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EFFECTS OF GLUTATHIONE AND GLUTATHIONE-S - TRANSFERASE ON AFLATOXIN B(,1) MUTAGENESIS IN THE AMES TEST

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EFFECTS OF GLUTATHIONE AND GLUTATHIONE-S-TRANSFERASE ON AFLATOXIN B_1 MUTAGENESIS IN THE AMES TEST

by

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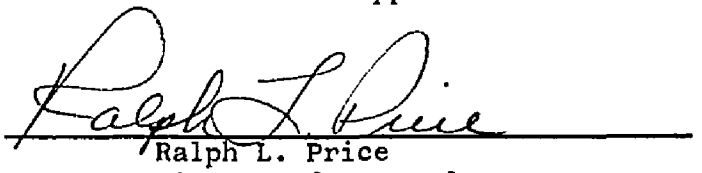

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ABSTRACT

The effects of glutathione (GSH) and the combination of GSH and glutathione-S-transferase (G-S-T) on aflatoxin B_1 (AFB_1) mutagenesis in the Ames test using Salmonella typhimurium strains TA 98 and TA 100 were tested. Ten concentrations of AFB_1 from 0 to 1.0 µg per plate added to a liver microsomal homogenate (S-9), and preplating incubation times of 0 and 30 minutes at 37° C were used. The mixtures of AFB_1 and S-9 mix were incubated prior to plating with and without bacteria, GSH and GSH+G-S-T. The addition of GSH and GSH+G-S-T caused a significant decrease in the mutagenesis with both strains at concentrations below 0.1 µg. At concentrations of 0.1 µg or greater, the lethal effect of AFB_1 was decreased. One explanation for these decreases is the formation of the AFB_1-GSH conjugate. This study shows that the AFB_1-GSH conjugation reaction is a detoxification reaction for AFB_1 when using the Ames test.

CHAPTER 1

INTRODUCTION

The Ames test, a short-term, in vitro, mutagenic assay, is used for the determination of chemically induced mutagenesis. This test is used for proposed food additives, drugs (for animal and human use), natural or environmental contaminates and current or proposed pesticides as an indicator of potential carcinogenicity and has proven to be a reliable assay (1,2,3).

Many compounds have been tested for mutagenicity using the Ames test and approximately 90% of those with positive results have shown to be carcinogenic in animal studies (4). Of these substances, many require bioactivation by a liver enzyme system showing that they are promutagens; these compounds have also been shown to require bioactivation prior to causing cancer (1,3). The majority of these substances which are bioactivated in the liver are then conjugated by substances such as glutathione (GSH) and glucuronic acid. The conjugation results in the addition of the GSH or glucuronic acid to the parent compound so that it could be excreted. Since this is a 2-phase process, bioactivation and conjugation, the conjugated metabolite should also be considered in the toxicity, mutagenicity or carcinogenicity testing. Few chemicals have been tested to determine if the conjugation of these compounds increases or decreases the mutagenesis with the Ames test.

Aflatoxin, a group of secondary metabolites of the molds Aspergillus flavus and Aspergillus parasiticus, have been a major concern not only because of the health hazards but also because of their occurrence in foods and feeds. Aflatoxin is found naturally in four forms, B_1, B_2, G_1 and G_2, and is known to contaminate a variety of agricultural commodities. Of the forms known, aflatoxin B_1 (AFB_1), is not only the most common but has recently been termed as the most potent, naturally-occurring cancer-producing substance known (5). Aflatoxin B_1 is a potent mutagen with the Ames test; recently, a direct correlation between the mutagenic action of AFB_1 and its carcinogenicity has been shown (1).

Aflatoxin has been shown to be a promutagen and procarcinogen, indicating that it requires activation before it can cause adverse effects. Many carcinogenic or mutagenic substances have been shown to require this activation. Examples are benzo-(a)-pyrene, dibromoethane, and dichloroethane (5,6). People are exposed to many of these substances in many ways. For example, charbroiling of certain foods can result in the formation of benzo-(a)-pyrene or complex substances resulting from the cooking of foods in general. For example, protein-sugar derivatives of tyrosine have been found to be mutagenic in the Ames test (7,8,9).

Understanding how these substances are metabolized and what makes them mutagenic or carcinogenic is important. Determining the effects of conjugation on the mutagenesis or carcinogenic activity can be just as important. For example, the dibromoethane (EDB)-GSH conjugate has been tested for mutagenesis with the Ames test. Results of this study showed

that reaction with GSH actually increased the mutagenesis. Further studies indicated that the EDB could react either directly with the GSH or be activated by the cytochrome-P-450 system and then react with GSH. It was found that the direct reaction resulted in an increase in the activity due to a GSH mediated reaction between the EDB and the DNA.(10).

The purpose of this study was to determine if the addition of GSH affected the incidence of frame-shift or base-pair substitution mutations induced by AFB_1. The enzyme, glutathione-S-transferase (G-S-T), was added to a portion of the samples containing the GSH to determine the effect of the enzyme, which would catalyze the formation of the AFB_1- GSH conjugate.

CHAPTER 2

LITERATURE REVIEW

Aflatoxin

Most aflatoxin show various biological effects to animals or humans consuming commodities contaminated with them. After extensive research the following effects have been reported:

1. Cytotoxicity
2. Mutagenicity
3. Carcinogenicity
4. Teratogenicity
5. Inhibition of DNA, RNA, lipid and protein synthesis
6. Inhibition of membrane transport system
7. Covalent binding to macromolecules.

More recently there have been reports that AFB_1 exposure can increase the susceptibility to hepatitis (11). It has also been linked to Reye's Syndrome, but a direct casual effect is difficult to determine (11).

Because of the unavoidable occurrence of this mycotoxin, it is important that metabolism and the mode of action are understood.

Biotransformation of Aflatoxin

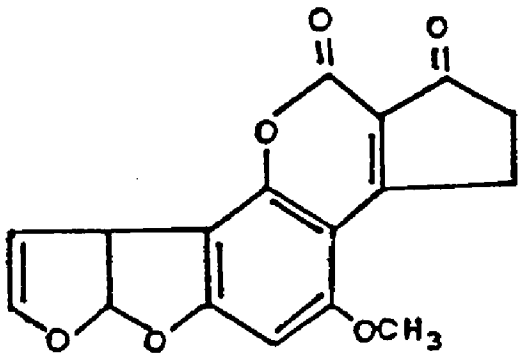
Aflatoxin is metabolized by the mixed function oxidase system of the liver, the cytochrome-P-450. The cytochrome-P-450 activates the

AFB_1 to the 8,9-epoxide form (Figure 1), which has been shown to be biologically active. Through the epoxide form the AFB_1 can covalently bind to DNA, RNA, and other macromolecules, an event which may cause numerous adverse effects. It is also metabolized by reduction, hydroxylation, hydration, O-demethylation and epoxidation (Table 1) (12).

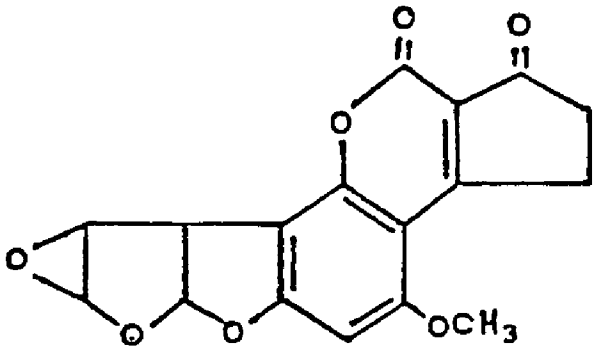
Role of Glutathione in AFB_1 Metabolism *in vivo*

Once the AFB_1 has been activated by the cytochrome-P-450 system to form the AFB_1-8,9,-epoxide, research has shown that the major conjugate formed is the AFB_1- GSH conjugate. This is the result of the AFB_1-8,9,-epoxide reacting either directly with or aided by the enzyme, glutathione-S-transferase (G-S-T), with the GSH (15, 16). The AFB_1- GSH conjugate, 8,9-dihydro-8-(S-glutathionyl)-9-hydroxy-AFB_1, has been identified as the major biliary metabolite (13).

A study by Loury et. al. (14) showed that pretreatment of rats with phenobarbital (PB) resulted in a decrease in AFB_1 toxicity and an increase in the amount of the AFB_1- GSH conjugate formed. Phenobarbital pretreatment not only induced the cytochrome-P-450 system but also increased the activity of the enzyme G-S-T (15, 16). This resulted in increased formation of the AFB_1- GSH conjugate. There was a direct correlation between the increase in G-S-T activity and the decrease in tumorogenic activity of the AFB_1 in rat studies. McBodile et al. (17) showed a decrease in hepatotoxic effects of AFB_1 in rats pretreated with PB. They determined from this work that the role of G-S-T was important in the detoxification of AFB_1 *in vivo* because the hepatic concentration of the GSH was not increased by the PB treatment.



AFLATOXIN B_1



AFLATOXIN B_1 -8,9- OXIDE

Figure 1. Structures of AFB_1 and AFB_1-8,9-oxide

Table 1. Examples of AFB_1 Metabolites

Reaction	Metabolite
Reduction	Aflatoxicol
Hydroxylation	AFM_1, AFQ_1
Hydration	AFB_2
o-demethylation	AFP_1
Epoxidation	AFB_1-8,9-oxide

Raj et al. (18) determined that there is a G-S-T which has a high affinity for AFB_1, which is also induced by PB. Since they found no increase in the GSH levels, they concluded that the rate of the conjugation reaction between the AFB_1-8,9-oxide and the GSH is dependent on the amount of G-S-T present rather than on the amount of GSH.

Role of Glutathione in AFB_1 Metabolism *in vitro*

Loury et al. (14) showed a decrease in the cytoxicity of AFB_1 in rat liver cultures which were treated with PB. The results showed that not only were the levels of the cytochrome-P-450 increased but so were the levels of the G-S-T.

The effects of GSH in the Ames test are currently being explored. Wong and Hsieh (19) showed that addition of GSH to the S-9 fraction of the Ames test did decrease the mutagenesis of AFB_1. However, they also changed the assay from a bilayer assay to a trilayer assay. The GSH and AFB_1 along with the S-9 were added in one layer of top agar, while the bacteria was added in another. This allowed for the AFB_1-8,9-oxide-GSH conjugation to readily occur. They suggested that the addition of the GSH to the same layer as the bacteria would not allow enough time for the formation of the AFB_1-GSH because of the presence and permeability of the bacteria.

Mutagenic and Carcinogenic Activity of AFB_1

Reactive intermediates of AFB_1 have been found to be the forms that bind to the bases ofthe DNA. As mentioned earlier, in rats the AFB_1-8,9-oxide can be conjugated or it can covalently bind to different

cellular macromolecules causing mutations, inhibition of protein, DNA, or RNA synthesis, teratogenesis or carcinogenesis. A direct correlation between the mutagenic and carcinogenic activity has been established (16,17). In rats, the form of AFB_1 that causes mutagenesis has also been shown to cause carcinogenesis. Aflatoxin B_1 is termed a procarcinogen or promutagen indicating that it requires biotransformation or activation to become biologically active (24).

Salmonella/Mammalian-Microsome Mutagenic Assay

Due to the high correlation between carcinogenesis and mutagenesis a number of short-term microbial assays for chemically induced mutagenesis have been developed. The best known and most widely used is the Salmonella/mammalian-microsome assay or the Ames test. This assay was developed as a screen for chemical mutagens. However, recently it is proven reliable not only as a screening assay but also as a comparative test for carcinogenicity (1,3,4,6).

In this assay, chemicals are tested using several histidine-requiring mutant strains of Salmonella typhimurium on petri plates containing minimal growth nutrients and trace amounts of histidine and biotin. The strains are selected for sensitivity to specific chemical structures and incubated with or without rat liver homogenate containing the cytochrome-P-450 system (20). Mutagenesis is measured by the number of bacterial colonies which revert from the histidine-requiring to the wild type (non-histidine-requiring) because of the test chemical.

The strains used in the assay were developed from Salmonella typhimurium LT_2. Strains TA 1535, TA 1536, TA 1537 and TA 1538 are

histidine-dependent mutations containing different genes, his G46, his C207, his C3076 and his D3052, respectively. Through these genes the bacteria becomes histidine-requiring. These strains also have a deep rough or rfa mutation which eliminates the lipopolysaccharide from the cell membrane. This makes the bacteria more permeable and therefore more susceptible to chemically induced damage. In addition the deletion of the uvrB region of the chromosome eliminates the DNA excision repair system and causes increased sensitivity of the organism to DNA alterations (20). Strains TA 100 and TA 98 were developed from strains TA 1535 and TA 1538, respectively. McCann et al. (21) reported that alterations of the R (repair) factor is effective in detecting mutagens which were thought to be weak or negative. The strains containing the pkM101 plasmid have an enhanced error-prone DNA repair system and are the strains most commonly used.

TA 100 detects DNA base-pair substitution and TA 98 detects frame-shift mutagens. A base-pair substitution mutation results when one base in the DNA is substituted for another. There are 2 types of base-pair substitutions: 1) transition - a purine is substituted for a purine or a pyrimidine for a pyrimidine and 2) transversion - a purine is substituted for a pyrimidine or vice versa. A frame-shift mutation results when one base is either deleted or added to the DNA resulting in a shift in the reading codon.

S-9 Activation for the Ames Test

Because of the large number of chemicals which are procarcinogens, such as aflatoxins, in vitro activation of these compounds is

important. If the activation is not included or is inadequate, false negatives can result. Most chemicals requiring activation are activated by the mixed-function oxidase system of the liver. Therefore, a liver microsomal homogenate from rats treated with Arcolor 1254 to induce the cytochrome-P-450 system has been accepted as the standard method (3,20). This homogenate is called the S-9 fraction and is essential to this assay. An adequate and effective amount of S-9 to be added should be pre-determined for each chemical to be tested. Excessive S-9 fraction may depress the metabolic activity of most chemicals (9). The S-9 mix includes S-9 fraction and NADP and glucose-6-phosphate as cofactors for the P-450 reaction (Table 2). Elimination of these cofactors for the P-450 reaction will result in decreased sensitivity of the assay or in false negatives.

Evaluation of the Ames Test

There are many advantages to using the Ames test when comparing it to other mutagenic assays. The Ames test is an inexpensive, short-time, reliable assay (1). However, one of its disadvantages is that it is normally used to test one substance at a time and only the reactive intermediate of that substance. Even though, it is important to know the effects of a substance and its reactive intermediate, it is equally important to know the effect of the conjugated compound or a mixture of substances.

Being able to test a mixture of substances for mutagenicity is of major importance since many of the substances tested are normally

Table 2. Components of S-9 Mix

Component	Concentration/ml S-9
D-glucose-6-phosphate	5 umoles
NADP (Na salt)	4 umoles
$MgCl_2$	8 umoles
KCl	33 umoles
Na-K phosphate buffer, pH 7.4	100 umoles
S-9 Fraction	100 ul

found in combination with AFB_2, AFG_1 and AFG_2. Therefore, testing mixtures of substances with the Ames test would be important in determining if the other substances in the mixture would have an additive, synergistic or competitive effect on the mutagenesis.

Expanding the Ames test in this manner would allow for a better overall evaluation of the test substances and would increase the effectiveness of this assay.

CHAPTER 3

MATERIALS AND METHODS

Sample Preparation

One mg of pure crystaline aflatoxin (Sigma Chemical) was dissolved in 1ml DMSO and then diluted to a concentration of 1ng/ul with distilled water. Concentrations of aflatoxin used were 0, 0.02, 0.04, 0.06, 0.08, 0.1, 0.25, 0.5, 0.75, 1.0 ug per plate.

Glutathione (Sigma Chemical) was dissolved in distilled water to a final concentration of 5mM/500ul and G-S-T (Sigma Chemical) was dissolved in phosphate buffer, pH 7, to a final dilution of 2 units/100ul.

Mutagenic Assay

Salmonella typhimurium strains TA 98 and TA 100 were generously supplied by Dr. Dennis Hseih, University of California, Davis. Strains were checked for alterations and stored according to the procedure of Ames et al. (20).

Standard plate assays were carried out according to the procedure of Ames et al. (20) (Appendix A) with the following modifications. GSH (5mM/plate) and the combination of GSH and G-S-T (2 units/plate) were added to the S-9 mix and sample. One half of the samples were incubated for 30 minutes at 37^{o}C prior to plating and of those half were incubated with the bacteria and half were incubated without the bacteria (Figure 2). This was done for each strain, TA 98 and TA 100, and each concentration

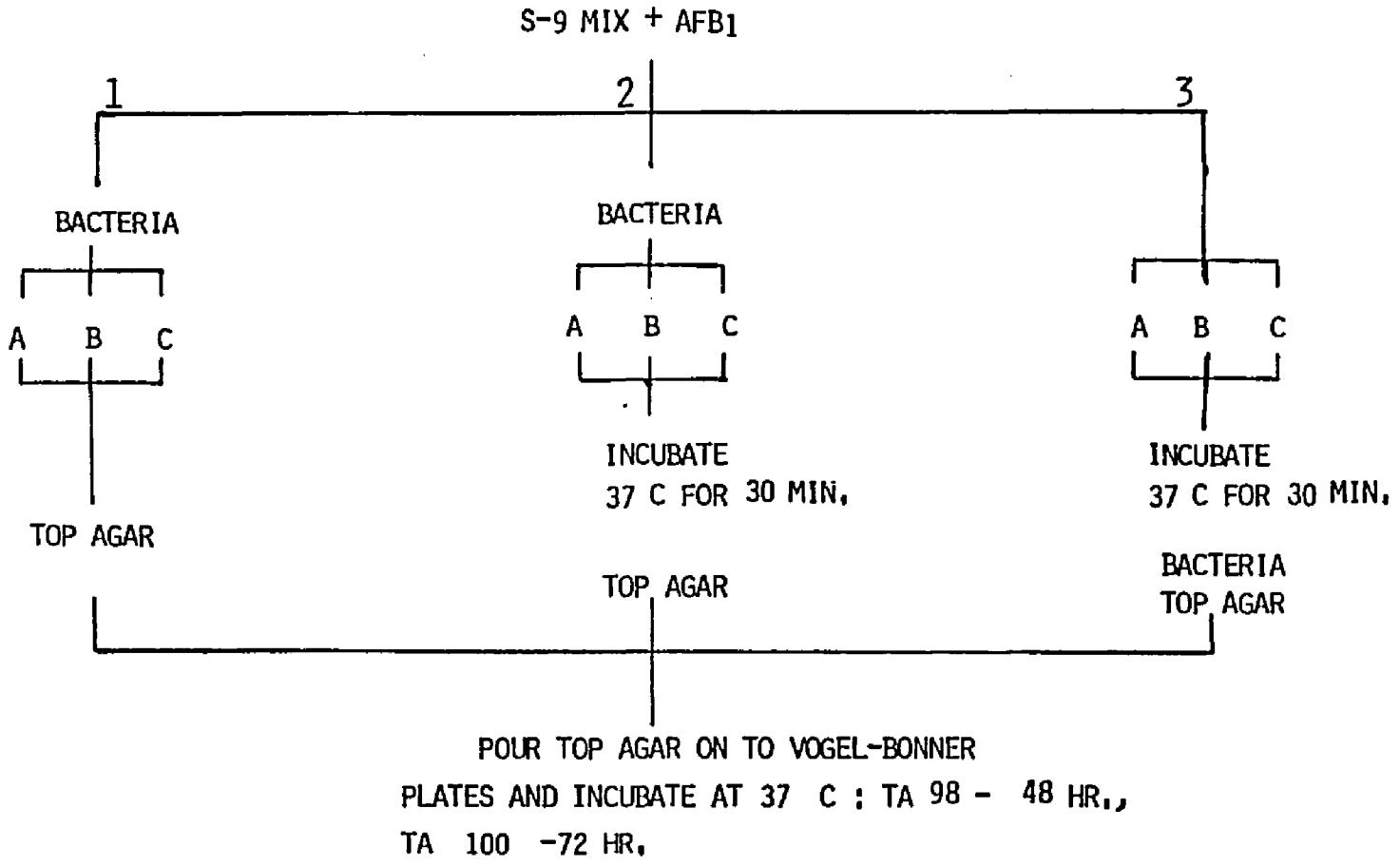


Figure 2. Flow Chart for Ames Test Modification, A–AFB_1, B–AFB_1 + GSH, C–AFB_1 = GSH + G-S-T.

was done in triplicate. All samples were, randomly coded prior to plating to prevent any bias in final analysis.

All safety precautions were taken in the handling of the bacteria and the AFB_1 (Appendix B)

Statistical Analysis of Data

Statistical analysis of data was done using a computer program to calculate a three-way analysis of variance. Means were separated using the least significant difference at $p<0.05\%$ (22).

CHAPTER 4

RESULTS

The effects of GSH and the combination of GSH and G-S-T on the AFB_1 mutagenesis and lethality to Salmonella typhimurium strains TA 98 and TA 100 was tested. This was done by varying the AFB_1 concentration, and by incubating the GSH and/or the GSH and G-S-T with and without bacteria for 30 minutes at 37 C prior to plating.

The results of this study show that the addition of the GSH to the S-9 fraction of the Ames test did decrease both the mutagenesis and lethality to both strains, TA 98 and TA 100. Incubating the S-9 containing AFB_1, GSH or GSH+G-S-T with and without bacteria prior to plating had no effect with the TA 100; however, there were significant differences in the mutagenesis at the higher concentrations with the TA 98. The decreases indicate that the conjugation of the AFB_1-8,9-oxide with GSH is a metabolic detoxification reaction.

Preliminary Testing

Preliminary testing was done using TA 98 to determine optimum preplating incubation time. The AFB_1 (0.1ug/plate), 0.2ml S-9, with and without 5mM GSH and 2 units G-S-T were incubated prior to plating with and without bacteria for 0, 30, 60 and 90 minutes. The times chosen were based on work done by Kado et al. (23) in which the sensitivity of the Ames test was increased when the sample, S-9 mix and bacteria

were incubated together for 90 minutes at 37°C prior to plating. The amount of GSH added was based on the average amount in the normal rat liver which contains between 2 and 7 mM GSH. The results show no significant differences between the 30 and 60 minutes incubation within each group, but show a sharp increase in the number of revertants after 90 minutes (Figure 3). When the AFB_1 alone was incubated without bacteria prior to plating, there was a significant decrease in revertants after 30 minutes. The addition of GSH and G-S-T resulted in a significant decrease in the mutagenesis of AFB_1 due to the conjugation of GSH to the reactive intermediate, AFB_1-8,9-oxide. Formation of the AFB_1- GSH conjugate prevented binding of the epoxide to the DNA receptor site in the cell.

Effects on TA 98: Formation of Frame-shift Mutagens

The effects of GSH and GSH+G-S-T on the mutagenesis of AFB_1 with TA 98 without preplating incubation are shown in Figure 4. The results show a significant difference in the mutagenesis at all concentrations. At concentrations below 0.1ug/plate, the samples containing only AFB_1 show a constant increase in mutagenesis with respect to AFB_1 concentration; however, at concentrations above 0.1ug the AFB_1 was lethal to the bacteria. At the lower concentrations samples containing the GSH and GSH+G-S-T had significantly lower mutagenesis. At AFB_1 concentrations above the 0.1ug usually lethal, there was an increase in mutagenesis in those containing the GSH and the GSH+G-S-T.

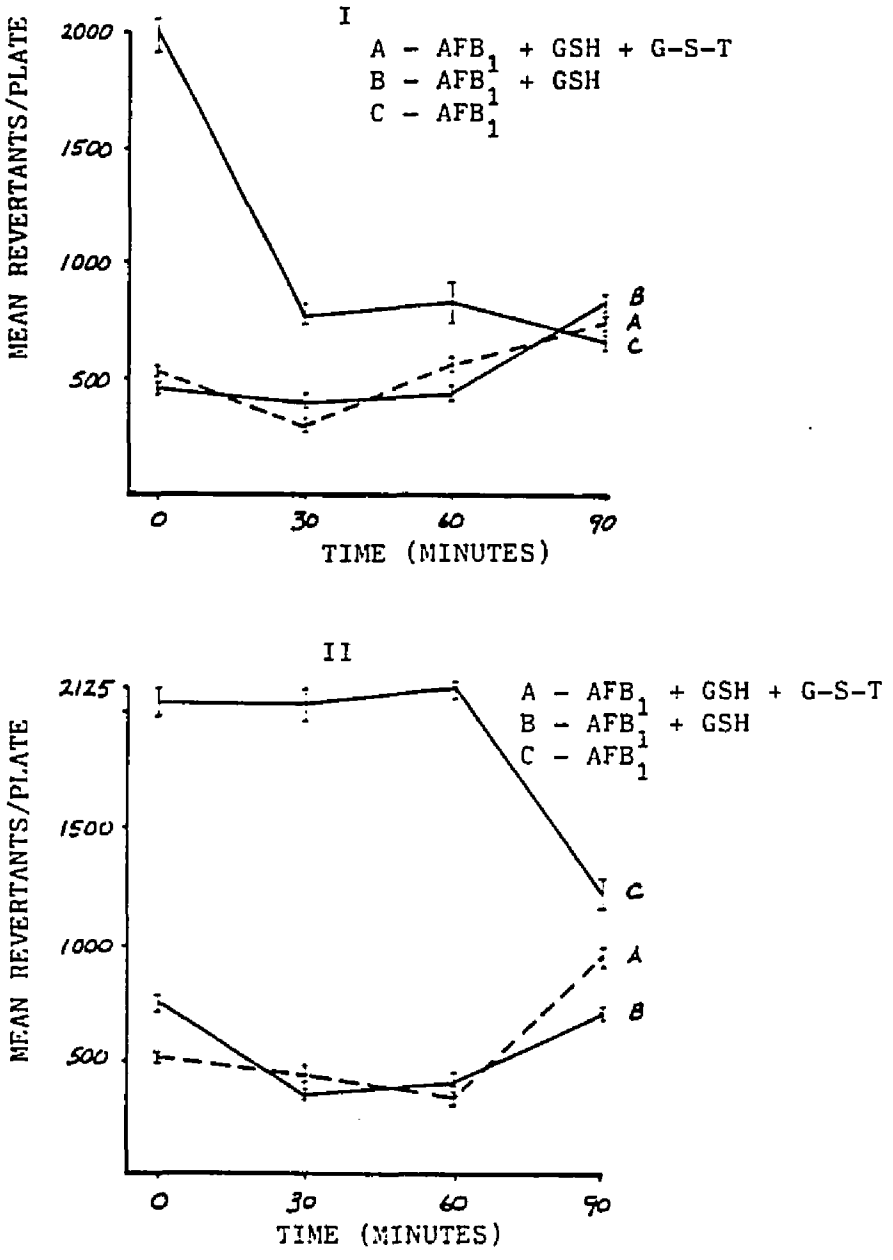


Figure 3. AFB_1 Mutagenesis With TA 98: Determining the Optimum Preplating Incubation for S-9 Mix and GSH or GSH + G-S-T. I S-9 Incubated With Bacteria, II S-9 Incubated With Bacteria Without Bacteria.

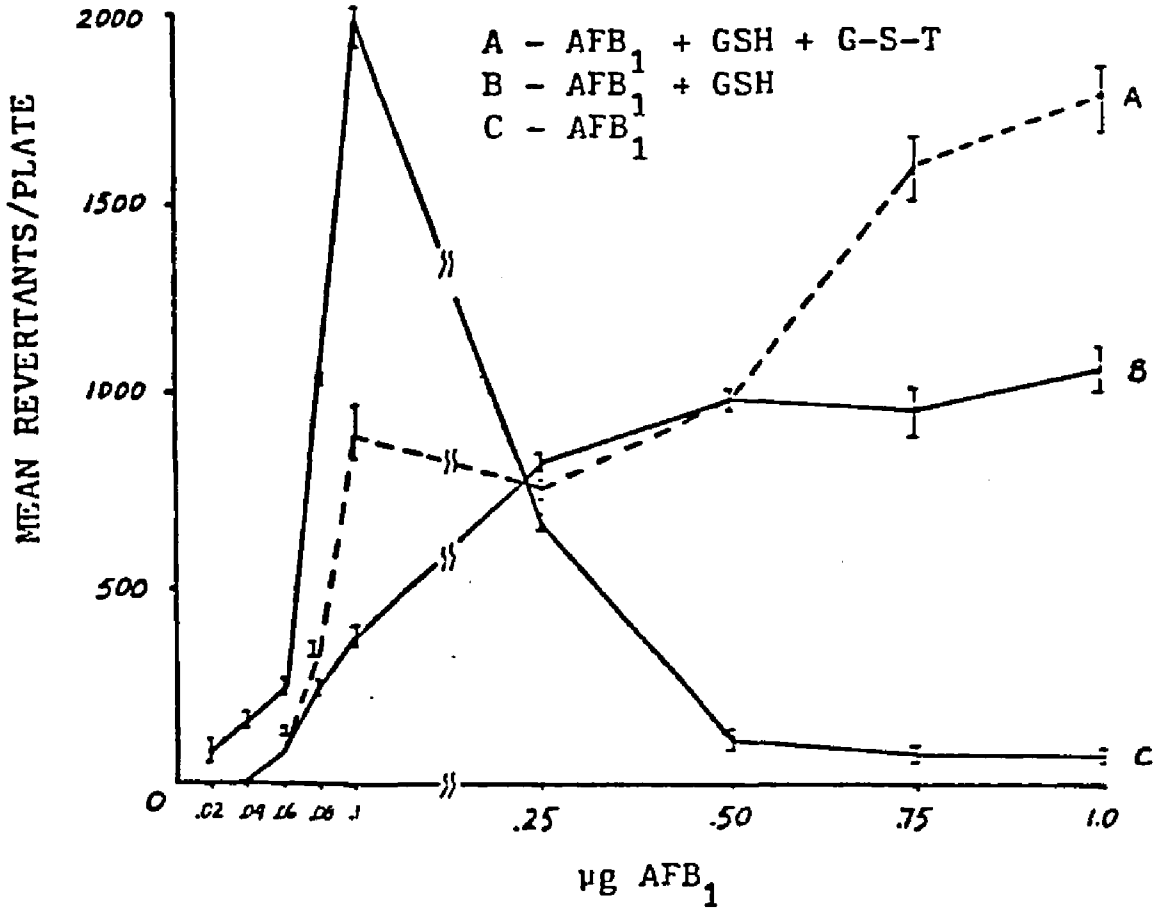


Figure 4. AFB_1 Mutagenesis With TA 98: No Incubation of S-9 Prior to Plating and Addition of GSH and GSH + G-S-T.

Figure 5 shows results from the samples which were incubated prior to plating with bacteria. The plates which contained only AFB_1 had the same type of response as those which were not incubated prior to plating. The effect of the GSH on AFB_1 mutagenesis was not as significant as those which were not incubated prior to plating. There was a decrease in the mutagenesis; however, the GSH was not as effective. Those plates containing GSH+G-S-T show a constant increase in mutagenesis over concentration and a significant decrease in the lethality of the AFB_1 to the bacteria. The overall effects of the GSH and GSH+G-S-T were not as significant when compared to the samples that were not incubated prior to plating.

Figure 6 shows results from the samples that were incubated for 30 minutes prior to adding bacteria and then plating. The samples that contained only AFB_1 showed lethal concentrations when compared to those which were run at time zero. The samples containing the GSH showed less lethality than those containing the AFB_1 alone. There was little difference between those that were incubated prior to plating for 30 minutes with bacteria or those that were not. There were no significant differences between any samples containing only the GSH. At lower concentrations, the samples that contained GSH and G-S-T showed lower mutagenesis but as the AFB_1 concentration increased so did the mutagenesis. Mutagenesis was higher in the GSH+G-S-T samples than those containing only GSH at higher concentrations.

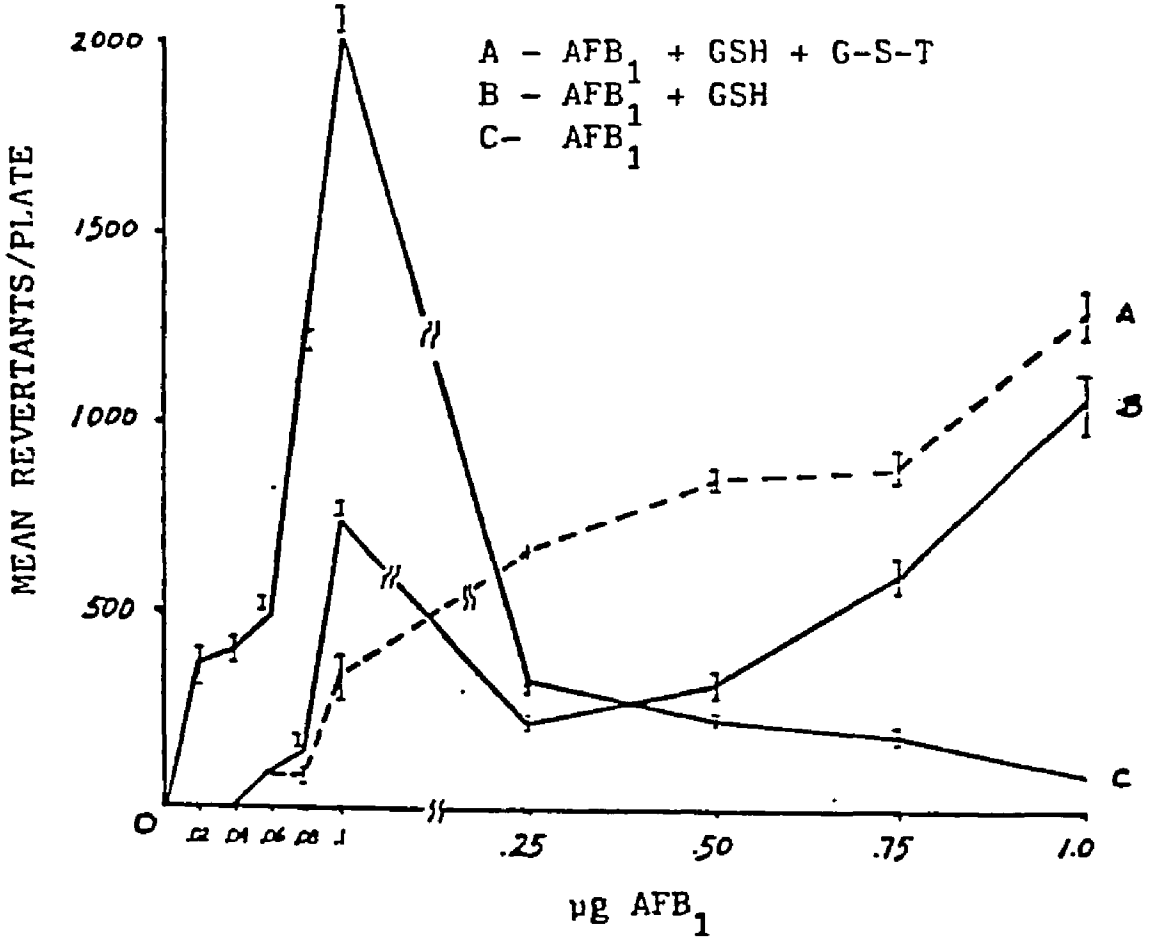


Figure 5. AFB_1 Mutagenesis With TA 98: S-9 With AFB_1 Alone, AFB_1 + GSH or AFB_1 + GSH + G-S-T Incubated With Bacteria at 37°C for 30 Minutes Prior to Plating.

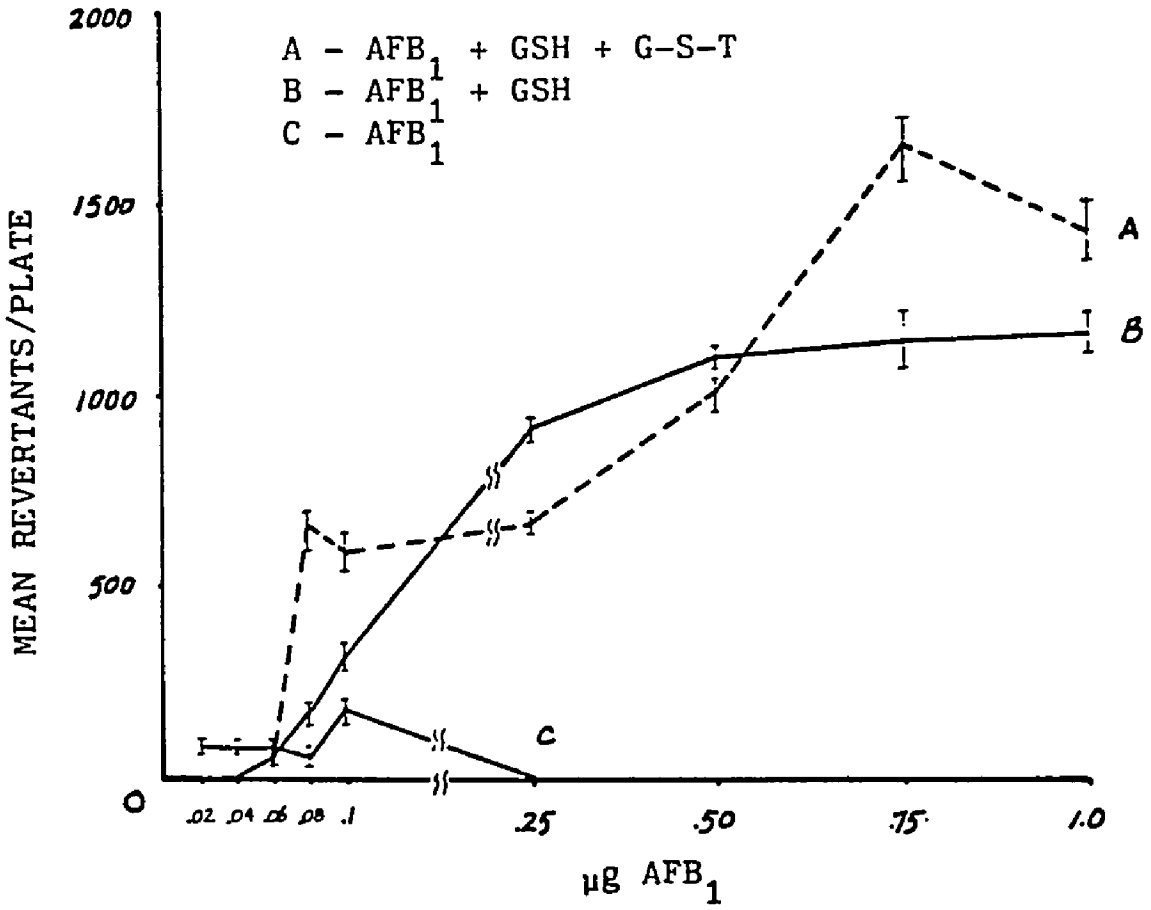


Figure 6. AFB_1 Mutagenesis With TA 98: S-9 With AFB_1 Alone, AFB_1 + GSH or AFB_1 + GSH + G-S-T Incubated Without Bacteria at 37°C for 30 Minutes Prior to Plating.

Effects with TA 100: Formation of Base-pair Substitution Mutagens

Figure 7 shows the results from the samples which were not incubated prior to plating, these show a significant difference in the mutagenesis, depending on the presence of GSH. There was no significant difference between those containing the GSH or the GSH+G-S-T. At concentrations above 0.10 ug/plate the AFB_1 was lethal to the bacteria; however, those containing the GSH showed higher revertant counts indicating that some of the AFB_1-8,9-oxide was bound and inactivated by the GSH which lowered the levels of AFB_1 available to produce lethality.

Figure 8 shows results obtained from those samples that were incubated prior to plating with the bacteria at 37°C for 30 minutes. Those samples containing only AFB_1 show the same overall effects as those which were not incubated prior to plating. The same was found to be true with the samples that contained the GSH and GSH+G-S-T. The results from those containing the GSH show a slow constant increase in the mutagenesis over all concentrations. In a similar manner, at the higher concentrations, those samples that were not incubated prior to plating and contained GSH decreased the lethal effects to the bacteria. There were no significant differences between those samples that contained GSH and those that contained the GSH+G-S-T.

Figure 9 shows the results obtained from the samples which were incubated prior to plating at 37°C for 30 minutes without bacteria. Those plates containing only AFB_1 show lethality to the bacteria at all levels when compared to those that were not incubated prior to plating

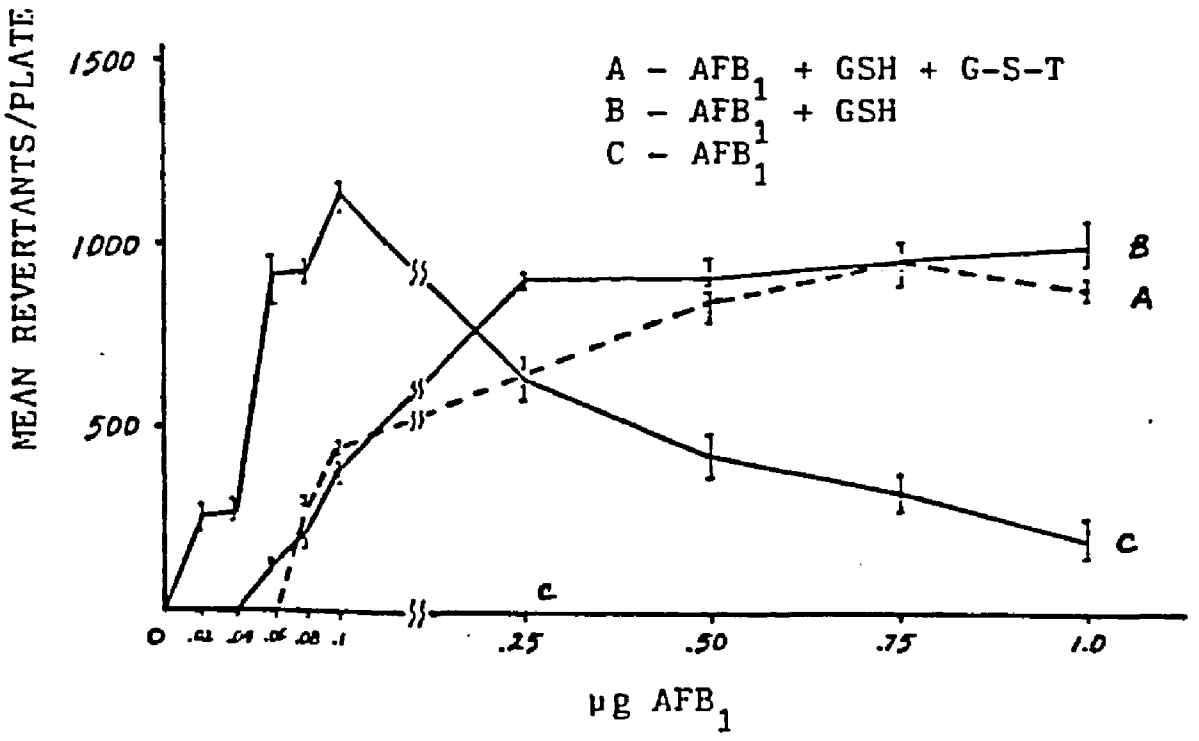


Figure 7. AFB_1 Mutagenesis With TA 100: No Incubation of S-9 Prior to Plating and Addition of GSH and GSH + G-S-T.

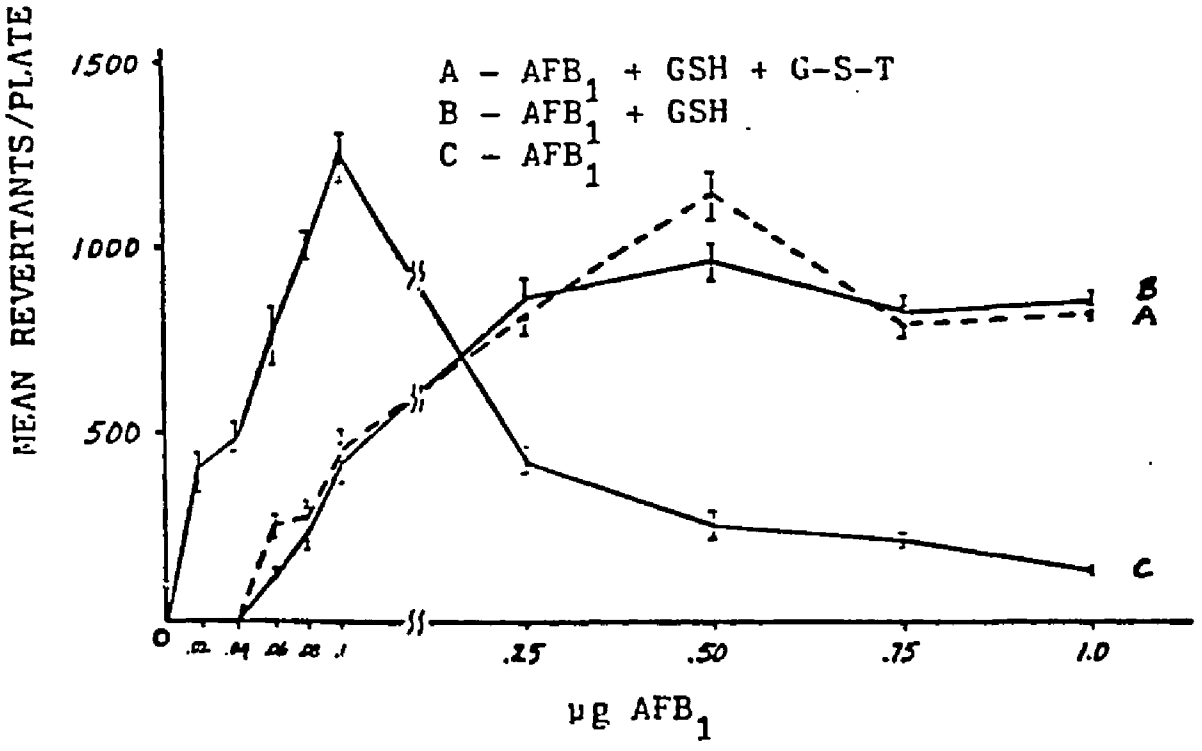


Figure 8. AFB_1 Mutagenesis With TA 100: S-9 With AFB_1 Alone, AFB_1 + GSH or AFB_1 + GSH + G-S-T Incubated With Bacteria at 37°C for 30 Minutes Prior to Plating.

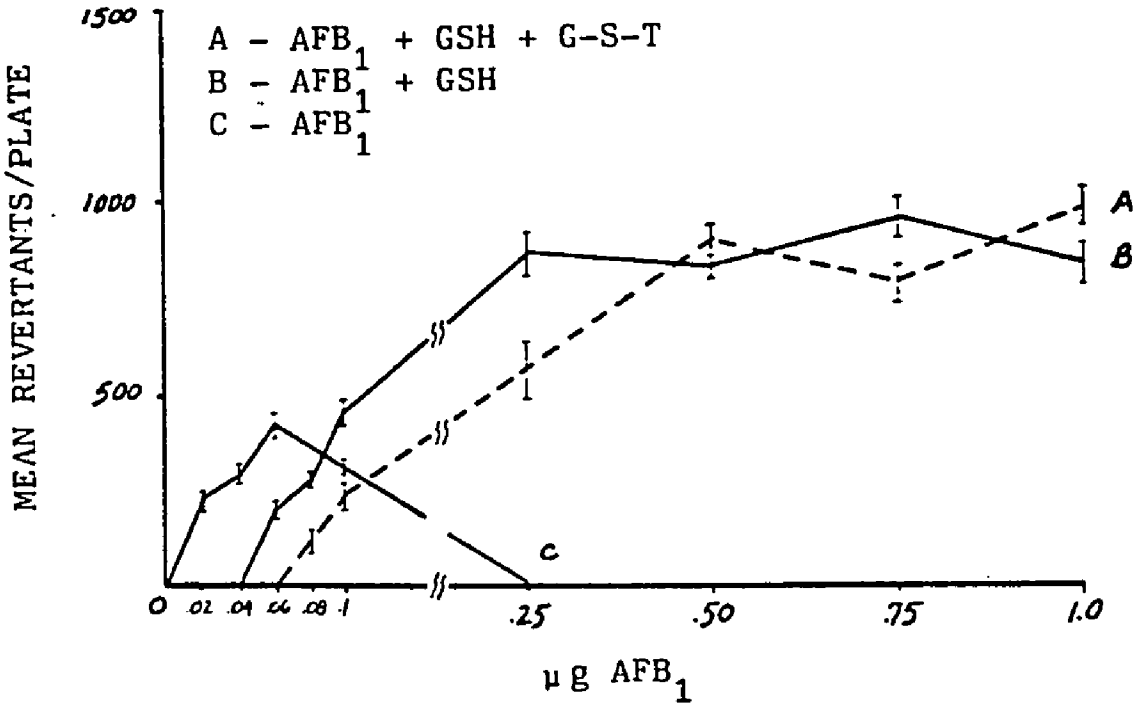


Figure 9. AFB_1 Mutagenesis With TA 100: S-9 With AFB_1 Alone, AFB_1 + GSH or AFB_1 + GSH + G-S-T Incubated Without Bacteria at 37°C for 30 Minutes Prior to Plating.

or those that were incubated with the bacteria included. This indicated that the amount of AFB_1-8,9-oxide produced by the S-9 at concentrations of .06ug and above is sufficient for lethality. The samples containing GSH with and without the G-S-T show a constant increase in mutagenesis as the concentration increased, differences were not significant. Lethality to the bacteria was decreased by both the GSH and the G-S-T treatments.

CHAPTER 5

DISCUSSION

The presence of GSH and G-S-T in the S-9 mix of the Ames test not only decreased AFB_1 mutagenesis but also decreased the AFB_1 lethality to the bacteria over all concentrations. The effects of the GSH and G-S-T varied depending on the bacterial strain used and the type of mutant formed, whether or not the S-9 mix was incubated prior to plating with or without the bacteria and with or without GSH and GSH+G-S-T.

The results from this study show that the AFB_1-GSH conjugate formed by a reaction between AFB_1-8,9-oxide and GSH is a metabolic detoxification reaction when using this assay. The AFB_1-8,9-oxide is the reactive metabolite formed by the action of the cytochrome-P-450 on AFB_1 and it is this form that has been shown to be mutagenic. The conjugation reaction is catalyzed by the enzyme G-S-T in an *in vivo* system. In this study the effect of the G-S-T on the AFB_1-GSH conjugation was dependent on the amount of S-9 mix used in the assay. This is shown by Figures 4 and 7. Figure 4 shows the results obtained using TA 98 and Figure 7 shows results obtained from TA 100. When testing AFB_1 with the Ames test, strain TA 98 requires 0.25 ml S-9 mix and TA 100 requires 0.5 ml S-9 mix. This results in different enzyme levels for AFB_1 activation and indicates the S-9 fraction contains a sufficient amount of G-S-T for the GSH conjugation of AFB_1-8,9-oxide when using TA 100. This also shows the importance of the amount of S-9 mix used in the assay.

The effects of S-9 mix preplating incubation, with AFB_1 and with and without GSH and GSH+G-S-T, depended on whether or not the bacteria were present. The decreases seen when the bacterial culture was included could be due in part to limited growth nutrients and the amount of AFB_1-8,9-oxide present when the bacteria was added. When no GSH or G-S-T was included the AFB_1 was lethal at all concentrations. However, when either the GSH or the GSH-G-S-T was present the lethality was decreased which is shown by the increase in mutagenesis. When the bacteria was added to the S-9 mix after preplating incubation the results obtained were not significantly different than those obtained without preplating the S-9 mix.

One of the major criticisms of the Ames test and other in vitro mutagenic assays is that they ignore whole animal protective mechanisms (25,26). One of these protective mechanisms is conjugation of a reactive metabolite by GSH. By incorporating GSH into the S-9 mix of the Ames test, the results obtained in this study were similar to those obtained from animal testing (24). The addition of GSH and G-S-T changed the dose/response relationship between AFB_1 and the resulting mutagenic activity. At concentrations above 0.1 µg AFB_1 per plate, total bacterial death was observed; however when GSH and G-S-T were added total bacterial death did not occur. This reduction is due to the AFB_1-GSH reaction which decreased the total available AFB_1-8,9-oxide. There was, however, sufficient levels of the AFB_1-8,9-oxide to cause mutagenesis.

This study shows that it is possible to modify the Ames test to test compounds not only the activated forms but also those resulting from protective biochemical reactions and therefore, bring the results closer to those observed in animal studies. This study also shows that the Ames test can be modified to test more than one substance at a time. This is important because some substances, such as aflatoxin, appear in more than one form and these different forms are found existing together. Because of the number of substances which appear as mixtures in nature, it is important to determine their effects on one another as potential health hazards.

Since the Ames test is one of the more common mutagenic assays used, being able to incorporate other biochemical reactions or test a mixture of substances could increase the reliability of this assay for evaluating chemical mutagens (25). Using these modifications alone may result in more false negatives being reported, therefore, they should not be used in place of the standard assay but with it in order to determine if the mutagenic response is affected.

CHAPTER 6

CONCLUSIONS

The results from this study show that the addition of GSH and G-S-T to the S-9 mix of the Ames test decreases both AFB_1 mutagenesis and AFB_1 lethality to Salmonella typhimurium strains TA 98 and TA 100. The effects of additional G-S-T to catalyze the AFB_1-GSH conjugation reaction was dependent upon the amount of S-9 mix used in the assay. Incubation of the S-9 mix, AFB_1, GSH, and GSH-G-S-T prior to plating depended on whether or not the bacterial cultures were included with the S-9 mix. Incubation prior to plating with bacteria showed higher lethality than those which were not incubated and those that were incubated prior to plating without bacteria.

This study also shows that the Ames test can be modified to test more than one substance and also that it can be modified to include more than one biochemical reaction products in the same test. This modification should be used with the standard assay in order to prevent any false negatives.

APPENDIX A

AMES ASSAY METHODOLOGY

Stock solutions:

The following solutions are prepared using distilled water, sterilized and stored in the refrigerator. Label everything; contents, date, and initials.

0.4 M Magnesium Chloride ($MgCl_2 \cdot 6H_2O$) mw 203.31, 8.31gm/100ml.

1.65 M Potassium Chloride (KCl) mw 5.6, 12.32gm/100ml.

0.2 M Sodium Phosphate buffer ($Na_2HPO_4 + NaH_2PO_4$) mw 120 and 142, 2.84gm and 2.4gm/100ml together in the same water).

The following are to be made using sterilized water and vials, again make sure everything is properly labeled.

1.0M Glucose-6-phosphate mw 282.1, 0.282gm/ml (prepare just prior to running the test).

0.1M NADP mw 765.4, 0.0765gm/ml (prepare just prior to running the test).

0.1M Histidine mw 191.6, 0.1916gm/ml (refrigerate)

0.5mM Histidine mw 191.6, 0.0096gm/10ml (refrigerate)

0.5mM Biotin mw 244.31, 0.0021gm/10ml (refrigerate)

Crystal violet, 1mg crystal violet/1ml water (room temperature)

Ampicillin 8mg/ml 0.2N Sodium Hydroxide (refrigerate)

Culture mediums:

The following are made using distilled water and sterilized prior to use:

Nutrient Broth (per 100ml water)-0.8gm Nutrient Broth + 0.5gm NaCl

Nutrient Broth Agar (per 100ml)-0.8gm Nutrient Broth + 0.5gm NaCl + 1.5gm Agar. After sterilization, pour plates before agar cools, 10ml/plate. Let plates set till hard and store at room temperature till use.

Vogel-Bonner Medium-1.0gm Magnesium Sulfate, 10gm Citric Acid, 50gm Potassium, Phosphate, 17.5gm Sodium Ammonium Phosphate and 67ml water. Dissolve and sterilize (refrigerate)

Vogel-Bonner Plates (per 125ml)-2.5ml Vogel-Bonner Medium (1.5%), 12.5ml 20% glucose solution (2%) and 110ml Water Agar (1.875gm Agar/110ml water), sterilize each component separately. Transfer VBM and glucose solution to water agar using sterile pipets. Swirl mixture (do not shake), pour plates, (30ml/plate), let harden (store at room temperature till used).

Top Agar (per 100ml)-0.6gm Agar + 0.5gm NaCl. Mix and sterilize in autoclave 20psi, 250^{o}F for 20 minutes, cap securely and store at room temperature. When sterilizing, all flasks are loosely capped to prevent breakage.

Test strains:

Salmonella typimurium TA 98 and TA 100. All bacteria cultures are to be stored at -80^{o}C.

Growing cultures:

1. Prepare Nutrient Broth in 2-25ml erlenmeyer flasks, one for each strain and sterilize.

2. After cooling broth, innoculate in the following manner: using a sterile wooden applicator, scrape the frozen culture and transfer to broth. Replace culture in freezer immediately after innoculation. Label flasks for each strain. Cap flasks with gas permeable stopper (cotton plug or tube cap), agitate in the incubator at medium speed for 16 hours maximum at $37^{o}C$.

3. After removing the culture from the incubator make master plates and new frozen cultures. Take sample from cultured broth and using a spectrophotometer measure bacterial growth. An absorbance of 0.8 at 650nm is a count of 10^{6} cells (do not use if less). Make master plates using Vogel-Bonner plates (VBP). Add 0.1ml-0.1M histidine, 0.1ml-0.5mM biotin and 0.1ml Ampicillin to plate surface and dry over night. After the plate has dried streak plate with bacteria, using a sterile innoculating loop. Incubate the plates for 24 hours at $37^{o}C$. To make frozen cultures transfer 1.6ml cultured broth to 1 dram vial and add 0.14ml DMSO, cap tightly and freeze. One is to be used for further testing instead of original because the freeze-thaw cycle can damage the bacteria. The other is to be used as a master culture.

Checking bacteria:

These tests are run prior to and concurrently with the Ames test to make certain that the bacteria has not been altered in any way.

1. Histidine requirements: Using VB plates, 2/culture, label the plates with the following information strain number, with and without histidine.

a. With histidine--add directly to plate 0.1ml, 0.1M histidine and 0.1ml, 0.5mM biotin (sterile solutions) and using a sterile spatula spread and let dry overnight. Then streak the plate with culture strain to be tested. Incubate at 37°C for 24-48 hours.

b. Without histidine--streak the VBP, as is, with strain to be tested and incubate for 24-48 hours at 37°C.

A positive result is growth with histidine and no growth without. This is the result you want to obtain. Growth can normally be detected by plate cloudiness after 24 hours.

2. Crystal violet (Rfa): Use Nutrient Broth Plates (NBP) and label with strain and streak with bacteria. Using sterile forceps, place sterile filter disk into crystal violet and carefully place onto disk where bacteria has been placed, incubate at 37°C for 24 hours. A positive test is a zone of inhibition around the disk.

3. Ampicillin resistance (R-factor): Use NBP and label with strain and AMP. Using a sterile loop, streak a line of ampicillin on one plate and let dry overnight. After drying, streak lines of bacteria over the ampicillin and incubate at 37°C for 24 hours. A positive test is growth over the ampicillin. resistance to ampicillin indicates loss of the R-factor.

Ames test:

1. Preparation of S-9 mix. This must be prepared immediately prior to running the Ames test and can be kept on ice for up to 24 hours. To each 2ml S-9 fraction add 0.4ml MgC12; 0.4ml KCl; 0.1ml NADP; 0.8ml G-6-P; 10.0ml Sodium phosphate buffer; 6.7ml sterilized water.

2. Preparation of top agar: add to 100ml sterilized molten top agar 5ml of 0.5mM histidine and 5ml of 0.5mM biotin and swirl to mix, do not shake.

3. Using culture tubes containing 2ml top agar add the following: 0.1ml bacteria; 0.1ml sample (or less depending on sample toxicity, record amount); 0.5ml S-9 mix for TA 100 and 0.25ml for TA 98. Mix and pour onto VB plate. Immediately tilt and turn plate quickly to insure the top agar completely covers the plate as evenly as possible. Incubate for 36-72 hours at 37°C. This is to be done in duplicate for each sample per strain.

4. Control plates: There are positive and negative controls. Positive control: These plates are set up as mentioned except that a standard (AFB_1) is used in place of the sample. Negative control: These plates are set up without sample or standard. All control plates are run in triplicate for each strain. Incubate at 37°C for 37-48 hours. These are to be run with each set of samples.

5. After incubation remove from the incubator and count the revertant colonies. The number of colonies from the negative plates are averaged and subtracted from the sample plates for that particular strain and results recorded. The number subtracted is called the spontaneous revertants.

APPENDIX B

SAFETY PRECAUTIONS

Ames Test: Handling bacteria

1. All regular laboratory safety precautions must be taken - lab coats, gloves, etc.

2. Work under a fume hood, especially if working with volatile compounds.

3. Clean working area before and after running the Ames test with 70% ethanol.

4. All glassware containing only bacteria and broth is autoclaved for 20-25 minutes at 250^{o}F/20 psig prior to disposal, strains TA 98 and TA 100 are heat sensitive. The glassware is then washed with hot, soapy water and autoclaved before using.

5. Most equipment used is disposable (culture tubes, gloves, pipet tips, etc.) and are discarded along with any carcinogenic samples through Risk Management.

6. Glassware used for this test should not be used for chemical analysis due to interferring chemical residues.

Aflatoxin B_1

1. Always wear gloves when working with aflatoxin.

2. Work area is cleaned with hot, soapy water containing bleach, then wiped down with acetone.

3. In case of a spill, the area is cleaned as stated in #3 and cleaning material discarded through Risk Management.

4. The AFB_1 crystalline vial was not opened, but the DMSO was injected into the vial and the desired amount removed with a sterile syringe. Extra caution must be taken because of the carcinogenic potential of this substance.

LIST OF REFERENCES

1. Taylor, S. L., 1982. Mutagenesis vs. Carcinogenesis. J. Food Tech. 36(4):65.

2. Casiano, D. A., 1982. Mutagenesis Assay Methods. J. Food Tech. 36(4):48.

3. Ames, B. N., F. D. Lee, W. E. Durston, 1973. An Improved Bacterial Test System for the Detection and Classification of Mutagens and Carcinogens. Proc. Nat. Acad. Sci. 70(4):782.

4. Purchase, I. F. H., E. Longstoff, J. Ashby, J.A. Styles, D. Anderson, P. A. Lafevre and F. R. Westwood, 1976. Evaluation of six short-term tests for detecting organic chemical carcinogens and recommendations for their use. Nature 264:624.

5. Council for Agricultural Science and Technology, 1979. Aflatoxin and other mycotoxins: An agricultural perspective. CAST, Ames, Iowa.

6. McCann, J., E. Choi, E. Yamasaki, and F. D. Lee. 1973. Detection of Carcinogens as Mutagens in the Salmonella/Microsome Test: Assay of 300 Chemicals. Proc. Nat. Acad. Sci. 72(12)5135.

7. Pariza, M. W., 1983. Carcinogenicity/Toxicity Testing and the Safety of Foods. J. Food Tech. 37(1):84.

8. Spingarn, N. E., C. T. Garvie-Gould, and L. A. Slocum, 1983. Formation of mutagens in sugar-amino acid model systems. J. Agric. Food Chem. 31(2):301.

9. Sugimura, T. and M. Nagao, 1979. Mutagenic Factors in Cooked Foods. CRC Critical Reviews in Toxicology. P. 189.

10. Ozawa, N. and F. P. Guengerich, 1983. Evidence for formation of an S-[2-(N -guanyl)ethyl]glutathione adduct in glutathione-mediated binding of the carcinogen 1, 2-dibromoethane to DNA. Proc. Nat. Acad. Sci. 80:5266.

11. Ciegler, A. and J. W. Bennett, 1980. Mycotoxins and Mycotoxicosis. BioScience 30(8):512.

12. Gurtoo, H. L. and T. C. Campbell, 1974. Metabolism of aflatoxin B_1 and metabolism-dependent and independent binding to rat hepatic microsomes. Mol. Pharmacol. 10:776.

13. Degen, G. H. and H. G. Neumann, 1978. The major metabolite of aflatoxin B_1 in the rat is a glutathione conjugate. Chem.-Biol. Interact. 22:239.

14. Loury, D. J., D. P. H. Hsieh, and J. L. Byard, 1984. The effect of phenobarbitol pretreatment on the metabolism, covalent binding and cytotoxicity of aflatoxin B_1 in primary cultures of rat hepatocytes. J. Tox. and Environ. Health 13:145.

15. Kaplowitz, N. J., J. Kuhlendamp and G. Clifton, 1975. Drug induction of hepatic glutathione-s-transferase in male and female rats. Biochem J. 146:351.

16. Lotlikar, P. D., S. Insetta, P. R. Lyons and E. C. Jhee, 1980. Inhibition of microsome-mediated binding of aflatoxin B_1 to DNA by glutathione-s-transferase. Cancer Letters 9:143.

17. McBodile, M. U. K., M. Hoschler and R. A. Neal, 1975. A possible protective role for reduced glutathione in aflatoxin B_1 toxicity: Effect of pretreatment of rats with phenobarbitol on 3-methylchonanthrene on aflatoxin toxicity. Toxicol. Appl. Pharmacol. 34:128.

18. Raj, H. E., K. Santhanam, R. P. Gupta and T. A. Venkitasabramanian, 1975. Oxidative metabolism of aflatoxin B_1: Observation of the formation of epoxide-glutathione conjugate. Chem.-Biol. Interact. 11:301.

19. Wong, J. J. and D. P. H. Hsieh, 1980. Inhibition of aflatoxin mutagenesis by conjugative metabolism. Presented at the 19th Annual Meeting of the Society of Toxicology.

20. Ames, B. N., J. McCann, and E. Yamasaki, 1975. Methods for detecting carcinogens as mutagens with the Salmonella/mammalian-microsome mutagenicity test. Mut. Res. 31:347.

21. McCann, J., N. E. Spingarn, J. Kaliori and B. N. Ames, 1975. Detection of carcinogens as mutagens: Bacterial tester strains with R-factor plasmids. Proc. Nat. Acad. Sci. 72(3):979.

22. Steel, R. G. D. and J. H. Torrie, 1960. Principles and procedures of statistics. McGraw-Hill Book Co., Inc., New York.

23. Kado, N. Y., D. Langley, and E. Eisenstadt, 1983. A simple modification of the Salmonella liquid-incubation assay. Increased sensitivity for detecting mutagens in human urine. Mut Res. 121:25.

24. Wong, J. J. and D. P. H. Hsieh, 1976. Mutagenicity of aflatoxins related to their metabolism and carcinogenic potential. Proc. Nat. Acad. Sci. 73:2241.

25. Cline, D., 1985. Mutagenicity in Drug Development: Interpretation and Significance of Test Results. Reg. Tox. and Pharm. 5(1):79.

26. Kier, L. D., 1985. Use of the Ames Test in Toxicology. Reg. Tox. and Pharm. 5(1):59.