	56.1	51.3	57.1	50.3
sd % of Pos	2.6	2.5	4.3	3.2	5.0	2.9	4.4	4.2
PhiP S9 Repetition								
Stercobilin	123	143	174	162	189	195	-	-
	131	159	180	172	192	181	-	-
	111	162	185	178	175	-	-	-
mean	122	155	180	171	185	188	-	-
sd	10.1	10.2	5.5	8.1	9.1	9.9	-	-
% of Pos	30.9	39.3	45.6	43.4	47.1	47.8	-	-
sd % of Pos	2.6	2.6	1.4	2.1	2.3	2.5	-	-
AFB1 +S9								
Stercobilin	302	191	180	175	201	207	-	-
	256	182	193	171	212	134	-	-
	262	165	181	169	195	134	-	-
mean	273	179	185	172	203	158	-	-
sd	25.0	13.2	7.2	3.1	8.6	42.1	-	-
% of Pos	111.3	73.0	75.2	69.9	82.5	64.5	-	-
sd % of Pos	10.2	5.4	2.9	1.2	3.5	17.2	-	-
Urobilin	71	76	71	82	78	92	104	116
	68	70	69	78	77	99	91	106
	70	71	79	79	61	102	112	118
mean	70	72	73	80	72	98	102	113
sd	1.5	3.2	5.3	2.1	9.5	5.1	10.6	6.4
% of Pos	22.0	22.9	23.1	25.2	22.8	30.9	32.3	35.8
sd % of Pos	0.5	1.0	1.7	0.7	3.0	1.6	3.4	2.0
AFB1 +S9 Repetition								
Stercobilin	196	209	175	243	204	176	-	-
	206	214	172	196	183	168	-	-
	167	166	153	190	175	154	-	-
mean	190	196	167	210	187	166	-	-
sd	20.3	26.4	11.9	29.0	15.0	11.1	-	-
% of Pos	62.6	64.8	55.0	69.2	61.8	54.8	-	-
sd % of Pos	6.7	8.7	3.9	9.6	4.9	3.7	-	-

Table 12: Single revertant numbers of stercobilin and urobilin towards TNFone and PhiP induced genotoxicity in *TA98*

12.1.2 *Positive control values TA98 for Stercobilin*

	TNFone	PhiP +S9	PhiP +S9 Repetition	AFB1 +S9	AFB1 +S9 Repetition
Positive I	467	463	425	291	321
Positive II	495	481	433	284	260
Positive III	396	527	323	162	328
mean	453	490	394	246	303
sd	51.0	33.0	61.3	72.5	37.4
% of Pos	100	100	100	100	100
sd % of Pos	11.3	6.7	15.6	29.5	12.3

Table 13: Positive control values for TNFone, PhiP and AFB1 in *TA98* (Stercobilin)

12.1.3 *Negative control values TA98 for Stercobilin*

	TNFone	PhiP +S9	PhiP +S9 Repetition	AFB1 +S9	AFB1 +S9 Repetition
Negative I	26	57	73	44	49
Negative II	33	59	80	47	51
Negative III	51	64	70	41	52
Negative IV	50	62	70	47	55
Negative V	43	52	70	63	48
Negative VI	39	51	67	61	42
mean	40	58	72	51	50
sd	9.8	5.2	4.5	9.2	4.4
% of Pos	8.9	11.7	18.2	20.6	16.3
sd % of Pos	2.2	1.1	1.1	3.7	1.5

Table 14: Negative control values for TNFone, PhiP and AFB1 in *TA98* (Stercobilin)

12.1.4 *Positive control values TA98 for Urobilin*

	TNFone	PhiP +S9	AFB1 +S9
Positive I	353	526	312
Positive II	341	472	331
Positive III	392	467	306
mean	362	488	316
sd	26.7	32.7	13.1
% of Pos	100	100	100
sd % of Pos	7.4	6.7	4.1

Table15: Positive control values for TNFone, PhiP and AFB1 in *TA98* (Urobilin)

12.1.5 *Negative control values TA98 for Urobilin*

	TNFone	PhiP +S9	AFB1 +S9
Negative I	30	55	55
Negative II	35	55	55
Negative III	38	42	42
Negative IV	25	66	66
Negative V	37	70	70
Negative VI	27	42	42
mean	32	55	55
sd	5.4	11.7	11.7
% of Pos	8.8	11.3	11.3
sd % of Pos	1.5	2.4	2.4

Table 16: Negative control values for TNFone, PhiP and AFB1 in *TA98* (Urobilin)

12.2 Antimutagenic/antioxidant assays with *TA102*

12.2.1 ***Single revertant numbers for TA102***

Mutagen	Bile pigment dose [µmol/plate]							
	2	1	0,5	0,1	0,05	0,01	0,005	0,001
TNF*one*								
Stercobilin	666	676	743	760	727	815	-	-
	686	693	658	774	688	991	-	-
	707	793	580	695	660	1022	-	-
mean	686	721	660	743	692	943	-	-
sd	20.5	63.2	81.5	42.2	33.7	111.6	-	-
% of Pos	56.4	59.2	54.2	61.0	56.8	77.4	-	-
sd % of Pos	1.7	5.2	6.7	3.5	2.8	9.2	-	-
Urobilin	625	509	685	769	783	1045	-	-
	642	591	589	721	792	882	-	-
	-	566	402	741	810	724	-	-
mean	634	555	559	744	795	884	-	-
sd	12.0	42.0	143.9	24.1	13.7	160.5	-	-
% of Pos	52.0	45.6	45.9	61.1	65.3	72.6	-	-
sd % of Pos	1.0	3.5	11.8	2.0	1.1	13.2	-	-
***t*-BuOOH**								
Stercobilin	1303	1412	1115	1416	1429	1462	-	-
	1293	1489	1137	1382	1475	1318	-	-
	-	1394	1178	1354	1501	1302	-	-
mean	1298	1432	1143	1384	1468	1361	-	-
sd	7.1	50.5	32.0	31.0	36.5	88.1	-	-
% of Pos	111.1	122.6	97.9	118.5	125.7	116.5	-	-
sd % of Pos	0.6	4.3	2.7	2.7	3.1	7.5	-	-
Urobilin	1268	1231	1130	1567	1430	1621	1043	1436
	1152	1199	1066	1039	1423	1506	1161	1489
	1012	1249	-	1580	1449	1159	883	1431
mean	1144	1226	1098	1395	1434	1429	1029	1452
sd	128.2	25.3	45.3	308.7	13.5	240.5	139.5	32.1
% of Pos	77.2	82.8	74.1	94.2	96.8	96.4	69.4	98.0
sd % of Pos	8.7	1.7	3.1	20.8	0.9	16.2	9.4	2.2
***t*-BuOOH +S9**								

Stercobilin	1221	1503	1643	1176	1231	1524	-	-
	1210	1393	1391	1230	1105	1578	-	-
	1123	1445	-	1118	1226	1531	-	-
mean	1185	1447	1517	1175	1187	1544	-	-
sd	53.7	55.0	178.2	56.0	71.3	29.4	-	-
% of Pos	51.2	62.6	65.6	50.8	51.4	66.8	-	-
sd % of Pos	2.3	2.4	7.7	2.4	3.1	1.3	-	-
Urobilin	384	482	495	553	583	521	648	697
	407	440	444	501	574	543	791	696
	621	370	285	502	324	481	629	593
mean	471	431	408	519	494	515	689	662
sd	130.7	56.6	109.5	29.7	147.0	31.4	88.6	59.8
% of Pos	57.1	52.2	49.5	62.9	59.9	62.5	83.6	80.3
sd % of Pos	15.9	6.9	13.3	3.6	17.8	3.8	10.7	7.2
AFB1 +S9								
Stercobilin	993	581	578	745	602	455	-	-
	1180	580	601	625	963	473	-	-
	1009	605	612	601	551	539	-	-
mean	1061	589	597	657	705	489	-	-
sd	103.7	14.2	17.3	77.1	224.6	44.2	-	-
% of Pos	105.2	58.4	59.2	65.2	70.0	48.5	-	-
sd % of Pos	10.3	1.4	1.7	7.7	22.3	4.4	-	-
Urobilin	312	442	452	491	394	481	367	473
	341	474	410	484	382	495	468	427
	363	392	399	462	422	461	455	406
mean	339	436	420	479	399	479	430	435
sd	25.6	41.3	28.0	15.1	20.5	17.1	54.9	34.3
% of Pos	44.3	57.1	55.0	62.7	52.3	62.7	56.3	57.0
sd % of Pos	3.3	5.4	3.7	2.0	2.7	2.2	7.2	4.5

Table 17: Single revertant numbers of stercobilin and urobilin towards TNFone, *t*-BuOOH and AFB1 induced genotoxicity in TA102

12.2.2 *Positive control values TA102 for Stercobilin*

	TNFone	*t*-BuOOH	*t*-BuOOH +S9	AFB1 +S9
Positive I	1306	1086	1992	1006
Positive II	1402	1250	2500	1003
Positive III	1375	-	2444	1015
mean	1218	1168	2312	1008
sd	160.6	116.0	278.5	6.2
% of Pos	100	100	100	100
sd % of Pos	13.2	9.9	12.0	0.6

Table 18: Positive control values for TNFone, *t*-BuOOH and AFB1 in *TA102* (Stercobilin)

12.2.3 *Negative control values TA102 for Stercobilin*

	TNFone	*t*-BuOOH	*t*-BuOOH +S9	AFB1 +S9
Negative I	371	341	480	421
Negative II	318	311	465	495
Negative III	235	323	428	415
Negative IV	369	329	382	433
Negative V	320	385	405	461
Negative VI	339	310	-	445
mean	325	333	432	445
sd	49.8	27.9	40.7	29.6
% of Pos	26.7	28.5	18.7	44.1
sd % of Pos	4.1	2.4	1.8	2.9

Table 19: Negative control values for TNFone, *t*-BuOOH, AFB1 in *TA102* (Stercobilin)

12.2.4 Positive control values TA102 for Urobilin

	TNFone	*t*-BuOOH	*t*-BuOOH +S9	AFB1 +S9
Positive I	1306	1601	905	721
Positive II	1402	1553	810	759
Positive III	1375	1291	758	811
mean	1218	1482	824	764
sd	160.6	166.9	74.5	45.2
% of Pos	100	100	100	100
sd % of Pos	13.2	11.3	9.0	5.9

Table 20: Positive control values for TNFone, *t*-BuOOH and AFB1 in *TA102* (Urobilin)

12.2.5 Negative control values TA102 for Urobilin

	TNFone	*t*-BuOOH	*t*-BuOOH +S9	AFB1 +S9
Negative I	371	392	237	303
Negative II	318	411	255	291
Negative III	235	333	292	290
Negative IV	369	371	223	259
Negative V	320	337	229	272
Negative VI	339	391	185	314
mean	325	373	237	288
sd	49.8	31.7	35.5	20.1
% of Pos	26.7	25.1	28.7	37.7
sd % of Pos	4.1	2.1	4.3	2.6

Table 21: Negative control values for TNFone, *t*-BuOOH and AFB1 in *TA102* (Urobilin)

12.3 Mutagenicity assays with *TA98* and *TA102*

12.3.1 ***Single revertant numbers for TA98 without S9***

Mutagen	**Bile pigment dose [µmol/plate]**	
	2	**0,01**
TNF*one*		
Stercobilin	47	36
	42	36
	39	36
mean	43	38
sd	4.0	2.9
% of Neg	76.4	67.5
sd % of Neg	7.2	5.2
Urobilin	54	53
	54	53
	47	71
mean	52	59
sd	4.0	10.4
% of Neg	84.2	96.2
sd % of Neg	6.6	16.9

Table 22: Single revertant numbers of stercobilin and urobilin towards TNFone induced genotoxicity in *TA98*

12.3.2 ***Positive control values TA98 without S9 for Stercobilin***

	TNFone
Positive I	417
Positive II	425
Positive III	434
mean	425
sd	8.5

Table 23: Positive control values for TNFone in *TA98* (Stercobilin)

12.3.3 ***Negative control values TA98 without S9 for Stercobilin***

	TNFone
Negative I	58
Negative II	59
Negative III	57
Negative IV	60
Negative V	51
Negative VI	50
mean	56
sd	4.3
% of Neg	100
sd % of Neg	7.6

Table 24: Negative control values for TNFone in *TA98* (Stercobilin)

12.3.4 ***Positive control values TA98 without S9 for Urobilin***

	TNFone
Positive I	462
Positive II	478
Positive III	442
mean	461
sd	18.0

Table25: Positive control values for TNFone in *TA98* (Urobilin)

12.3.5 *Negative control values TA98 without S9 for Urobilin*

	TNFone
Negative I	63
Negative II	62
Negative III	56
Negative IV	61
Negative V	60
Negative VI	66
mean	61
sd	3.3
% of Neg	100
sd % of Neg	5.4

Table26: Negative control values for TNFone in *TA98* (Urobilin)

12.3.6 *Single revertant numbers for TA102 without S9*

Mutagen	**Bile pigment dose [µmol/plate]**	
	2	**0,01**
TNFone		
Stercobilin	337	225
	349	243
	312	250
mean	333	239
sd	18.9	12.9
% of Neg	83.0	59.7
sd % of Neg	4.7	3.2
Urobilin	341	366
	353	335
	400	411
mean	365	371
sd	31.2	38.2
% of Neg	92.3	93.8
sd % of Neg	7.9	9.7
***t*-BuOOH**		
Stercobilin	337	225

	349	243
	312	250
mean	333	239
sd	18.9	12.9
% of Neg	83.0	59.7
sd % of Neg	4.7	3.2
Urobilin	341	366
	353	335
	400	411
mean	365	371
sd	31.2	38.2
% of Neg	92.3	93.8
sd % of Neg	7.9	9.7

Table 27: Single revertant numbers of stercobilin and urobilin towards TNFone and *t*-BuOOH induced genotoxicity in *TA102*

12.3.7 *Positive control values TA102 without S9 for Stercobilin*

	TNFone	**t-BuOOH**
Positive I	1106	1653
Positive II	1121	1641
Positive III	1109	-
mean	1112	1647
sd	7.9	8.5

Table 28: Positive control values for TNFone and *t*-BuOOH in *TA102* (Stercobilin)

12.3.8 *Negative control values TA102 without S9 for Stercobilin*

	TNFone	t-BuOOH
Negative I	397	397
Negative II	427	427
Negative III	395	395
Negative IV	402	402
Negative V	417	417
Negative VI	367	367
mean	401	401
sd	20.7	20.7
% of Neg	100	100
sd % of Neg	5.2	5.2

Table29: Negative control values for TNFone and *t*-BuOOH in *TA102* (Stercobilin)

12.3.9 *Positive control values TA102 without S9 for Urobilin*

	TNFone	t-BuOOH
Positive I	1092	1309
Positive II	1045	1151
Positive III	1107	1158
mean	1081	1206
sd	32.3	89.3

Table 30: Positive control values for TNFone and *t*-BuOOH in *TA102* (Urobilin)

12.3.10 *Single revertant numbers for TA98 with S9*

Mutagen	Bile pigment dose [µmol/plate]	
	2	**0,01**
Phip		
Stercobilin	49	47
	51	43
	49	51
mean	50	47
sd	1.2	4.0
% of Neg	98.3	93.1
sd % of Neg	2.3	7.9
Urobilin	51	41
	42	45
	50	40
mean	48	42
sd	4.9	2.6
% of Neg	94.4	83.2
sd % of Neg	9.8	5.2
AFB1		
Stercobilin	49	47
	51	43
	49	51
mean	50	47
sd	1.2	4.0
% of Neg	98.3	93.1
sd % of Neg	2.3	7.9
Urobilin	51	41
	42	45
	50	40
mean	48	42
sd	4.9	2.6
% of Neg	94.4	83.2
sd % of Neg	9.8	5.2

Table31: Single revertant numbers of stercobilin and urobilin towards PhiP and AFB1 induced genotoxicity in *TA98*

12.3.11 ***Positive control values TA98 with S9 for Stercobilin and Urobilin***

	PhiP	AFB1
Positive I	349	123
Positive II	385	151
Positive III	422	162
mean	385	145
sd	36.5	20.1

Table32: Positive control values for PhiP and AFB1 in *TA98* (Stercobilin and Urobilin)

12.3.12 ***Negative control values TA98 with S9 for Stercobilin and Urobilin***

	PhiP	AFB1
Negative I	56	56
Negative II	50	50
Negative III	48	48
Negative IV	50	50
Negative V	51	51
Negative VI	48	48
mean	51	51
sd	2.9	2.9
% of Neg	100	100
sd % of Neg	5.8	5.8

Table33: Negative control values for PhiP and AFB1 in *TA98* (Stercobilin and Urobilin)

12.3.13 *Single revertant numbers for TA102 with S9*

Mutagen	**Bile pigment dose [µmol/plate]**	
	2	**0,01**
t-BuOOH		
Stercobilin	361	313
	380	325
	391	307
mean	377	315
sd	15.2	9.2
% of Neg	102.7	85.7
sd % of Neg	4.1	2.5
Urobilin	292	307
	314	332
	306	343
mean	304	327
sd	11.1	18.4
% of Neg	82.7	89.1
sd % of Neg	3.0	5.0
AFB1		
Stercobilin	361	313
	380	325
	391	307
mean	377	315
sd	15.2	9.2
% of Neg	102.7	85.7
sd % of Neg	4.1	2.5
Urobilin	292	307
	314	332
	306	343
mean	304	327
sd	11.1	18.4
% of Neg	82.7	89.1
sd % of Neg	3.0	5.0

Table34: Single revertant numbers of stercobilin and urobilin towards *t*-BuOOH and AFB1 induced genotoxicity in *TA102*

12.3.14 Positive control values TA102 with S9 for Stercobilin and Urobilin

	t-BuOOH	AFB1
Positive I	1914	725
Positive II	2003	701
Positive III	1902	694
mean	1940	707
sd	55.2	16.3

Table 35: Positive control values for *t*-BuOOH and AFB1 in *TA102* (Stercobilin and Urobilin)

12.3.15 Negative control values TA102 with S9 for Stercobilin and Urobilin

	t-BuOOH	AFB1
Negative I	388	388
Negative II	404	404
Negative III	327	327
Negative IV	387	387
Negative V	355	355
Negative VI	344	344
mean	368	368
sd	29.9	29.9
% of Neg	100	100
sd % of Neg	8.1	8.1

Table 36: Negative control values for *t*-BuOOH and AFB1 in *TA102* (Stercobilin and Urobilin)

13 CURRICULUM VITAE

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