AFLATOXIN B_1 AND LIVER FUNCTION

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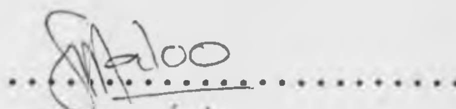
DECLARATION:

This Thesis is my original work and has not been presented for a degree in any other University.



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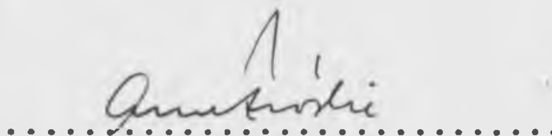

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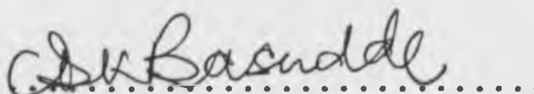

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DEDICATION

This work is dedicated to my wife Rashida and daughter Khadija.

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SUMMARY

Effects of aflatoxin B_1 and aflatoxin B_1 in combination with diethylmaleate, a glutathione depletor on liver function was studied by calculating the clearance (half life) in perfusate plasma and retention by the liver of a dye Sulphobromophthalein (BSP) using isolated liver perfusion technique.

When donor rats were pretreated with aflatoxin B_1 (3.0 mg/kg) intraperitonealy and perfused four hours later, an increase in the biological half life time (19.2 minutes) and % retention of the dye (41.3%) was noticed as compared to the controls having half life time (11.5 minutes) and % retention (19.3%). Bile volume output measured as µl/g liver weight upto 60 minutes of perfusion was reduced to 31 µl/g liver wt as compared to controls having 48 µl/g liver wt.

Pretreatment with diethylmaleate (DEM) 30 minutes prior to aflatoxin B_1 treatment showed marked increase in half life time of the dye (53.8 minutes) and % retention of (41.8%). Bile volume output was drastically reduced to 19 µl/g liver wt.

These results indicate that depletion of glutathione by diethylmaleate potentiates the effects of aflatoxin B_1 induced hepatotoxicity as seen by using isolated perfusion technique.

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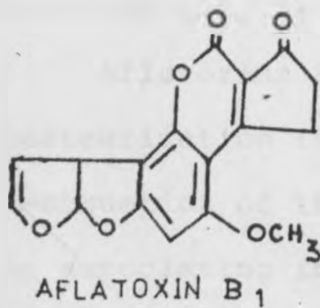
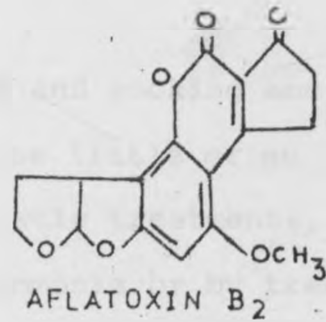
SECTION ONE

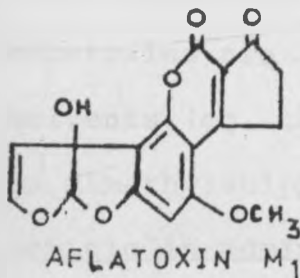
INTRODUCTION

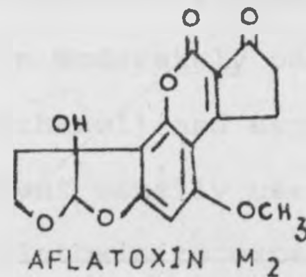
AFLATOXINS, THEIR CHARACTERISTICS, METABOLISM AND THEIR TOXIC EFFECTS

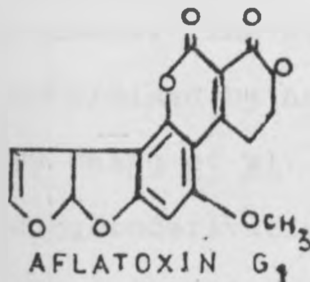
The four metabolites produced by *Aspergillus flavus* and *A. parasiticus* belong to the group of bisfurano-coumarin compounds. Upto today, 17 compounds of the bisfurano-coumarin derivatives have been isolated. Of these, the four major metabolites are aflatoxin B_1, being usually found in the highest concentrations and most toxic, followed by aflatoxin G_1.

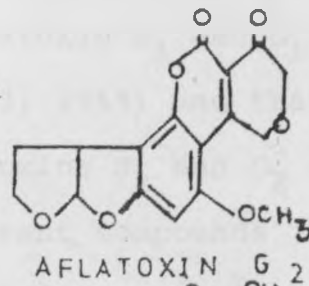


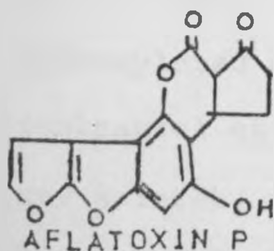












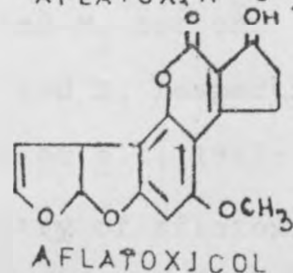


Fig 1: The structures of some aflatoxins and their metabolites

Aflatoxin B_2 and G_2 occur in lower concentrations. Aflatoxin M_1 and M_2, metabolites of aflatoxin B_1 and B_2 are excreted in milk and can also be detected in animal tissues. The structures of a number of aflatoxins and aflatoxin B_1 related metabolites are illustrated in Fig 1.

Aflatoxin B_1, a secondary metabolite of Aspergillus flavus group of fungi, is one of the most powerful hepatotoxins known. Its hepatotoxic and hepatocarcinogenic properties are well documented by Goldblatt, (1969).

Aflatoxins are heat stable and cooking and pasteurization temperatures cause little or no destruction of the toxins. Drastic treatments, such as autoclaving in presence of ammonia or by treatment with hypochlorite, totally destroys the aflatoxins. Aflatoxins are freely soluble in moderately polar solvents (eg. chloroform and methanol) and especially in dimethylsulphoxide, the solvent usually used as a vehicle in administration of aflatoxin to experimental animals. The structure of aflatoxin B_1 and G_1 were determined by Asao et al. (1963, 1965) and that of B_2 by Chang et al. (1963). Aflatoxins B_2 and G_2 are dihydroderivatives of their parent compounds (Hartley et al., 1963). Aflatoxins M_1 and M_2 are the hydroxylated metabolites of B_1 and B_2 respectively (Holzapfel et al., 1966; Masri. et al., 1967; Buchi and Weinreb, 1969). The chemistry of aflatoxins has

been reviewed by Roberts (1974).

METABOLISM

Like most foreign compounds, aflatoxin B_1 is also metabolised by the liver and its metabolites are excreted into bile. Bassir and Osiyemi (1967), using radiolabelled aflatoxin B_1, reported that maximum rate of biliary excretion of aflatoxin B_1 derived radioactivity in rats occurred 60-90 minutes following a single intraperitoneal dose, with the first 45-60 minutes being a latent period before appearance of any radioactivity in bile. Unger *et al.* (1977), using isolated liver perfusion technique, reported an uptake of 70% radiolabelled ^{14}C aflatoxin B_1 by the rat liver from the circulating perfusate within 5 minutes and the rate of biliary excretion of the derived radioactivity of parent compound reaching a maximum at 30 minutes. These findings indicate that aflatoxin B_1 is rapidly absorbed and metabolised by the liver. Ingestion of aflatoxin contaminated foodstuffs and absorption of the toxins from the alimentary tract is responsible for most field cases of aflatoxin - induced diseases in animals and man. In tissues, the highest concentration of ^{14}C ring labelled aflatoxin B_1 was found in the liver of experimental rats (Wogan *et al.*, 1967). Residues of the toxin were found in liver and muscle tissues of poultry and liver of pigs when fed on contaminated ration (Mintzlaff *et al.*, 1974).

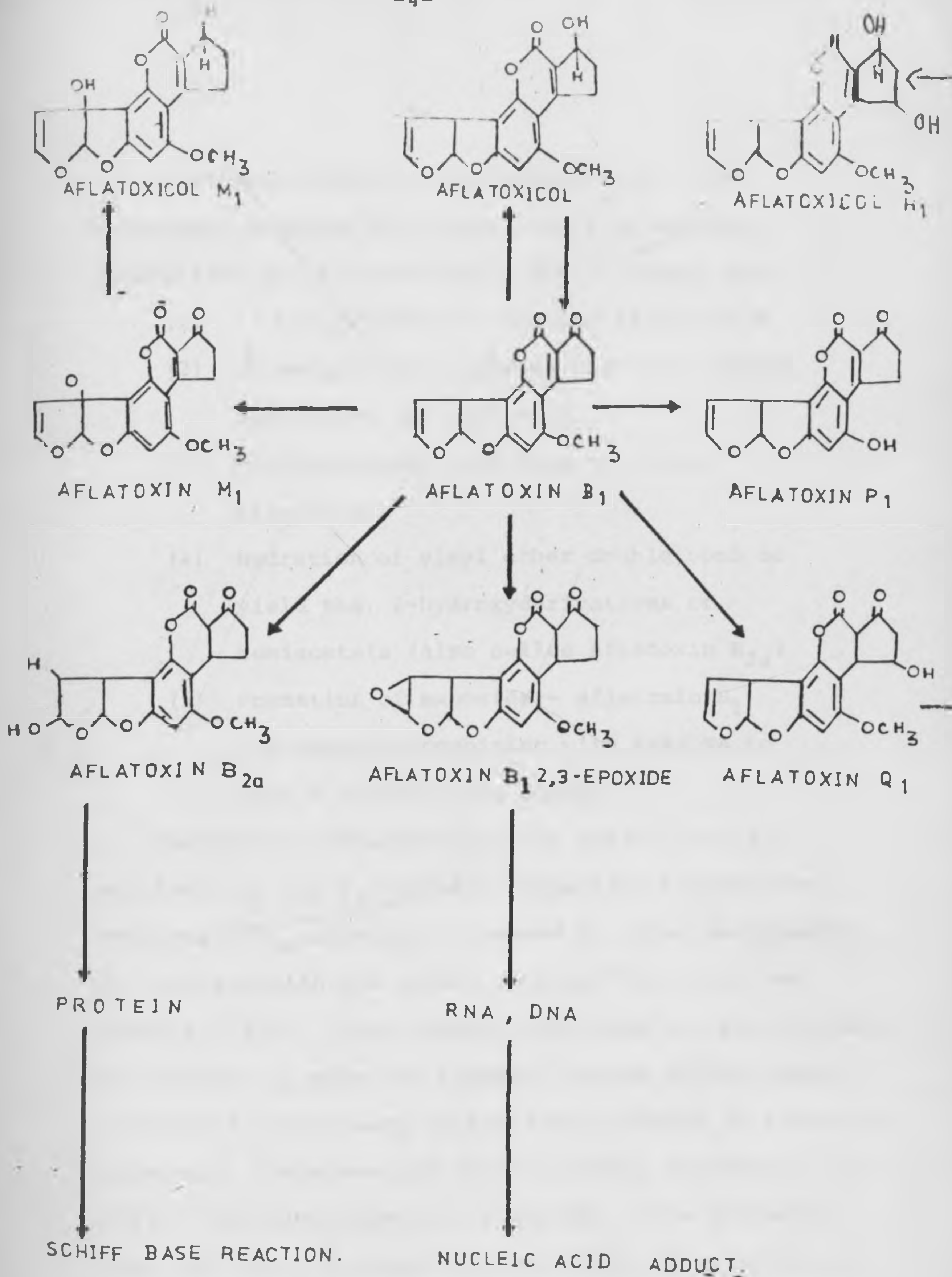


Fig 2: Aflatoxin B_1 metabolism in the liver.

Biotransformation of aflatoxin B_1 by liver microsomal enzymes (Patterson, 1973) to various metabolites are illustrated in Fig 2. These are:

(1) 4' hydroxylation - forming aflatoxin M_1

(2) 0' methylation - giving rise to a phenol derivative, aflatoxin P_1

(3) Cyclopentenone reduction - to form aflatoxicol

(4) Hydration of vinyl ether double bond to yield the: 2-hydroxyderivatives or hemiacetals (also called aflatoxin B_{2a})

(5) Formation of expoxide:- aflatoxin B_1 2,3-expoxide combining with RNA/DNA to form a nucleic acid adduct.

Metabolic transformation and activation of aflatoxin B_1 and G_1 to their respective hemiacetals (aflatoxin B_{2a} and G_{2a}) is caused by liver homogenates of certain avian and rodent species (Patterson and Roberts, 1970). These hemiacetals bind non specifically to proteins *in vivo* and probably become sufficiently reactive to cause many of the acute effects of aflatoxin poisoning (Patterson and Roberts, 1972; Patterson, 1973, 1977). Epoxide formation is thought to be the most important form of metabolic activation of aflatoxin B_1. It has been shown that this highly reactive epoxide metabolite binds covalently to DNA and induces mutation in a bacterial *in vitro* test system (Garner *et al.*, 1971, 1972; Ames *et al.*, 1973). Recovery of a

2, 3-dihydrodiol of aflatoxin B_1 following mild acid hydrolysis of the DNA adduct formed is assumed to be an indirect evidence of the formation of the reactive 2, 3-epoxide (Swenson et al., 1974, 1977). In view of the interaction with DNA, it is now generally accepted that the epoxide of aflatoxin B_1 is the bacterial mutagen and the proximal carcinogen. The major excretory route of aflatoxin B_1 and most of its metabolites is mainly through bile, (Bassir and Osiyemi, 1967) although excretion in milk, urine and expired carbondioxide has been reported.

Evidence regarding formation of aflatoxin B_1 epoxide by the rat liver microsomes which conjugated with glutathione to form aflatoxin - B_1 - epoxide - glutathione conjugate has been reported by Raj et al., (1975). Glutathione a tripeptide, is the most abundant of the free thiols found in tissues (Boyland and Chasseaud, 1970), and one of its biological functions is to conjugate histotoxic metabolites of certain drugs and toxicants that would otherwise bind to cellular macromolecules and initiate cell damage. These glutathione conjugates, which are water soluble, anionic, with molecular weights greater than 300, all possess factors which favour biliary excretion. Chasseaud (1974) mentioned the subtrates of this conjugation reaction which included large number of electrophillic substances such as insecticides, herbicides, diagnostic reagents (BSP), carcinogens and intermediates for many

chemical production processes. Glutathione effects its protective role with the help of a number of glutathione S-transferases. Boyland & Chasseaud,(1969) have shown that glutathione -S- epoxide transferase protects against the highly reactive intermediates generated from such compounds as polycyclic hydrocarbons by the microsomal mixed function oxidases. Depletion of hepatic glutathione by pretreatment with diethylmaleate (DEM) (Boyland and Chasseaud, 1970) markedly potentiates acetominophen - induced hepatic necrosis (Mitchell et al., 1973), whereas protection is offered when pretreatment with glutathione precursors is undertaken.A similar picture regarding protective role of glutathione has been shown by Jollow et al. (1974) where bromobenzene - induced hepatotoxicity was potentiated when glutathione was depleted. Likewise aflatoxin B_1, a hepatotoxin appears to owe at least part of its hepatotoxicity to cell damaging effects of hemiacetals (Patterson, 1972) and of transient epoxide metabolites (Campbell and Hayes, 1976). A protective role of glutathione in aflatoxin B_1 toxicity has been demonstrated by Mgbodile et al. (1975), where rats pretreated with a glutathione depletor showed marked hepatotoxicity. In goats, Hatch et al. (1979) observed a similar protective role of glutathione against aflatoxin B_1 toxicity. These results indicate that glutathione may participate in detoxyfying aflatoxin B_1 by conjugation.

The isolated liver perfusion technique was first carried out by Bernard in 1855 and has since been modified to suit various studies regarding the physiological functions of the liver including glycogen metabolism, amino acids metabolism and drug metabolism (Bartosek *et al*., 1973). This technique is also used to study the detoxification of foreign compounds. Aflatoxin B_1 study, regarding uptake, metabolism and excretion in bile, using isolated liver perfusion was carried out by Unger *et al*. (1977).

Sulphobromophthalein (BSP), a dye used in clinical and experimental evaluation of liver function is rapidly taken up by the hepatic parenchymal cells (Stege *et al*., 1975), conjugated primarily with glutathione via a thio ether linkage and excreted into bile against a concentration gradient (Javitt, 1971). Liver function can also be assessed by studying the clearance of BSP using isolated liver perfusion technique. Liang *et al*., (1978) used this technique to study the effects of uremic toxins on liver function by observing the clearance of BSP from perfusate and its excretion in bile.

The objectives of the present study were to assess liver function using BSP clearance and excretion with isolated liver perfusion technique, after exposure of liver donor rats to aflatoxin B_1 contaminated feed

stuff. A similar assessment was to be done after pretreatments with aflatoxin B_1, glutathione depletor and both glutathione depletor and aflatoxin B_1, prior to liver perfusion. Assessment of liver function with these pretreatments was done by finding

(a) Rate of elimination of BSP from perfusate by the liver

(b) Retention of BSP after a given time interval of perfusion

(c) Cumulative bile volume after a given time interval of perfusion.

SECTION TWO

LITERATURE REVIEW

Aflatoxins came into the limelight in the early 1960's when a severe outbreak of an unknown disease called "Turkey X disease" led to the deaths of more than 100,000 young poults in England. Similar outbreaks were also reported in Kenya and Uganda (Blount, 1961; Asplin and Carnaghan, 1961).

Turkey X disease was characterised by loss of appetite, lethargy and weakness of the wings. Affected birds died within a week. Post mortem examinations showed liver haemorrhages, necrotic hepatic lesions and frequently swollen kidneys. Histopathology revealed degeneration of liver parenchyma and extensive proliferation of bile duct-epithelium (Blount,1961).

It was the work done by Asplin and Carnaghan (1961) that led to the discovery of the toxic agent which was found in both Brazilian and East African groundnuts. Extraction of the toxic agent was initially carried out at the Central Veterinary Laboratory, Weybridge, England. Ducklings used as test animals were found to be highly susceptible to the toxic agent (Asplin and Carnaghan, 1961). Later it was found that the toxic agent was produced by an isolate of the common mould *Aspergillus flavus* (Sargeant *et al.*, 1961). The toxic agent was isolated and identified as being four principle metabolites of the fungus *Aspergillus flavus*. These metabolites were named "aflatoxins" and enumerated B_1, B_2 and G_1,G_2 according to their

fluorescence under ultra violet light and their separation on thin layer chromatography. (Nesbitt *et al*., 1962, Hartley *et al*., 1963).

The environmental distribution of *A. flavus* is universal (Semeniuk, 1954), but not all strains of the fungi produce aflatoxins. The production of aflatoxins further varies with geographical and seasonal factors depending on the conditions under which crops are grown, harvested and stored. Aflatoxins have been found in the following commodities

Oilseeds/meals - groundnuts, cottonseed copra, sunflower and soyabeans

Crude Vegetable Oils - groundnuts, olive and coconut

Cereal grains/meals - maize, sorghum, rice, wheat, barley, millet and oats.

Treenuts - pistachio, Brazilnuts, almonds, walnuts, pecans, and filberts.

Fruits - figs

Dairy Products - milk, butter, cheese - as metabolite of aflatoxin B_1 which is known as aflatoxin m_1.

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The production of aflatoxins is mainly dependent on the moisture content of the crops and the temperature conditions under which crops are stored. Diener *et al*. (1969) established a moisture content in equilibrium with a relative humidity of 85% ($aw^* = 0.85$) as the lower limit for growth of *A. flavus* and for production of aflatoxins. Davies *et al*. (1971) have reported

* aw water activity

minimum, optimum and maximum temperatures for aflatoxin production as 12°C, 27°C, and 40-42°C respectively. Northolt et al. (1976) concluded that detectable quantities of aflatoxin B_1 were not formed at water activity value (aw) below 0.83 and temperatures below 10°C.

The aflatoxin producing mould is traditionally considered to be a storage mould and aflatoxin contamination can therefore be prevented by proper storage of the cereal crops. It has recently been established that infection by mould and toxin production may also occur in unharvested cereal crops (Dickens and Satterwhite, 1973). Infection may also be aided by insect damage to crops Sellscohop et al., 1965; Stephenson and Russel, 1974). It is however considered that the post harvest production of aflatoxins is generally the most important.

The presence of *A. flavus* mould growth on a commodity is not necessarily indicative of aflatoxin contamination, but neither does the absence of visible mould growth assure freedom from aflatoxin contamination. Thus, finding *A. flavus* mould growth can only be a presumptive indication of aflatoxin contamination. However analytical determination must be undertaken to establish the presence of toxins (Global perspective on Mycotoxins, FAO/WHO/UNEP, 1977). Analytical assessment of aflatoxin contamination can be done by biological and chemical assays. Bioassay techniques currently available

are not suitable for routine screening purposes and also their detection-level limits their use for low concentrations of aflatoxin contamination. A commonly used bioassay method is chick embryo-developed by Horwitz *et al*. (1975). Several other bioassay procedures have been developed and reviewed by Goldblatt, (1969) and Ciegler *et al*. (1971). Chemical assays, however are more accurate and much quicker compared to bioassay techniques, though not specific when analysing for routine screening purposes. The various methods developed for the analysis of aflatoxins involve five basic steps i.e. extraction, lipid removal, clean up, separation and quantification. The most widely used method, the Contamination Branch (CB) method (Eppley, 1966) is the official method of the Association of Analytical Chemists. Any sample to be analysed is first extracted with chloroform. The remaining lipids and aflatoxins are then transferred to a silica gel column where the lipids are selectively eluted with hexane. Pigments are then eluted with absolute diethylether and the aflatoxins finally eluted with 3% methanol in chloroform. Separation is done on thin layer chromatography plates and the intensity of the fluorenscence of the sample under ultra violet light is compared with that of the standards and quantification done accordingly.

Analytical procedures have been developed for various oilseeds cereals and dairy products. These

methods are well documented by Jones,(1972). Qualitative mini column techniques have also been developed for field conditions for a wide range of commodities. (Holaday, 1976). Recently, methods using high pressure liquid chromatography have been developed for mycotoxin analysis. These methods are more sensitive and accurate and can be applied to a number of mycotoxins (Panalaks *et al*., 1977).

Several analytical methods for the detection of aflatoxin residues in animal tissues have also been developed and their detection limits evaluated (Jemmali and Murthy, 1976).

AFLATOXICOSIS

The term aflatoxicosis implies the disease condition caused by the action of the poison i.e. the toxin upon its entrance into the host's body. (Forgacs, *et al*., 1962).

In the early 1950's, before the discovery of the toxic agent, cattle, pigs and dogs died in the USA from eating mouldy corn (Seibold *et al*., 1952; Sippel *et al*., 1953; Newberne *et al*., 1955; Bailey *et al*., 1959). Outbreaks also occurred in pigs, fed on groundnut meal which was later found to be contaminated (Loosemore and Harding, 1961). It was later proved that the causative agent was aflatoxins in the feedstuff (Newberne *et al*., 1966).

Outbreaks of hepatitis in man and dogs occurred in India due to aflatoxicosis, where toxic hepatitis was associated with the consumption of heavily

aflatoxin contaminated maize (Krishnamachari *et al.*, 1975a). Dogs, feeding on food remnants in the affected villages manifested a disease characterised by jaundice, ascites and frequently death (Krishnamachari, *et al.*, 1975b).

In Kenya, between July and October 1978, a great number of canine deaths due to aflatoxicosis were reported from Nairobi, Mombasa, Malindi, Eldoret and Nakuru (Price *et al.*, 1978). Analysis of different commercially prepared dog feed, using thin layer chromatography, revealed aflatoxin levels ranging from nil to 3,000 ug/kg (Price *et al.*, 1978). Clinical and pathological findings were also described by Price *et al.* (1978).

Susceptibility and clinical and pathological changes to aflatoxicosis varies between animals, and in general young animals are more susceptible to the disease than adults. The order of susceptibility in domesticated mammals as estimated by feeding contaminated rations is as follows: Young piglets, 3 to 12 weeks old are most susceptible followed by pregnant sows, calves, fattening swine, mature cattle and sheep (Allcroft, 1969). Clinical signs, gross and microscopic lesions associated with acute and chronic aflatoxicosis in animals have been reviewed by Edds (1973); Newberne (1973) and Butler (1974).

Generally the clinical signs of acute intoxication in most species studied include lack of

appetite, weight loss, unthriftiness, icterus, neurological disorders including convulsions and death. Pathological changes are most common in the liver whereby the liver is pale or discoloured and hepatic sections show diffuse centrilobular necrosis with fat accumulation within the hepatocytes. Chronic aflatoxicosis induce liver changes characterised by marked bile duct proliferation and periportal fibrosis leading to cirrhosis. The clinical picture is dominated by marked icterus (Newberne, 1973).

The table below shows a wide range of dietary aflatoxin concentration causing toxicosis in various animal species. It also shows susceptibility in various age groups.

Table 1: Dietary aflatoxin concentration causing toxicosis from Edds.G.T., 1973

Species	Age	Aflatoxin	Duration of feeding	Effects
Calves	Weanling	0.22-2.2	16 weeks	stunting, death, liver damage
Steers	2 years	0.22-0.66	20 weeks	liver damage
Cows	2 years	2.4	7 months	liver damage clinical illness
Pigs	Newborn	0.234	4 days (to sow)	stunting
Pigs	2 weeks	0.17	23 days	anorexia, depression, icterus Ascites, stunting
Pigs	4-6 weeks	0.41-0.69	3-6 months	stunting, liver damage
Chickens	1 week	0.84	10 weeks	stunting, liver damage
Ducks	unknown	0.3	6 weeks	liver damage, death

Carcinogenicity

Aflatoxins, besides being hepatotoxic, are also known to be carcinogenic. Long-term exposures to low concentrations of aflatoxin in diet of animal feeds causes the development of hepatoma, cholangiocarcinoma, and hepatocellular carcinoma (Newberne, 1973). The carcinogenesis of aflatoxins has been reviewed by Wogan (1973, 1977). A concise report on aflatoxin carcinogenesis in experimental animals has been published by WHO (1979).

Effects in Man: Epidemiological Studies

Liver carcinogenesis:

Available epidemiological data, primarily from regions of sub Saharan areas of Africa and South East Asia support the positive correlation of aflatoxin ingestion and human cancer in population studies in which estimates of aflatoxin intake and incidence of primary liver cancer were made concurrently. Studies in Uganda (Alpert *et al*., 1971), Swaziland (Keen *et al*., 1971) and Thailand (Shank *et al*., 1972) revealed positive indications between the frequency of aflatoxin contamination of foods at markets and in home stores, and the frequency of liver cancer in the study areas.

Studies in Kenya (Peers *et al*., 1973), Mozambique (Van Rensburg *et al*., 1974) and Swaziland (Peers *et al*., 1976) have shown positive relationship between the actual aflatoxin concentration of the meals about to be eaten and the incidence of primary liver cancer in

areas from which the meal samples were taken. Linsell and Peers (1977) have reported a possibility of viral involvement (Hepatitis B virus) in the aetiology of aflatoxin-associated liver cancer in these areas. Hepatitis B infection is common in countries with a high incidence of primary liver cancer and those individuals suffering from liver cancer also showed evidence of prior exposure to hepatitis B virus. (Vogel *et al*., 1970; Reys and Sequeira, 1974; Prince *et al*., 1975; Chainuvati *et al*., 1975). Nevertheless the present evidence favours aflatoxin as a possible major disease cause in primary liver cancer, but hepatitis B virus may well be a cofactor in the aetiology (Peers and Linsell, 1977).

Acute toxicity data are relatively scarce in relation to human exposure, but there are reported epizootics of "apparent" aflatoxicosis (Shank *et al*., 1971, Krishnamachari *et al*., 1975a).

SECTION THREE

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

Albino rats obtained from Veterinary Research Laboratories, Kabete weighing between 170g to 300g were used as liver donars for the isolated liver perfusion experiments as well as blood donars for the preparation of perfusate solutions. All rats were fed on laboratory chow and water *ad libitum* and kept under average room temperatures of 25°C. Rats used for the aflatoxin feeding trials were fed on aflatoxin contaminated rations only.

APPARATUS

A perfusion chamber consisting of upper unit, middle perfusator dish and lower oxygenator unit (Fig.3).

REAGENTS

All reagents used were analytical grade (Analar)

(1) Kreb Ringer's bicarbonate buffer consisting of:-

(a) Sodium chloride	Pronalysis	-	Mark Darmstadt
(b) Calcium chloride $2H_2O$	"	-	"
(c) Potassium chloride	"	-	"
(d) Sodium bicarbonate	"	-	"
(e) Magnesium chloride $6H_2O$	"	-	"
(f) Sodium dihydrogen phosphate	"	-	"

(2) Bovine albumin - Sigma Chemicals

(3) Heparin from Nyegaard and Co., Norway, diluted to 1000 I.E./ml in saline.

(4) Sodiumhydroxide made upto 10% solution in distilled water.

(5) Sodium chloride for preparation of physiological saline (pH 7.2)

(6) Hydrochloric acid diluted to make a 10% solution

(7) Sodium hydroxide solution of 0.1N.

DRUGS used were:-

Sulphobromophthalein (BSP), Koch Laboratories

Methoxyflurane (Penthrane[R]) - inhalant anaesthetic, Abbot Laboratories

Aflatoxin B_1 - Sigma Chemicals USA

Dimethylsuphoxide (DMSO), Merck Darmstadt

Diethylmaleate (DEM) given by Department of Pharmacology and Toxicology, Veterinary College of Norway.

For the contaminated feed experiments

Commercial pig finishing feed with 500 µg aflatoxin B_1/kg and home made maizemeal with 3000 µg aflatoxin B_1/kg were used.

PERFUSION METHOD (APPARATUS DESIGN)

This method was based on the general principles described by Miller *et al.* (1951) but with some modifications. A semi-schematic presentation of the perfusator is shown in Fig 3 and it consists of three main parts.

(1) The upper unit is a reservoir with a wide outlet for overflow securing a constant pressure of 16 cm of water. From the reservoir, a perfusate reaches the liver through a coiled glass tube which at the outlet is connected to

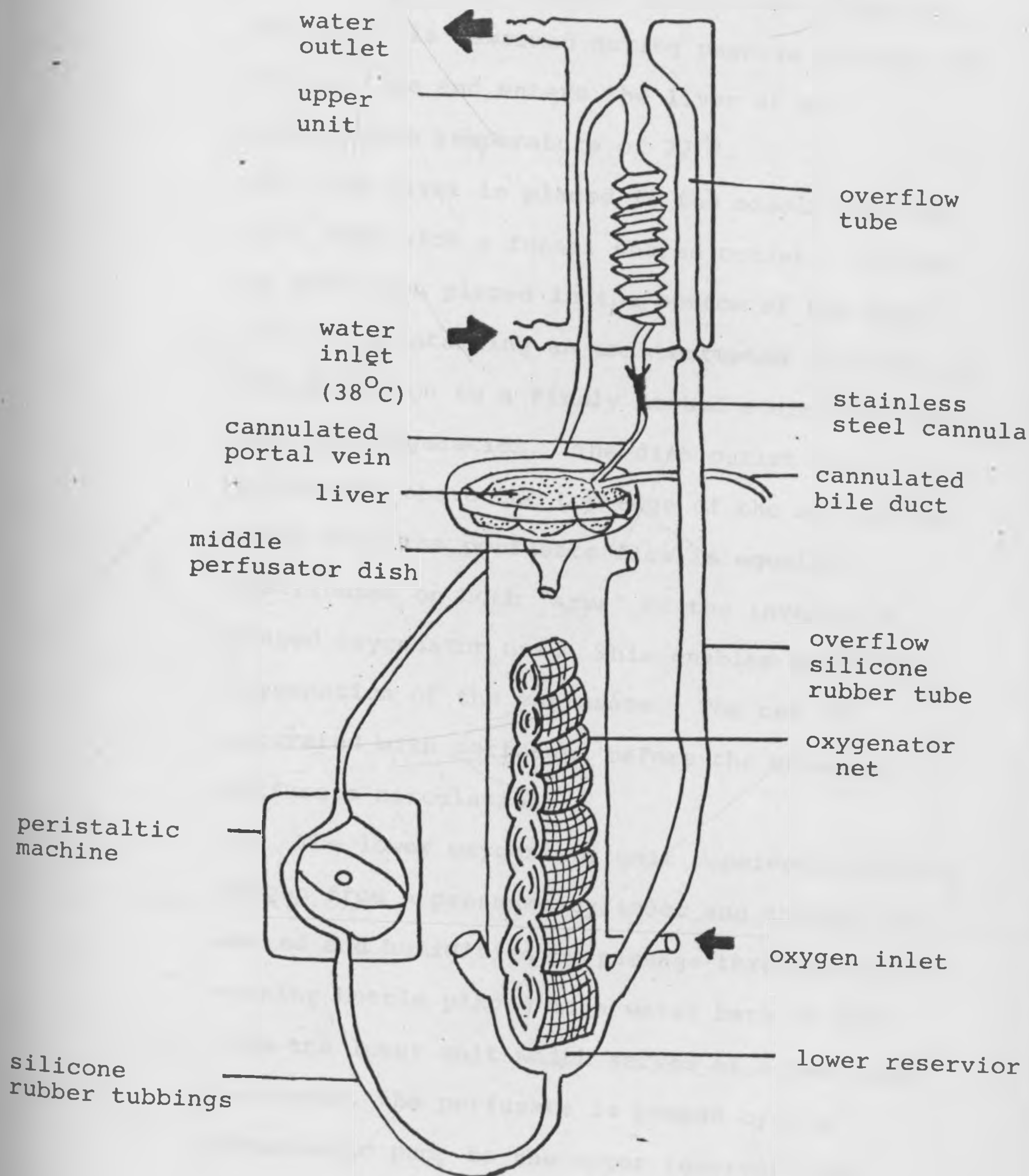

Fig 3: A semi schematic diagram of liver perfusion apparatus.

a stainless steel cannula. The whole upper unit is surrounded by a water jacket maintained at 38°C, but is rewarmed during passage through the coiled tube and enters the liver at an approximate temperature of 37°C.

(2) The liver is placed in the middle portion, in a dish with a funnel shaped outlet. A piece of wire-mesh placed in the bottom of the dish, aids in maintaining an uninterrupted flow through the outlet on to a finely meshed stainless steel net for oxygenation. The dish outlet is positioned above the top edge of the net in such a way that the perfusate flow is equally distributed on both "arms" of the inverted V shaped oxygenator net. This enables sufficient oxygenation of the perfusate. The net is saturated with perfusate before the onset of perfusate circulation.

(3) The lower oxygenator unit receives medicinal oxygen from a pressure cylinder and the gas is warmed and humidified by passage through a gas washing bottle placed in a water bath at 38°C. From the lower unit which serves as a perfusate reservoir, the perfusate is pumped by a peristaltic pump to the upper reservoir and into the coiled glass tube. Both upper and lower reservoirs are available for collection of perfusate samples as well as for addition

of substances. When not in use, the openings in these reservoirs are covered using parafilm.

PREPARATION OF PERFUSATE SOLUTION

Blood was drawn from the abdominal aorta of ether anaesthesised rats using heparinised syringes. About 12 to 15mls of heparinised blood was filtered through cotton gauze and diluted with two parts of bovine albumin - Kreb Ringer's bicarbonate buffer to one part of whole blood. Kreb Ringer's solution was prepared by dissolving 19.2g of sodium chloride, 0.48g of potassium chloride, 0.8g of calcium chloride and 0.24g of magnesium chloride in distilled water to a final volume of two (2) litres. Bicarbonate buffer was prepared by dissolving 3.0g of sodium bicarbonate and 0.25g of sodium dihydrogen phosphate in distilled water to a final volume of 500mls. These stock solutions were frozen at $-20^{o}C$ and could be used upto one month.

For the preparation of the perfusate solution, 50mls of the Kreb Ringer's solution and 10mls of the bicarbonate buffer, both at room temperature, were added to 1.5gm of bovine albumin and stirred thoroughly to a clear solution. The albumin buffer solution was adjusted to a pH of 7.4 using a few drops of 0.1 N sodium hydroxide solution.

Thirty mls of perfusate solution was added to the perfusate chamber which included the volume required to saturate the oxygenator net. The rest of

the perfusate solution was used to replendish the flowing perfusate after known volumes had been withdrawn as samples. The perfusate was circulated in the perfusion chamber for about 15 to 20 mins to attain a required temperature of 37°C prior to the assembling of the liver.

SURGICAL PROCEDURE

The weighed donor rat was anaesthesised using methoxyflurane. The rat's abdomen was cut open with a "U" shaped transverse incision. Bile duct was identified and exposed after displacing the intestines to the left side of the abdominal cavity. A snip incision, 15 to 20 cm from the duodenal end of the duct was made and the duct cannulated using the small diameter polyethylene cannula and secured with a single ligature. A check was made to ensure free flow of bile through the cannula. Loose connective tissue and fat deposits covering the iliolumbalis dextral vein were dissected to expose the vein. 0.15ml of heparin solution was injected into the vein and the injection site clamped to prevent bleeding. This was done to avoid coagulation of blood especially in the liver. Similarly the portal vein was located and cannulated with the polyethylene cannula of bigger diameter. The cannula was initially filled with perfusate solution prior to cannulation, so as to avoid air bubbles entering the portal vein and secured using a cotton thread ligature. After cannulation, prompt

removal of the liver was done by exercising all liver attachments to the abdominal cavity, diaphragm, stomach, spleen, kidney and intestines. The small perfusion pump, to which the portal cannula was attached, was switched on immediately after severing the vena cava.

The liver was supplied with perfusate solution by the small perfusion pump prior to being fixed on to the exit cannula of the upper unit of the perfusion chamber. The liver was then placed on the middle perfusator dish while perfusate flowed freely out of the exit cannula at 37°C. The portal cannula was cut and the perfusator steel cannula inserted into it. By this connection technique, no air bubbles were formed. During perfusion, the perfusate solution entered the liver via portal vein and left the liver through the cut ends of the vena cava (hepato caval sinus). The liver was positioned in such a way, that compression of the portal branches was avoided and even distribution of the perfusate to all lobes of the liver maintained. The bile duct cannula was also positioned to avoid any kinking of the bile duct which could hinder proper bile flow. The liver was allowed to stabilise for 30 minutes before the start of experiments. The flow of perfusate at the end of the stabilisation time was recorded as drops per min.

Sulphobromopthalein (BSP) was used to study the liver function of the perfused livers. Using a concentration of 3mg/ml, a dose of 12mg/kg body weight

of the rats was added to lower reservoir at the start of the experiment.

COLLECTION OF SAMPLES

Perfusate samples of lml were taken at 5 mins, 10 mins, 15 mins, 30 mins 45 mins 60 mins, and even upto 75 and 90 minutes time intervals and transferred into respective centrifuge tubes. At every removal of the perfusate samples, an equal volume of perfusate solution was used to replendish the circulating perfusate. Similarly, bile volume samples were collected at 15 mins, 30 mins, 45 mins, 60 mins, 75 mins and 90 mins time intervals. At the end of every hour after the start of the perfusion, 1 ml of perfusate solution was added to the liver surface to keep the surface moist and 1 ml of distilled water added to the lower reservoir to allow for the loss of water due to evaporation. At the end of the perfusion, the flow rate was again recorded. These recordings of drops per minute enabled the judgement of the degree of alteration in the flow rate of the perfusate.

SAMPLE PREPARATION

The perfusate samples collected were centrifuged and the clear supernatant transferred to smaller tubes. Two rows of eight tubes, each labelled 5 mins upto 90 mins were set up. 4.8mls of Dilution solution I* were pippeted into each of the tubes of the first row (alkaline solution). Similarly, 4.8mls of Dilution solution II** were pippeted into individual tubes of the second row (acidic solution). 0.2ml of the

supernatant from each sample was added to all tubes of both rows. The first row (alkaline) liberated the purple colour of sulphobromophtalein (BSP) whereas the second (acidic) row did not. A third row of six tubes for bile samples labelled 15 mins upto 90 mins was set up with 5mls of Dilution solution I, and 10 μl of bile from each sample was added to each dessignated tube. Both basic and acidic blanks were prepared using Dilution solutions I and II respectively. These blanks were used for setting the zero reading of the spectrophotometer. All tubes were thoroughly shaken with the test tube shaker prior to spectrophotometery readings.

* Dilution solution I : - 100mls of physiological saline plus 5mls of 10% hydroxide solution (alkaline solution)

** Dilution solution II.- 100mls of physiological saline plus 5mls of 10% hydrochloric acide (acidic solution).

SPECTROPHOTOMETRY READINGS

Optical densities of both perfusate and bile samples were recorded at a wavelength of 580 nanometres (nm). The optical density of BSP in perfusate supernatant samples was corrected for hemolysis by subtracting the optical densities recorded for acidic solutions from the

optical densities of alkaline solutions. The optical densities of the samples represented the amount of BSP in 0.2ml of perfusate which was calculated from the standard curve (Fig. 4). Therefore the total amount of BSP in circulating perfusate (30mls) at a particular time interval was calculated by multiplying the amount in 0.2ml by a factor of 150. This total amount of BSP at a designated time interval was then expressed as a percentage of the initial amount added to the circulating perfusate.

Bile volumes at each designated time interval were measured using micropippetes and estimated to the nearest µl. Similarly, amount of BSP in bile samples was calculated from the standard curve of BSP (Fig.5). This amount of BSP corresponded to 10µl of bile. Therefore total BSP in bile at a particular time interval was calculated in relation to the bile volume measured at that time interval. The excretion of BSP in bile was then expressed as percentage of the initial amount added to the circulating perfusate.

PREPARATION OF STANDARD CURVE OF SULPHOBROMOPATHALEIN

A standard solution of BSP (0.1mg/ml) was first prepared using distilled water as solvent. Ten clean test tubes labelled T_1 to T_{10} were arranged and to each tube were added various volumes of Dilution solution I and standard solution of BSP (Table 2).

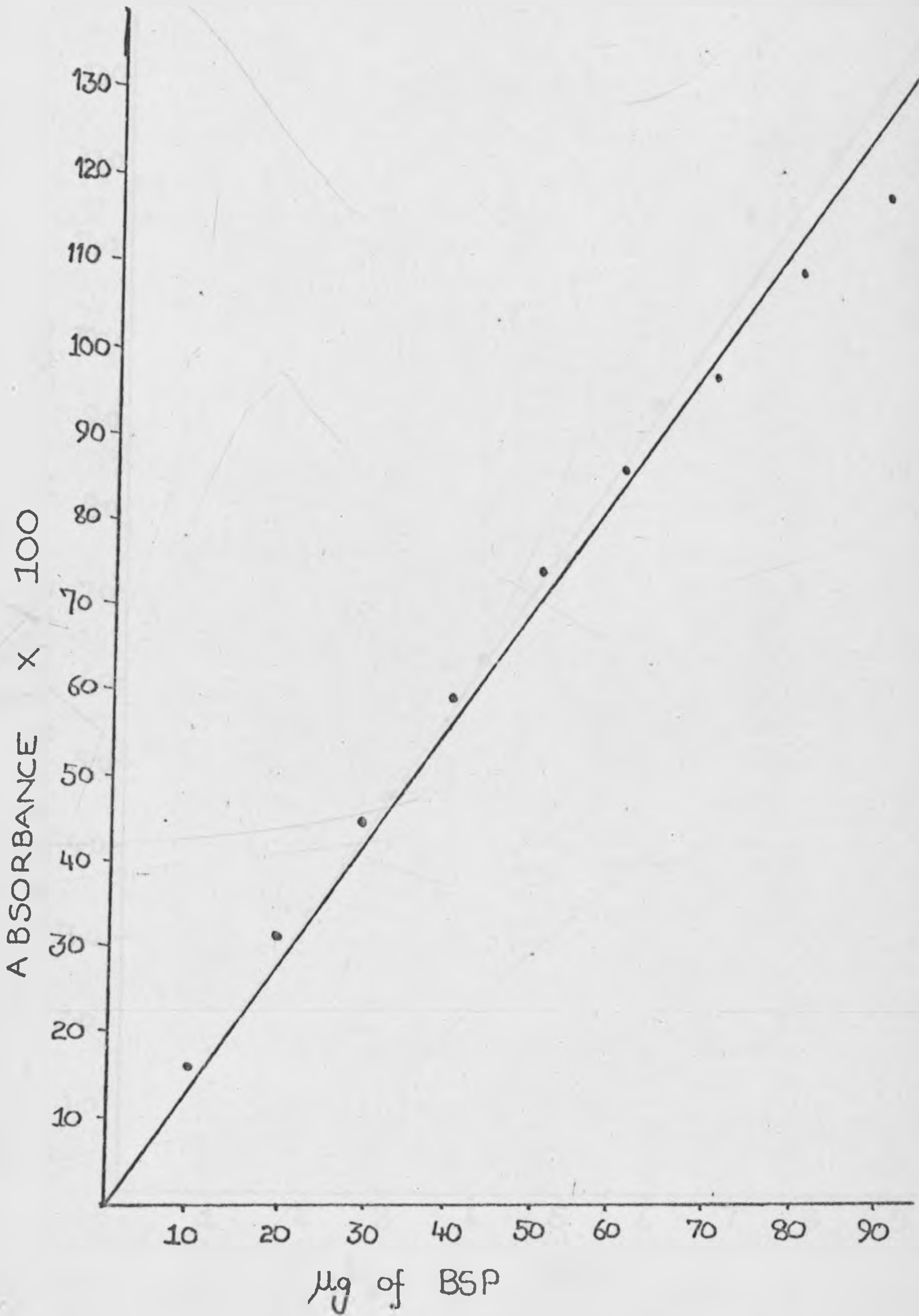

Figure 4: Standard curve of BSP for bile samples.

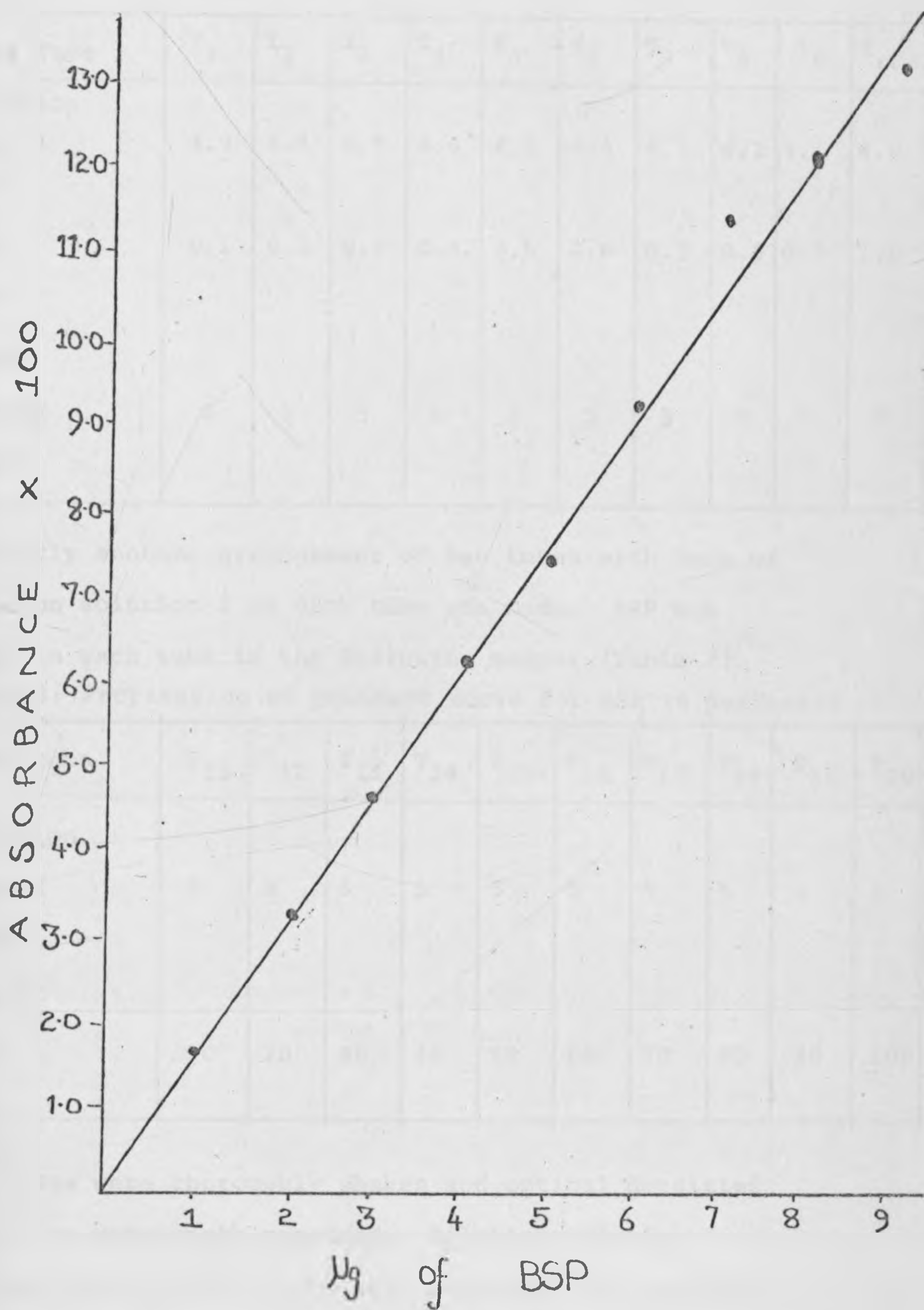

Figure 5: Standard curve of BSP for perfusate samples.

TABLE 2: Preparation of Standard curve for BSP in bile.

Test Tube	T_1	T_2	T_3	T_4	T_5	T_6	T_7	T_8	T_9	T_{10}
Dilution Soln I (ml)	4.9	4.8	4.7	4.6	4.5	4.4	4.3	4.2	4.1	4.0
BSP (ml)	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0
TOTAL VOLUME (ml)	5	5	5	5	5	5	5	5	5	5

Similarly another arrangement of ten tubes with 5mls of Dilution solution I in each tube was made. BSP was added in each tube in the following manner (Table 3)

TABLE 3: Preparation of Standard curve for BSP in perfusate.

Test tube	T_{11}	T_{12}	T_{13}	T_{14}	T_{15}	T_{16}	T_{17}	T_{18}	T_{19}	T_{20}
Dilution Soln I (ml)	5	5	5	5	5	5	5	5	5	5
BSP (µl)	10	20	30	40	50	60	70	80	90	100

All tubes were thoroughly shaken and optical densities at 580 nm wavelength measured. Dilution solution I was used as a blank to set zero reading. The optical densities recorded were plotted on two graphs.

To assess the rat's liver function by studying the clearance in perfusate and excretion in bile of BSP using isolated liver perfusion technique, three parameters, rate of elimination of BSP from perfusate, the percentage (%) retention of BSP by the liver and the volume of bile excreted were calculated.

(1) <u>Rate of elimination of BSP from perfusate</u>

This was expressed as half life ($t\frac{1}{2}$) in minutes and the remaining percentages of BSP in the perfusate were plotted against time in minutes on a semilogarithmic scale. The best fitting points for the rate of elimination of BSP for individual rats of all control as well as experimental groups were obtained by using a logarithmic curve fit programme. This programme obtained from a Helwett Packard 97 calculator, gave the best regression lines from which individual half lives were calculated.

(2) <u>The Percentage (%) BSP retained by the liver</u>

This value was calculated after 60 minutes of perfusion. The percentage of BSP still remaining in the perfusate after 60 minutes of perfusion was added to the total amount of BSP, expressed as percentage, excreted in bile upto 60 minutes. This added value was then subtracted from the initial amount of BSP administered into the perfusate at the start of the experiment (i.e. 100%). The difference obtained was therefore the percentage of BSP retained by the liver after 60 minutes.

(3) Cumulative bile volume

The amount of bile volume was governed by the size of the perfused liver. Therefore, the bile volume was expressed as μl per gram liver weight ($\mu l/g$ liver wt).

STATISTICAL ANALYSIS

Student's 't' test was performed for all statistical analysis of the experimental and control groups. Significance level of 95% was used for all groups.

EXPERIMENTAL DESIGN

CONTROLS

Seven liver donor rats fed on laboratory chow and water *ad libitum* were used to study liver function as assessed by BSP clearance and excretion using isolated liver perfusion technique.

EXPERIMENT 1

Two groups of rats, Group Ia and Ib, were fed on aflatoxin contaminated ration prior to liver perfusion.

(1) Group Ia: Rats were fed on commercial sow and weaner meal containing approximately 500 μg of aflatoxin B_1/kg of feed. The duration of feeding was for two weeks prior to liver perfusion.

(2) Group Ib: Aflatoxin B_1 contaminated home-made maizemeal containing 3000 μg of aflatoxin B_1/kg of feed was fed to five rats for a period of 10 days. One of the rats died during the surgical liver removal, thus only four rat livers were perfused. The duration of feeding with contaminated rations was short for both

groups due to inavailability of more contaminated ration.

EXPERIMENT 2

Aflatoxin B_1, at a dose of 3 mg/kg was dissolved in 0.2ml of dimethylsulphoxide (DMSO) and injected intraperitoneally (i.p) to liver donar rats 4 hours prior to liver perfusion. Seven liver donar rats (Group II) were used for the isolated liver perfusion.

EXPERIMENT 3

Rats were injected with a glutathione depletor, diethylmaleate (DEM) at a dose of 0.6ml/kg i.e. (Group III). This glutathione depletor dosage was used according to Mitchel *et al*. (1973) who observed a lowering of hepatic glutathione to about 6% of normal within 30 minutes without causing any harm to the liver. Perfusion of the livers of pretreated rats was carried out 30 minutes after treatment. One perfusion experiment failed due to power failure, thus only six replications were performed.

EXPERIMENT 4

Rats were pretreated with DEM (0.6ml/kg i.p) 30 minutes before being treated with aflatoxin B_1 (3.0 mg/kg i.p). Liver perfusion of these pretreated rats was carried out 4 hours later. Of the seven rats pretreated, only five liver perfusions were successful. In most cases, the cause of failure of liver perfusion was poor perfusion flow rate, lack of bile formation or death of the rat during surgery.

SECTION FOUR

RESULTS

CONTROLS

The rate of elimination of BSP from the perfusate expressed as half life of elimination (half life, $t\frac{1}{2}$) gave the mean value of 11.5 $\pm$ 2.5 mins. (Table 4). The individual values for half lives were calculated from the most linear sections of the rate of elimination graphs, drawn in the semi logarithmic scale (Fig. 6). These linear sections ran to about 60 minutes of perfusion time after which "tailing effects"* were observed (Fig. 6). The tailing effects became prominent when % BSP values were below 10%. Similar observation was noticed from the experimentally treated groups. As a result, all data were calculated within 60 minutes of perfusion. The mean value for % BSP retained after 60 minutes of perfusion was 19.3% $\pm$ 10%. Deviation within the group is high and is illustrated on a bar chart (Fig. 14). Cumulative bile volume expressed as µl/g liver weight excreted upto 60 minutes of perfusion showed a mean value of 48 µl/g liver weight $\pm$ 17 (Table 4).

* Tailing effects are discussed in the discussion.

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Table 4: shows results of groups of rats used as controls.

CONTROLS			
Rate	Mean Value ($\bar{x}$)	Standard Deviation (s)	Number of Replications (n)
Rate of elimination of BSP (Half life) $t_{1/2}$ mins	11.5 min	2.5 min	7
% BSP retained by liver after 60 minutes of perfusion	19.3%	10.0%	7
Cumulative bile volume µl/g liver weight excreted upto 60 minutes of perfusion	48	17	7

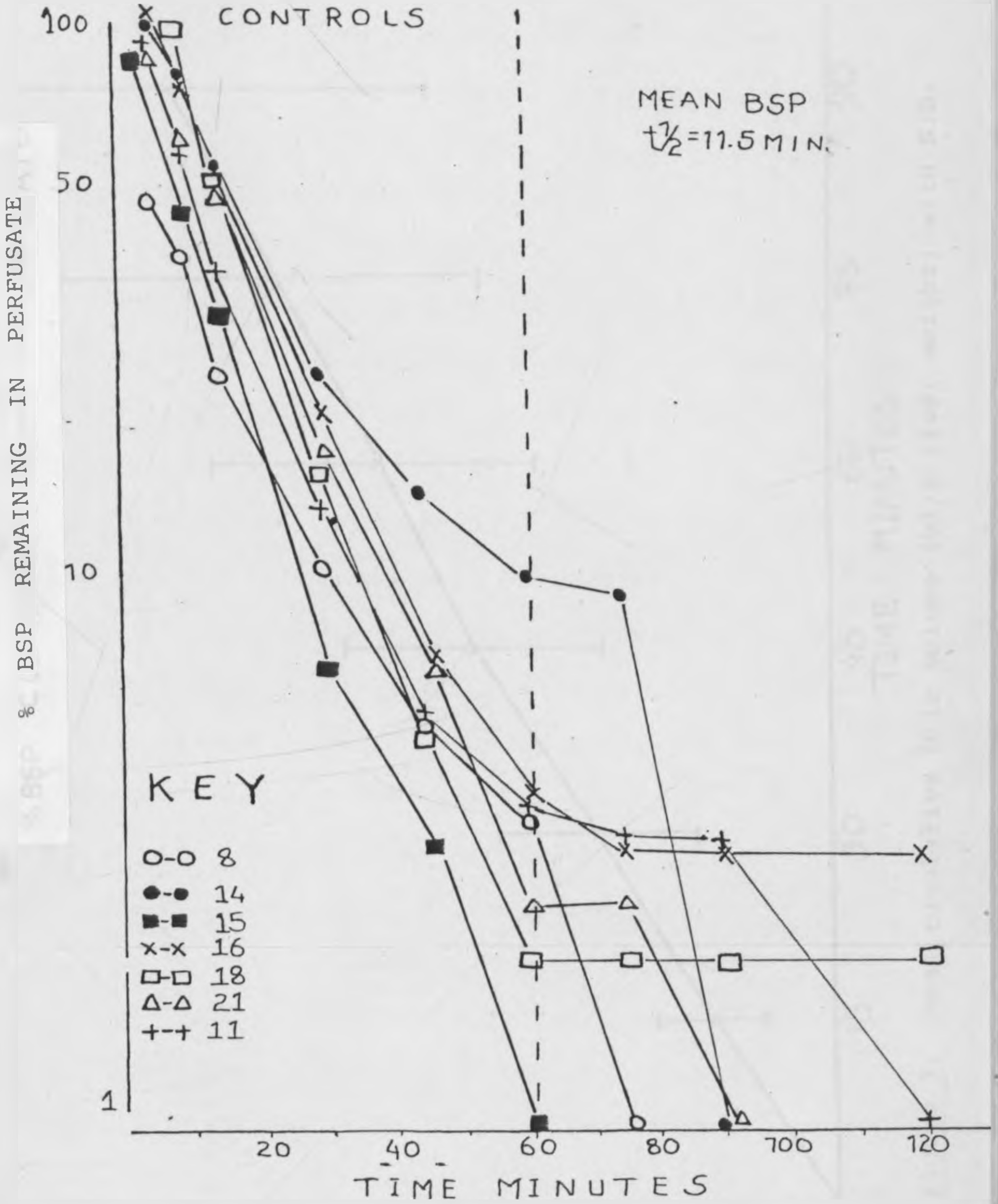

Figure 6: Rate of elimination of BSP from perfusate of the control Group.

* Notice the "tailing effects" observed after 60 minutes.

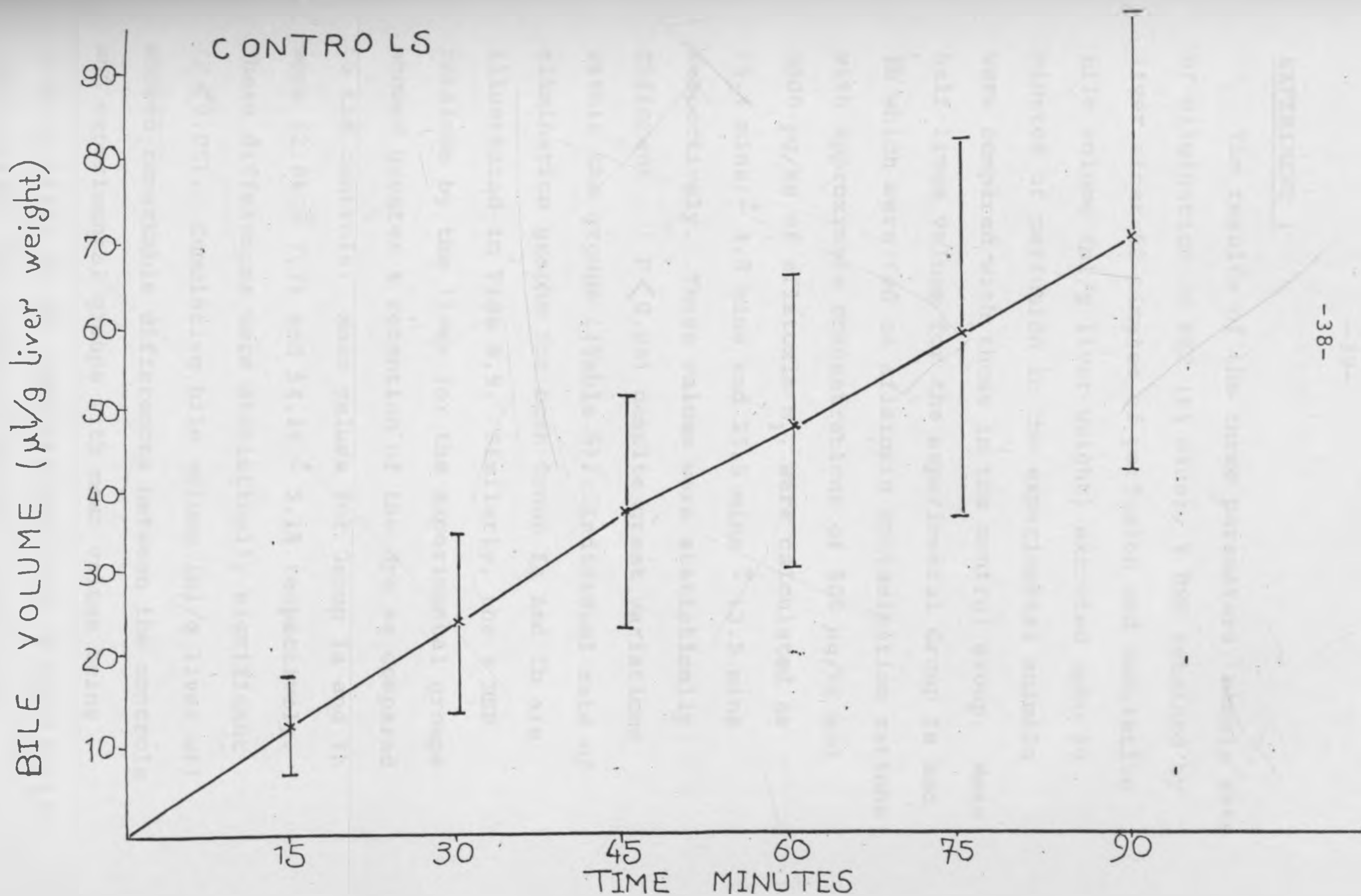

Figure 7: Mean cumulative bile volume (ul/g liver weight) with S.D.

EXPERIMENT 1

The results of the three parameters, namely rate of elimination of BSP (t½ mins), % BSP retained by liver after 60 minutes of perfusion and cumulative bile volume (µl/g liver weight) excreted upto 60 minutes of perfusion in the experimental animals were compared with those in the control group. Mean half lives values for the experimental Group Ia and Ib which were fed on aflatoxin contamination rations with approximate concentrations of 500 µg/kg and 3000 µg/kg of aflatoxin B_1, were calculated as 15.4 mins $\pm$ 3.8 mins and 25.5 mins $\pm$ 13.5 mins respectively. These values were statistically different ($P < 0.05$) despite great variations within the groups (Table 5). Individual rate of elimination graphs for both Group Ia and Ib are illustrated in Figs. 8,9. Similarly, the % BSP retained by the liver for the experimental groups showed greater % retention of the dye as compared to the controls. Mean values for Group Ia and Ib were 42.8% $\pm$ 7.7% and 54.2% $\pm$ 5.1% respectively. These differences were statistically significant ($P < 0.05$). Cumulative bile volume (µl/g liver wt) showed remarkable differences between the controls and experimental groups with mean values being 48 µl/g liver wt for controls and 15 µl/g and 17 µl/g liver weight for Groups Ia and Ib respectively.

The experimental groups thus showed great reduction in bile volume (Fig.10). Variation within the groups was very high. Inspite of these variations statistical analysis revealed significant differences.

This experiment showed that liver function of rats fed on aflatoxin contaminated feed was affected as carried out by isolated liver perfusion technique. This experiment also confirms that the feed was toxic to a certain degree to rat livers.

Table 5: results of two groups of rats fed on different levels of aflatoxin contaminated feed prior to liver perfusion.

Group Ia: fed on aflatoxin contaminated feed (500 μg/kg AFB_1) for 2 weeks.

Group Ib: fed on aflatoxin contaminated feed (3000 μg/kg AFB_1) for 10 days.

	Group Ia				Group Ib			
	MEAN VALUE ($\bar{x}$)	STANDARD DEVIATION (s)	NO. OF REPLI-CATIONS (n)	P VALUE	MEAN VALUE ($\bar{x}$)	STANDARD DEVIATION (s)	NO. OF REPLI-CATIONS (n)	P VALUE
Rate of elimination of BSP (Half Life) t½ minutes	15.4	3.8	5	**	25.5	13.5	4	**
% BSP retained in liver after 60 minutes of perfusion	42.8	7.7	5	**	54.2	5.1	4	**
Cumulative bile volume μl/g liver weight excreted upto 60 minutes of perfusion	15	6	5	**	17	13	4	**

** = $P < 0.05$

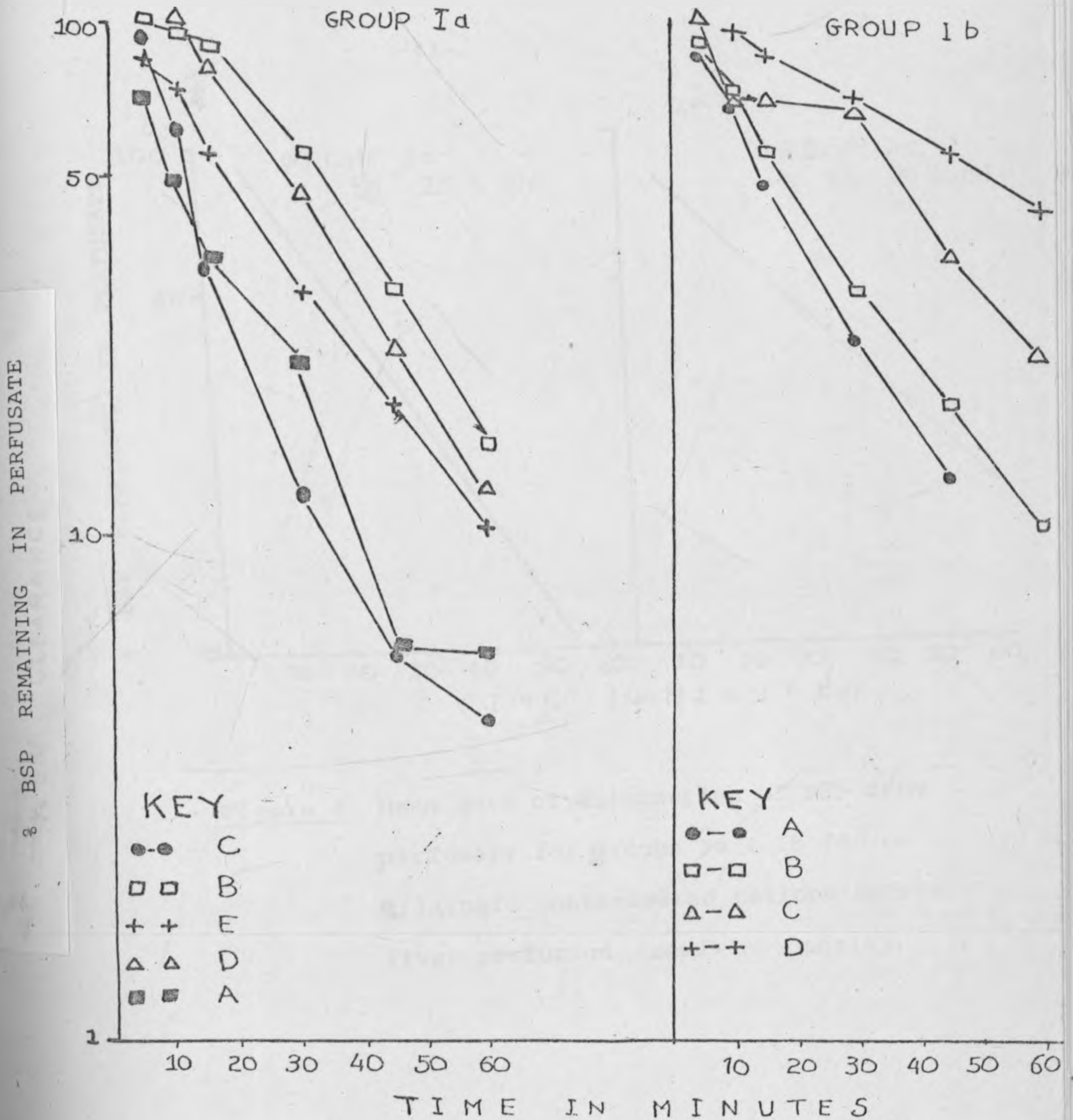

Figure 8: Individual rate of elimination of BSP from perfusate for groups Ia and Ib which were fed on aflatoxin contaminated feed (500 ug/kg and 3000 ug/kg aflatoxin B_1 respectively) prior to liver perfusion (semi-log scale).

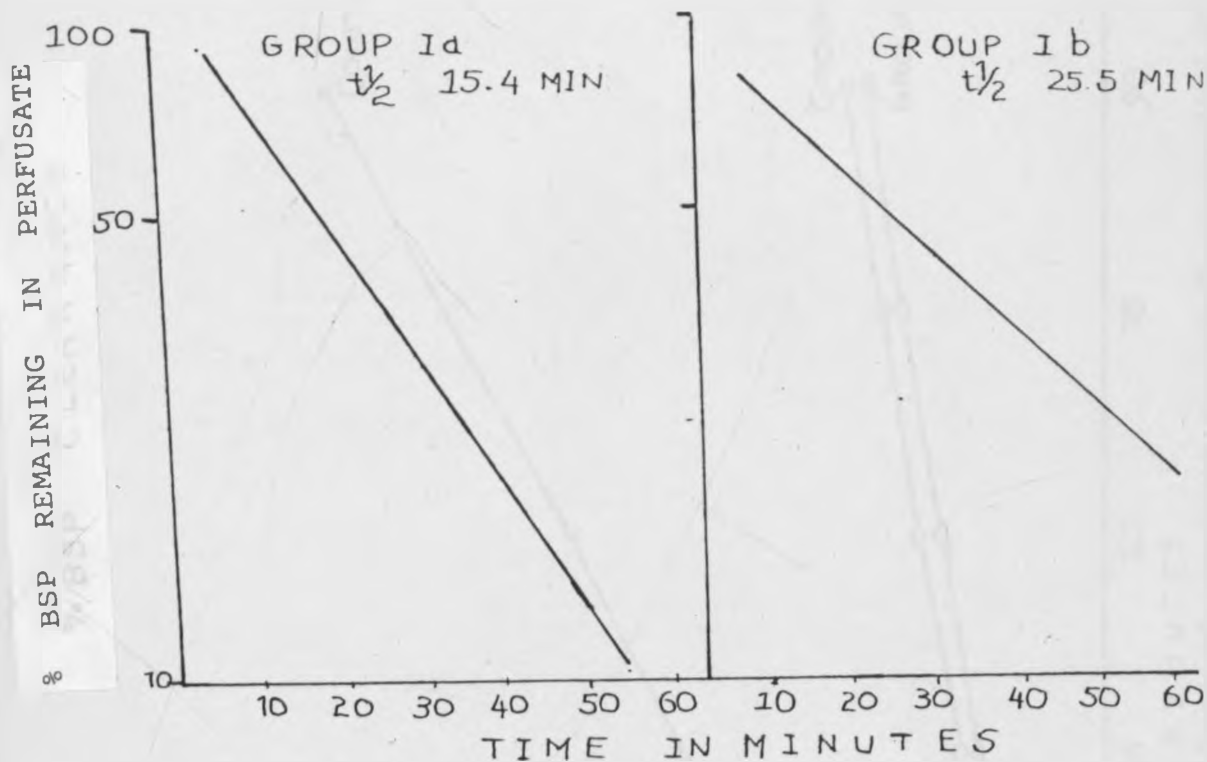

Figure 9: Mean rate of elimination of BSP from perfusate for groups Ia & Ib fed on aflatoxin contaminated rations before liver perfusion. (semi-log. scale).

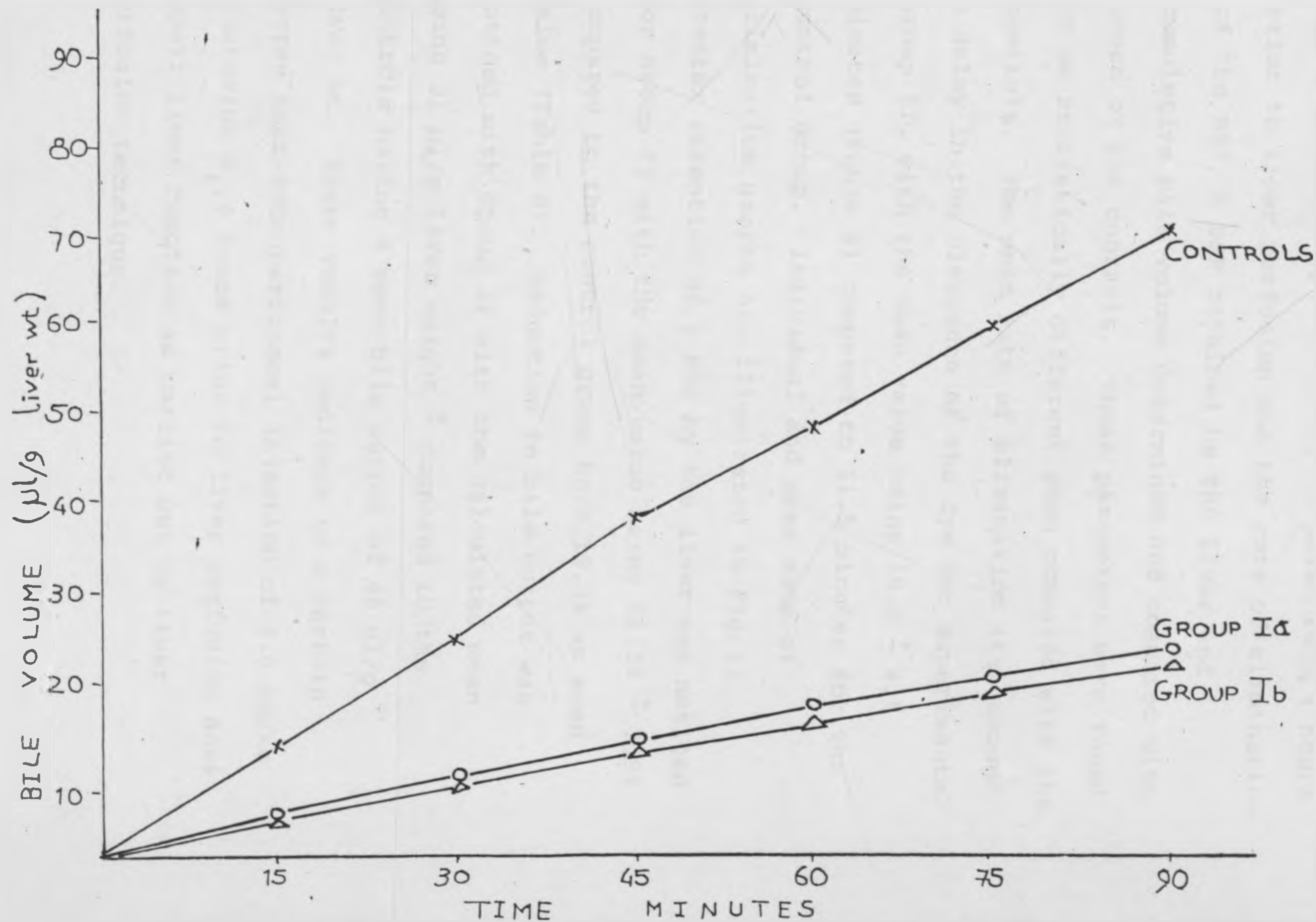

Figure 10: Mean cumulative bile volume of the control and experimental groups Ia and Ib.

EXPERIMENT 2

In this experiment 3.0 mg/kg of aflatoxin B_1 was administered intraperitoneally to seven rats, 4 hours prior to liver perfusion and the rate of elimination of the BSP, % BSP retained by the liver and cumulative bile volume determined and compared with those of the controls. These parameters were found to be statistically different when compared with the controls. The mean rate of elimination (t½) showed a delay in the clearance of the dye for experimental group II, with the mean value being 19.2 ± 4.5 minutes (Table 6) compared to 11.5 minutes for the control group. Individual and mean rate of elimination graphs are illustrated in Fig. 11. Greater retention of % BSP by the liver was noticed for Group II with the mean value being 41.3% ± 7.9% compared to the control group have 19.3% as mean value (Table 6). Reduction in bile output was noticed with Group II with the calculated mean being 31 µl/g liver weight ± compared to the controls having a mean bile output of 48 ul/g liver wt. These results indicate to a certain degree that intraperitoneal injection of 3.0 mg/kg aflatoxins B_1, 4 hours prior to liver perfusion does impair liver function as carried out by liver perfusion technique.

Table 6: shows results of groups of rats (Group II) pretreated with aflatoxin B_1 i.e. 4 hours prior liver perfusion and compared with controls.

	C O N T R O L S			G R O U P II			
	MEAN VALUE ($\bar{x}$)	STANDARD DEVIATION (s)	NO. OF REPLICA-TIONS (n)	MEAN VALUE ($\bar{x}$)	STANDARD DEVIATION (s)	NO. OF REPLICA-TIONS (n)	P. VALUE
Rate of elimination of BSP (Half Life) $t_{1/2}$ minutes	11.5	2.5	7	19.2	4.5	7	**
% BSP retained in Liver after 60 minutes of perfusion	19.3%	10.0	7	41.3	7.9	7	**
Cumulative bile volume (µl/g liver weight) excreted upto 60 minutes of perfusion	48	17	7	31	9.	7	**

** = $P < 0.05$

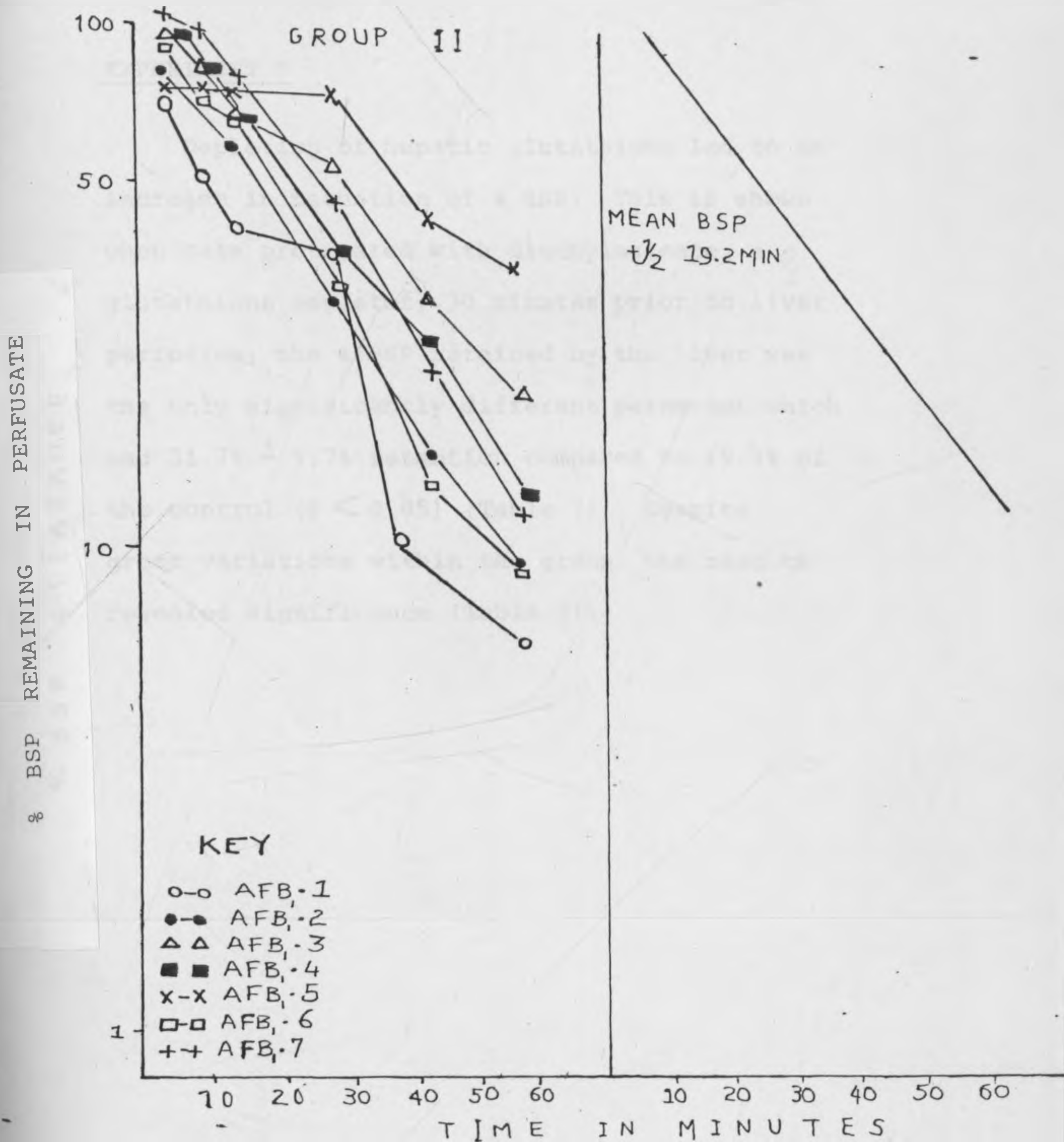

Figure 11: Individual and mean rate of elimination of BSP from perfusate for group II which was pretreated with 3.0 mg/kg aflatoxin B_1 ip. 4 hours prior to liver perfusion. (semi-log scale

EXPERIMENT 3

Depletion of hepatic glutathione led to an increase in retention of % BSP. This is shown when rats pretreated with diethylmaleate, a glutathione depletor, 30 minutes prior to liver perfusion, the % BSP retained by the liver was the only significantly different parameter which had 31.7% $\pm$ 9.7% retention compared to 19.3% of the control ($P < 0.05$) (Table 7). Despite great variations within the group, the results revealed significance (Table 7).

Table 7: shows results of group of rats (Group III) pretrated with diethylmaleate 30 minutes prior to liver perfusion.

	C O N T R O L S			G R O U P III			
	MEAN VALUE ($\bar{x}$)	STANDARD DEVIATION (s)	NO.OF REPLI-CATIONS (n)	MEAN VALUE ($\bar{x}$)	STANDARD DEVIATION (s)	NO. OF REPLI-CATIONS (n)	P VALUE
Rate of elimination of BSP (Half Life) t½ mins	11.5	2.5	7	12.6	3.5	6	
% BSP retained in liver after 60 minutes of perfusion	19.3	10.0	7	31.7	9.7	6	**
Bile volume ul/g liver weight excreted upto 60 minutes of perfusion	48	17	7	43	7	6	**

** = $P < 0.05$

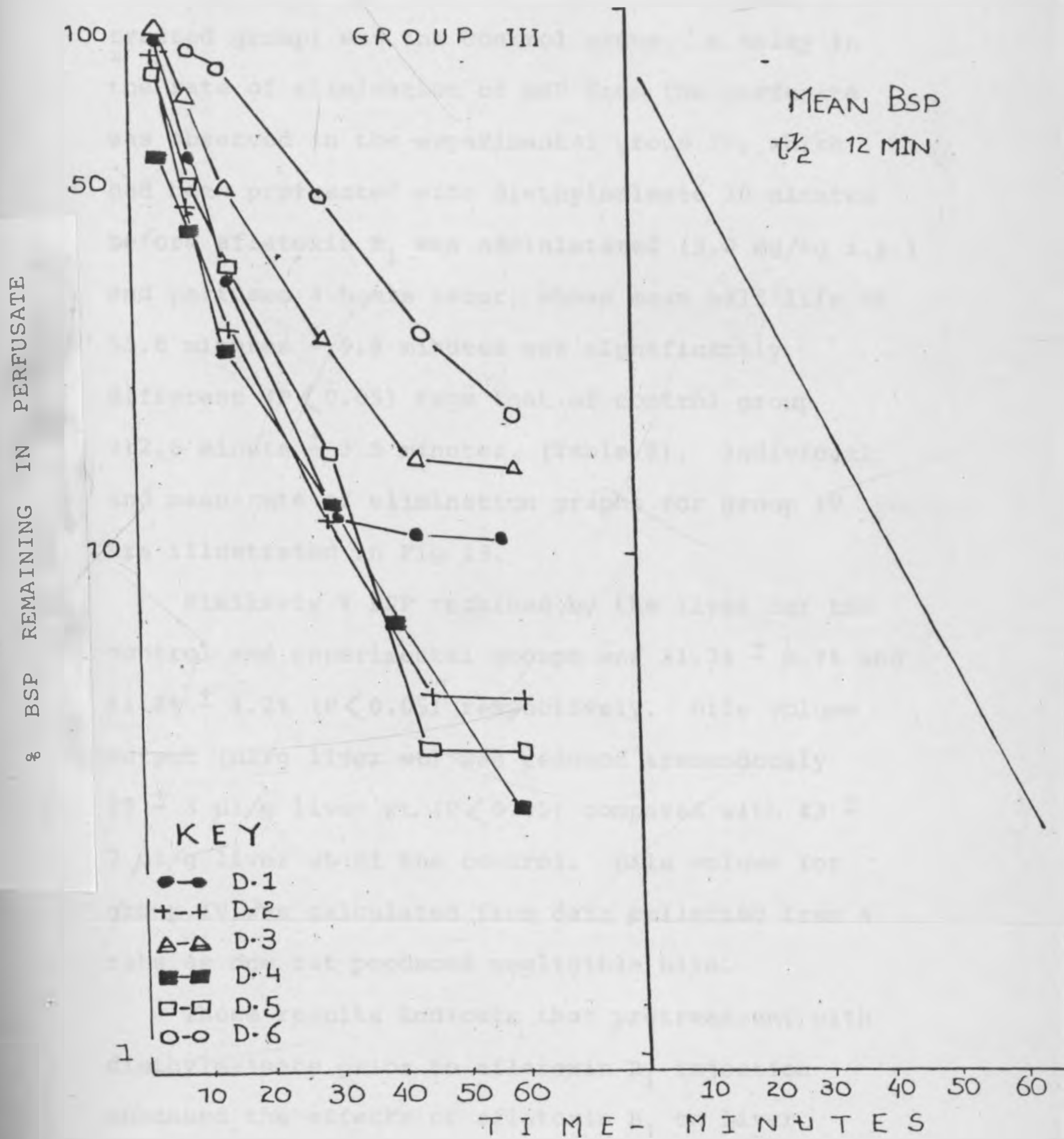

Figure 12: Mean and individual rate of elimination of BSP from perfusate of group III which was pretreated with diethylmaleate 30 minutes prior to liver perfusion. (semi-log. scale).

EXPERIMENT 4

In this experiment, Group III (diethylmaleate treated group) was the control group. A delay in the rate of elimination of BSP from the perfusate was observed in the experimental group IV, which had been pretreated with diethylmaleate 30 minutes before aflatoxin B_1 was administered (3.0 mg/kg i.p.) and perfused 4 hours later, whose mean half life of 53.8 minutes $\pm$ 9.8 minutes was significantly different ($P < 0.05$) from that of control group (12.6 minute $\pm$ 3.5 minutes. (Table 8). Individual and mean rate of elimination graphs for group IV are illustrated in Fig. 13.

Similarly % BSP retained by the liver for the control and experimental groups was 31.7% $\pm$ 9.7% and 41.8% $\pm$ 4.2% ($P < 0.05$) respectively. Bile volume output (µl/g liver wt.) was reduced tremendously 19 $\pm$ 3 µl/g liver wt. ($P < 0.05$) compared with 43 $\pm$ 7 µl/g liver wt. of the control. Bile volume for group IV was calculated from data collected from 4 rats as one rat produced negligible bile.

These results indicate that pretreatment with diethylmaleate prior to aflatoxin B_1 injection enhanced the effects of aflatoxin B_1 on liver function as carried out by isolated liver perfusion technique.

Table 8 : shows results of group of rats (Group IV) pretreated with diethylmaleate 30 minutes prior to aflatoxin B_1 injection (i.p) and perfused 4 hours later and compared with diethylmaleate group.

	DIETHYLMALEATE			GROUP IV			
	MEAN VALUE ($\bar{x}$)	STANDARD DEVIATION (s)	NO. OF REPLICATIONS (n)	MEAN VALUE ($\bar{x}$)	STANDARD DEVIATION (s)	NO. OF REPLICATIONS (n)	P. VALUE
Rate of elimination of BSP (Half Life)	12.6	3.5	6	53.8	9.8	5	**
% BSP retained in liver after 60 minutes of perfusion	31.7	9.7	6	41.8	4.2	5	**
Cumulative bile volume (ul/g) liver weight excreted upto 60 minutes of perfusion	43	7	6	19	2.5	4	**

** = $P < 0.05$

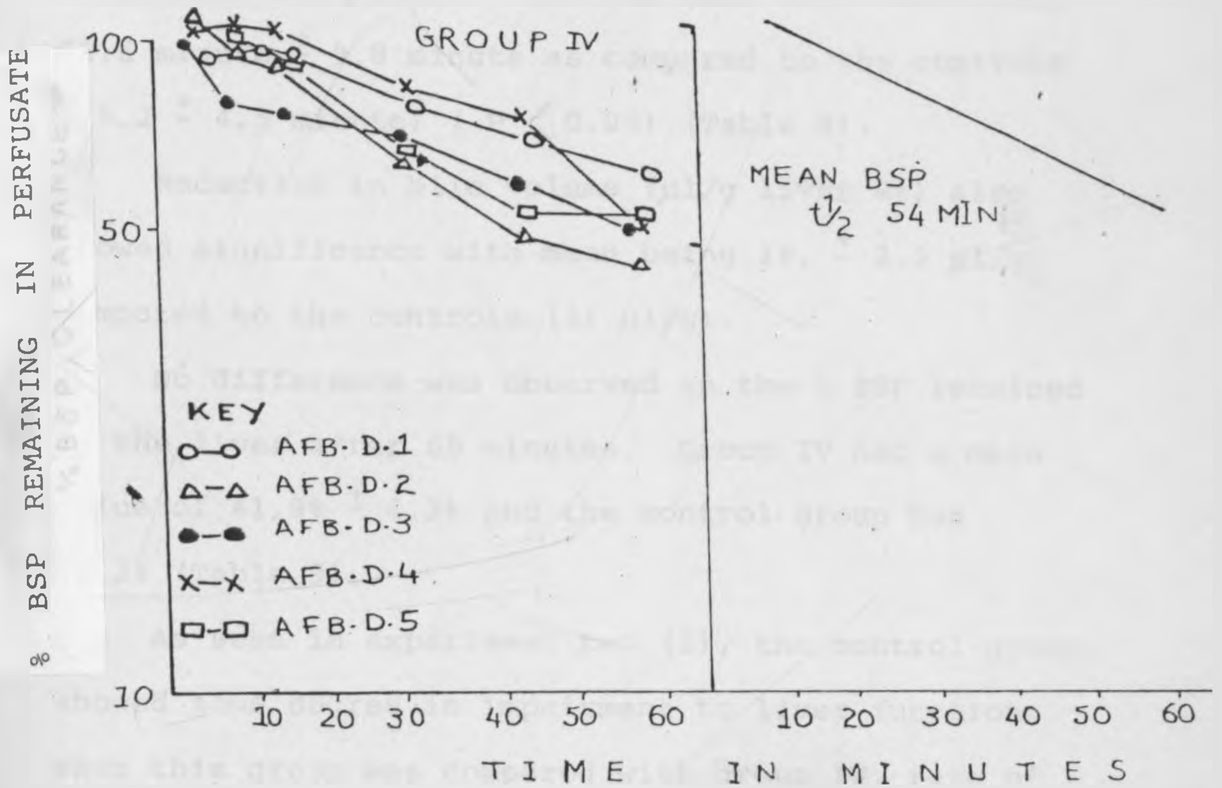

Figure 13: Individual and mean rate of elimination of BSP from perfusate for group IV which was pretreated with diethylmaleate 30 minutes before being treated with aflatoxin B_1 (3.0 mg/kg i.p) and perfused 4 hours later. (semi-log. scale).

EXPERIMENT 5

In this experiment, group II (aflatoxin B_1 injected animals) represented the control and all parameters of group IV were tested against this group. For group IV, which was pretreated with diethylmaleate 30 minutes prior to being injected with aflatoxin B_1 and perfused 4 hours later, the rate of elimination of BSP was much slower with the mean half life being 53.8 minute $\pm$ 9.8 minute as compared to the controls (19.2 $\pm$ 4.5 minute) ($P < 0.05$) (Table 9).

Reduction in bile volume (µl/g liver wt) also showed significance with mean being 19. $\pm$ 2.5 µl/g compared to the controls (31 µl/g).

No difference was observed in the % BSP retained by the liver after 60 minutes. Group IV had a mean value of 41.9% $\pm$ 4.2% and the control group had 41.3% (Table 9).

As seen in experiment two (2), the control group showed some degree in impairment to liver function. When this group was compared with group IV, rate of elimination of BSP, and bile volume where statistically significant indicating a potentiated effect of aflatoxin B_1 activity on liver function when pretreated with a glutathione depletor, diethylmaleate prior to aflatoxin B_1 injection.

Table 9 : shows results of group IV pretreated with diethylmaleate 30 minutes prior to being injected with aflatoxin B_1 and perfused 4 hours later and compared with aflatoxin B_1 treated group (Group II).

	GROUP II			GROUP IV			P VALUE
	MEAN VALUE ($\bar{x}$)	STANDARD DEVIATION (s)	NO. OF REPLIC-ATIONS (n)	MEAN VALUE ($\bar{x}$)	STANDARD DEVIATION (s)	NO. OF REPLIC-ATIONS (n)	
Rate of elimination of BSP (Half Life)	19.2	4.5	7	53.8	9.8	5	**
% BSP retained in liver after 60 minutes of perfusion	41.3	7.9	7	41.9	4.2	5	**
Cumulative bile volume (µl/g liver wt) excreted upto 60 mins of perfusion	31	9	7	19	2.5	*4	**

** = $P < 0.05$

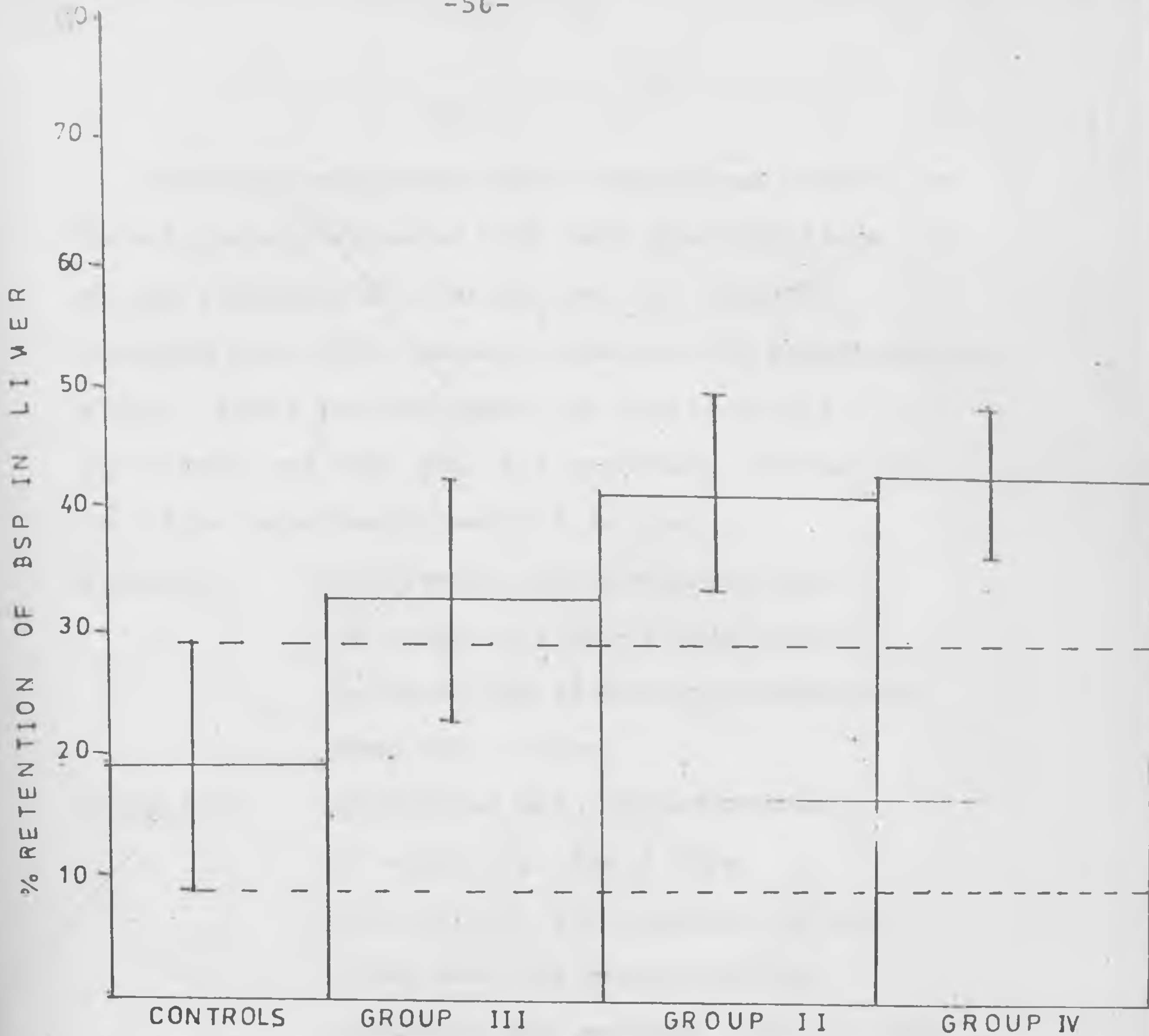

FIG 14 MEAN % RETENTION IN LIVER AFTER 60 MINS OF PERFUSION WITH S.D.

GROUP III - Rats pretreated with diethylmaleate 30 mins prior to liver perfusion.

GROUP II - Rats pretreated with aflatoxin B_1 4 hrs prior to liver perfusion.

GROUP IV - Rats pretreated with diethylmaleate 30 mins before being treated with aflatoxin B_1 and perfused 4 hrs later.

CONTROLS - Rats used as controls.

Various experiments were carried out, where the animals were pretreated with both phenobarbitone, an enxyme inducer and aflatoxin and the results compared with those animals treated with phenobarbitone alone. These results showed no statistically difference and were thus not reported. The groups for these experiments were as follows:-

Group VI: pretreated with phenobarbitone (8 mg/kg i.p) for 3 days prior to being fed aflatoxin contaminated feed for 10 days.

Group VII: pretreated with phenobarbitone (8 mg/kg i.p) for 3 days. Aflatoxin B_1 was injected 24 hours after the last phenobarbitone treatment and perfused 4 hours later.

SECTION FIVE

DISCUSSION

In the present experiments, the technique for the detection of liver injury, studied by observing the BSP elimination from perfusate and the excretion in bile using isolated liver perfusion, was found to be suitable. This method did verify the liver toxicity of the meal which had been fatal to dogs, but despite these findings, the method had its own limitations. High degree of variation for all the three parameters calculated was encountered within all the experimental groups as well as the control group. Among the various problems responsible for these variations were those factors in the technique itself such as

(1) The surgical preparation, whereby if greater time elapsed between removal of liver from the rat's abdomen and assembling it on the perfusion machine, it could lead to certain degree of liver death.

(2) The collection of blood from rats for the preparation of perfusate as well as the pumping action of the peristaltic pump that could lead to a greater degree of hemolysis which inturn causes improper oxygenation of liver tissue leading to deranged liver function.

(3) Inadaptability of the liver to the _in vitro_ perfusion system despite giving thirty minutes

stability time before the start of the experiment, this could also be a factor responsible for individual variations. Rate of elimination lines drawn on semilogarithmic scale are usually straight lines, but this was not the case as "tailing effects" were observed suggesting a survival time of the *in vitro* liver preparation to be about 60 minutes after the start of the experiment. These "tailing effects" could be attributed to these factors.

(a) Uncertainity about liver survival time using isolated liver perfusion technique and this could effect liver function, if liver survival is only for a shorter time period.

(b) Uncertainity about the sensitivity of analytical method for measuring BSP concentrations using spectrophotometry whereby optical densities of low concentrations of BSP were difficult to read. In most cases tailing effects were observed when concentrations of BSP fell below 10%. Therefore to eliminate these tailing effects and doubt's regarding liver survival time, all parameters throughout the experimental work were limited to the straight line sections of the rate of elimination graphs which ran upto 60 minutes of perfusion time.

In the present investigation it was found that when aflatoxin B_1 contaminated home made maizemeal, which was responsible for the death of four dogs

owned by a small scale farmer in Kiambu District, was fed orally to two groups of donar rats prior to liver perfusion, in concentrations of 500 µg/kg and 3000 µg/kg respectively, it was found to be hepatotoxic. The liver damaging properties of aflatoxin B_1 as assessed by liver function test using BSP clearance from perfusate plasma and percentage (%) BSP retention by the liver clearly showed delay in BSP half life times of both groups with values of 15.4 minutes and 25.5 minutes respectively, and an increases in % BSP retention values of 42.8% and 54.2% respectively. These values when compared to the control group having BSP half life of 11.5 minutes and % BSP retentions of 19.3% indicate hepatic disturbances.

Hepatic insults have been observed by Wogan and Newberne, (1967) who have shown that levels as low as 0.15 parts per million (ppm) in diets will induce a high incidence of hepatic carcinomas after a latent period of eighty two weeks. Acute toxicity of aflatoxin B_1 in rats has been described by Butler (1964), whereby a group of male rats were administered single doses of aflatoxin B_1 either orally or intraperitoneally. An LD_{50} by oral intubation of 7.2 mg/kg and 6.0 mg/kg by intraperitoneal route to male rats was reported. Majority of deaths occured 3 to 4 days after the single administration of B_1 and the lesions produced developed slowly as necrosis

was first observed after 36 to 48 hours and liver parenchymal regeneration was slow and accompanied by biliary proliferation.

Intraperitoneal injection of a single dose of aflatoxin B_1 at 3.0 mg/kg to rats effected their liver function as there was delay in the half life of BSP (19.2 minutes) as compared to controls, where the BSP half life was 11.5 minutes. Similarly % retention of BSP after 60 minutes was 41.3%, whereas the control group had a BSP % retention of 19.3%. Likewise reduction in bile volume was noticed, though being statistically significant, was not reduced markedly. These results indicate that aflatoxin B_1 did effect liver function within four hours prior to liver perfusion.

Further, it was found that when rats were pretreated with a combination of glutathione depletor, diethylmaleate, and aflatoxin B_1, four hours prior to liver perfusion, the hepatotoxic effect of aflatoxin B_1 was enhanced considerably. The slow rate of elimination of BSP from the perfusate, having a mean half life of 54 minutes and the % retention of BSP in the liver of 41.8%, together with the marked reduction in bile volume (19 µl/g liver wt) indicated the enhanced effects of aflatoxin B_1, leading to poor liver function of the combination pretreated group.

However, when only glutathione depletor was administered, 30 minutes before perfusion, the only significant parameter, when compared to the controls, was the % retention of BSP by the liver. This could be attributed to lower levels of glutathione available for conjugation with BSP before being excreted in bile.

For any foreign compound to be detoxified by the liver, it requires the passage of the compound from plasma, through hepatic parenchymal cell and finally excretion into bile. Thus hepatic uptake, hepatic metabolism and biliary excretion are major functions any foreign compound has to undergo before the compound or its metabolites are excreted in bile. Review by Klassen and Plaa (1975) mention various factors which govern the uptake, metabolism and excretion of foreign compounds. Aflatoxin B_1, a xenobiote and a hepatotoxin is metabolised mainly in the liver to various metabolites which are responsible for acute hepatotixic responses. These are hemiacetals (Patterson, 1973) and expoxides formed at 2, 3 double bond of aflatoxin B_1 (Shoental, 1970).

Sulphobromophthalein (BSP) is rapidly taken up by the liver cell, conjugated primarily with glutathione via a thioether linkage and excreted into bile (Javitt, 1971). In rats, the percentage of BSP excreted as glutathione conjugate is upto 69% (Klassen and Plaa, 1968). Depletion of hepatic glutathione with diethylmaleate pretreatment (Boyland and Chasseud,

1970) leads to reduced BSP-glutathione conjugate (BSP-GSH) excretion in bile. Results of experiment three show that pretreatment of donor rats with diethylmaleate, led to only increased percentage (%) retention of BSP, suggesting a lower excretion of the BSP conjugate due to reduction in the hepatic glutathione, despite bile volume output being the same as the control group. Likewise, no significant differences were observed in BSP half life of the pretreated group, indicating that diethylmaleate only depletes hepatic glutathione but does not alter or effect liver function.

One of the biological functions of glutathione is formation of conjugates with foreign compounds which are then excreted as mercapturic acids (Boyland and Chasseud, 1969). Conjugation with selected foreign compounds may lead to protection against biotransformed reactive metabolites which otherwise ellict hepatotoxic responses leading to hepatic necrosis (Mitchell et al.,1973). The protective role of glutathione has been showed by Mitchell et al, (1973), when pretreatment of mice with diethylmaleate markedly potentiated acetominophen induced hepatic necrosis, whereas pretreatment with glutathione precursor, cysteine protected against the drug induced hepatic necrosis. Mgbodile et al. (1975) also reported a possible protective role of glutathione in aflatoxin B_1 toxicity. Rats pretreated with

diethylmaleate prior to aflatoxin B_1 caused enhanced aflatoxin B_1 induced hepatotoxicity while on the other hand pretreatment with glutathione precursors prior to aflatoxin B_1 exposure reduced the hepatic necrosis. Depletion of hepatic glutathione by ethylmaleate potentiated the effects of aflatoxin B_1 in goats causing acute aflatoxicosis leading to death, but protection against the acute toxicity was observed when glutathione precursors were used prior to aflatoxin B_1 administration (Hatch et al. (1979). Similar results observed in this study clearly indicate that depletion of hepatic glutathione prior to aflatoxin B_1 exposure enhanced the aflatoxin B_1 induced toxic response. BSP half life value of 54 minutes as compared to that of aflatoxin B_1 pretreated goup ($t\frac{1}{2}$ 19.2 mins) elucidates the poor hepatic uptake. However no significant changes in the rate of elimination of BSP was noticed when rats were exposed to diethylmaleate alone. As hepatic glutathione levels were depleted, increase in % retention of BSP by the liver was observed, but when combination of a glutathione depletor and aflatoxin B_1 were used, % retention further increased. Greater retention of the dye could be attributed to a slower uptake as seen from the BSP rate of elimination, however marked reduction in bile volume (19 µl/g liver weight) of the combination pretreated group clearly indicated an enhancement of the aflatoxin B_1

activity. Bile volume, on the other hand, of the diethylmaleate group and aflatoxin B_1 group alone never showed appreciable reduction when compared with the controls. Whereas the cholestatic effect of the combination group together with the delay in half life time and increase in % retention suggest that liverfunction was affected markedly indicating a potentiated effect of aflatoxin B_1 induced hepatotoxicity. This enhancement of the induced toxicity suggest that glutathione possibly plays a role in detoxification of aflatoxin B_1 by forming a conjugate. Degen & Neumann (1978), have shown that when rats injected with radioactive aflatoxin B_1(^{14}C), half the dose eliminated into bile was mostly polar non extractable metabolites and among these, a glutathione conjugate was the main component which was tentatively identified as 2, 3-dihydro 2 (S glutathionyl) 3 hydroxy aflatoxin B_1 (AFB_1-GSH conjugate). Raj _et al_. (1975) gave evidence for the formation of aflatoxin B_1 epoxide by the rat liver microsomes which conjugated with glutathione to form the aflatoxin B_1 epoxide glutathione conjugate. The potentiation of aflatoxin B_1 induced toxic response on liver function may be due to the formation of epoxides which cannot conjugate with glutathione due to depletion with diethylmaleate.

SECTION SIX

CONCLUSION

The study enabled us to observe the effects of pretreatment with aflatoxin B_1, glutathione depletor, and both glutathione depletor and aflatoxin B_1 combined on liver function as assessed by BSP clearance in the perfusate, retention in the liver and excretion in bile in an isolated liver perfusion set up. These effects were:

(1) Hepatotoxicity of aflatoxin B_1: as observed by either oral or intraperitoneal pretreatment, caused impairment of liver function.

(2) Depletion of glutathione only increased retention of BSP by the liver as only low concentrations of glutathione were available for conjugation with BSP before excretion.

(3) Potentiantion of aflatoxin induced hepatoxicity when glutathione levels were depleted.

Aflatoxin B_1 is metabolised by the liver to various metabolites and one of this metabolite seems to be relatively more toxic, probably an aflatoxin B_1 2-3 epoxide which is thought to conjugate with glutathione before being excreted in bile. Therefore, depletion of glutathione caused potentiated effects of aflatoxin induced hepatotoxicity as the "toxic metabolite" is unable to conjugate with glutathione. However, further research is still needed in this field to study this particular route of metabolism of aflatoxin B_1.

Hepatotoxicity studies using isolated liver perfusion technique are not ideal as setting up an already *in vivo* toxic liver to an *in vitro* perfusion set up can lead to more experimental failures. However sub lethal radiolabelled aflatoxin B_1 would be an ideal substance to study its metabolism in the liver.

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APPENDIX I

WORKING DATA FOR THE CONTROL GROUP

μg BSP in 0.2 ml of perfusate

EXPT	0 min	5 min	10 min	15 min	30 min	45 min	60 min	75 min	90 min
8		7.9	6.3	4.15	1.7	0.9	0.6	0.1	0.09
14		15.0	12.0	8.4	3.45	2.1	1.5	1.4	0.0
15		19.0	10.0	6.45	1.5	0.7	0.2	0.1	0.0
16		16.5	11.0	6.4	2.4	1.0	0.7	0.6	0.6
18		20.5	14.5	11.0	3.9	1.6	0.6	0.6	0.0
21		19.0	14.0	8.15	3.6	1.25	0.6	0.6	0.6
11		-	24.0	12.5	3.7	1.15	0.55	0.4	0.4

μg BSP in 30 mls of perfusate

EXPT	BSP added	5 min	10 min	15 min	30 min	45 min	60 min	75 min	90 min
8	2550	1185	945	622.5	255	135	90	15	13.5
14	2280	2250	1800	1260	517.5	315	225	210	0.0
15	3300	2850	1500	967.5	255	105	30	15	0.0
16	2760	2475	1650	960	360	150	105	90	90
18	3600	3075	2175	1650	585	240	90	90	0.0
21	2760	2850	2100	1222.5	540	187.5	90	90	90
11	3780	-	3600	1875	555	172.5	82.5	60	0.0

% BSP remaining in perfusate

EXPT	0 min	5 min	10 min	15 min	30 min	45 min	60 min	75 min	90 min
8	100%	46.5	37.0	24.4	10.0	5.3	3.5	0.6	0.5
14	"	98.7	78.9	55.3	22.7	13.8	9.8	9.2	0.0
15	"	86.4	45.5	29.3	6.8	3.2	1.0	0.5	0.0
16	"	89.7	59.8	34.8	13.0	5.4	3.8	3.3	3.3
18	"	85.4	60.4	45.8	16.3	6.6	2.5	2.5	0.0
21	"	100.0	76.0	44.3	19.6	6.8	3.7	3.2	3.2
11	"	–	95.0	50.0	15.0	5.0	2.0	2.0	2.0

µg of BSP in 10µl of bile

EXPT	0 min	15 min	30 min	45 min	60 min	75 min	90 min
8	0.0	8.0	61.0	69.0	38.0	17.5	10.0
14	0.0	0.5	54.0	70.5	36.5	15.0	6.0
15	0.0	0.0	92.0	126.0	45.0	13.5	5.0
16	0.0	1.5	76.5	73.0	32.5	12.5	4.5
18	0.0	13.5	104.0	104.0	52.0	26.5	16.0
21	0.0	16.0	104.0	76.5	32.0	12.5	6.0
11	0.0	25.0	138.0	129.0	5 2.0	17.0	7.0

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Bile volume (μl)

EXP	0 min	15 min	30 min	45 min	60 min	75 min	90 min	liver wt (g)
8	-	114	86	90	60	71	64	8.0
14	-	103	112	108	100	103	92	6.5
15	-	95	101	120	95	101	110	10.0
16	-	127	110	126	92	110	124	6.0
18	-	81	118	75	81	64	38	15.0
21	-	83	101	87	78	82	74	7.0
11	-	85	111	101	88	83	78	10.0

Cumulative BSP (μg) excreted in bile

EXP	0 min	15 min	30 min	45 min	60 min	75 min	90 min	BSP added
8	0.0	91.2	615.8	1236.8	1464.8	1589.1	1630.0	2550
14	0.0	5.15	609.9	1371.3	1736.4	1890.8	1946.1	2280
15	0.0	0.0	929.2	2441.2	2868.7	3005.1	3060.1	3300
16	0.0	19.05	860.5	1780.4	2078.4	2216.9	2272.7	2760
18	0.0	109.4	1336.5	2116.5	2537.6	2707.2	2767.9	3600
21	0.0	132.8	1183.2	1848.8	2098.4	2200.9	2245.3	2760
11	0.0	212.5	1744.3	3047.1	3504.9	3646.0	3700.6	3780

Cumulative % excretion of BSP in bile

EXP	0 min	15 min	30 min	45 min	60 min	75 min	90 min
8	0	3.6	24.1	52.0	61.0	65.8	68.5
14	0	0.2	26.8	60.1	76.1	82.9	85.4
15	0	0	28.2	74.0	86.9	91.1	92.7
16	0	0.7	31.2	64.5	75.3	80.3	82.3
18	0	3.0	37.1	58.8	70.5	75.2	76.9
21	0	4.8	42.9	67.0	76.0	79.7	81.3
11	0	5.6	46.1	80.6	92.7	96.4	98.0

100% - % BSP excreted in bile

EXP	0 min	15 min	30 min	45 min	60 min	75 min	90 min
8	100%	96.4	75.9	48.0	38.0	34.2	31.5
14	100%	99.8	73.2	39.9	23.9	17.1	14.6
15	100%	100	71.8	26.0	13.1	8.9	7.3
16	100%	99.3	68.8	35.5	24.7	19.7	17.7
18	100%	97.0	62.9	41.2	29.5	24.8	23.1
21	100%	95.2	57.1	33.0	24.0	20.3	18.7
11	100%	94.4	53.9	19.4	7.3	3.6	2.0

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EXPERIMENT NO.	HALF LIFE ($t_{1/2}$)	% RETENTION BSP AFTER 60 MINUTES	CUMULATIVE BILE VOLUME ul/g LIVER WT AFTER 60 MINUTES
8	13.9	35.5	44
14	15.4	14.1	65
15	8.5	12.1	41
16	11.4	20.9	76
18	10.8	27.0	24
21	11.2	20.3	50
11	9.0	5.3	39
$\bar{x}$	11.5	19.3	48

APPENDIX II

WORKING DATA FOR EXPERIMENT 1 - GROUP Ia

µg BSP in 0.2 ml of perfusate

EXP	0 min	5 min	10 min	15 min	30 min	45 min	60 min	75 min	90 min
C	–	20.5	13.5	7.2	2.6	1.25	0.95	0.6	0.6
B		18.5	16.0	12.0	6.5	3.9	2.25	1.6	1.25
E		22.0	22.0	17.5	10.0	4.9	2.6	1.6	1.25
D		22.0	21.0	19.5	12.0	6.5	3.25	1.9	1.6
A		15.5	10.5	7.2	4.7	1.25	1.25	0.6	0.6

µg BSP in 30 ml of perfusate

EXP	BSP added	5 min	10 min	15 min	30 min	45 min	60 min	75 min	90 min
C	3300	3075	2025	1080	390	188	143	90	90
B	3300	2775	2400	1800	975	585	338	240	188
E	3300	3300	3300	2625	1500	735	390	240	188
D	3300	3300	3150	2925	1800	975	488	285	240
A	3300	2325	1575	1080	705	188	188	90	90

% BSP remaining in perfusate

EXP	0 min	5 min	10 min	15 min	30 min	45 min	60 min	75 min	90 min
C	100%	93.2	61.4	32.7	11.8	5.7	4.3	2.7	2.7
B	100.0	84.1	72.7	54.5	29.5	17.7	10.2	7.3	5.7
E	100.0	100.0	100	79.5	45.4	22.3	11.8	7.3	5.7
D	100.0	100.0	95.4	88.6	54.5	29.5	14.8	8.6	7.3
A	100.0	70.5	47.7	32.7	21.4	5.7	5.7	2.7	2.7

Bile volume (µl)

EXP	Liver wt (g)	15 min	30 min	45 min	60 min	75 min	90 min
C	11.0	68	78	72	53	53	52
B	14.0	32	32	34	26	18	20
E	12.0	49	42	56	44	51	18
D	13.0	47	41	41	52	50	46
A	17.0	71	68	48	40	33	28

µg BSP in 10 µl of bile

EXP	0 min	15 min	30 min	45 min	60 min	75 min	90 min
C		14.0	132.0	106.0	64.0	31.0	18.5
B		4.0	62.5	167.0	139.0	111.0	90.0
E		1.0	118.0	139.0	89.0	59.0	40.5
D		1.0	90.0	114.0	90.0	76.5	48.5
A		3.5	121.0	135.0	90.0	59.0	41.5

Cumulative BSP (ug) excreted in bile

EXP	BSP added	15 min	30 min	45 min	60 min	75 min	90 min
C	3300	95.2	1124.8	1888.0	1919.8	2084.1	2180.3
B	3300	12.8	212.8	780.6	1142.0	1341.8	1521.8
E	3300	4.9	500.5	1278.9	1670.5	1971.4	2044.3
D	3300	4.7	373.7	841.1	1309.1	1691.6	1914.7
A	3300	24.9	847.7	1495.7	1855.7	2050.4	2166.6

Cumulative % excretion of BSP in bile

EXP	0 min	15 min	30 min	45 min	60 min	75 min	90 min
C		2.8	34.1	57.2	58.2	63.2	66.1
B		0.3	6.4	23.7	34.6	40.7	46.1
E		0.1	15.2	38.8	50.6	59.7	61.9
D		0.1	11.3	25.5	39.7	51.3	58.0
A		0.7	25.7	45.3	56.2	62.1	65.7

100% - % BSP excreted in bile

EXP	0 min	15 min	30 min	45 min	60 min	75 min	90 min
C	100.0	97.2	65.9	42.8	41.8	36.8	33.9
B	100.0	99.7	93.6	76.3	65.4	59.3	53.9
E	100.0	99.9	84.8	61.2	49.4	40.3	38.1
D	100.0	99.9	88.7	74.5	60.3	48.7	42.0
A	100.0	99.3	74.3	54.7	43.8	37.9	34.3

EXP NO	HALF LIFE ($t_{\frac{1}{2}}$)	% RETENTION OF BSP AFTER 60 MINUTES	CUMULATIVE BILE VOLUME (µl/g liver wt) AFTER 60 MINUTES
C	11.5	37.5	25
B	19.1	55.2	9
E	16.2	37.6	16
D	18.8	45.5	14
A	11.5	38.1	13
$\bar{X}$	15.4	42.8	15

APPENDIX III

WORKING DATA FOR EXPERIMENT 1 - GROUP 1b

µg BSP in 0.2 ml of perfusate

EXP	0 min	5 min	10 min	15 min	30 min	45 min	60 min	75 min	90 min
A		14.0	11.0	7.8	3.9	2.1	0.0	0.0	0.0
B		18.5	16.0	12.0	6.5	3.9	2.3	1.6	1.3
C		15.0	10.5	10.5	10.0	5.2	3.3	2.0	2.0
D		18.5	17.5	15.0	12.5	10.0	7.8	5.9	3.4

µg BSP in 30 mls of perfusate

EXP	BSP added	5 min	10 min	15 min	30 min	45 min	60 min	75 min	90 min
A	2460	2100	1650	1170	585	315	0	0	0
B	3300	2775	2400	1800	975	585	337	240	188
C	2280	2250	1575	1575	1500	780	487	533	293
D	2760	2775	1625	2250	1875	1500	1170	885	533

% BSP clearance from perfusate

EXP	0 min	5 min	10 min	15 min	30 min	45 min	60 min	75 min	90 min
A	100	85.0	67.1	47.6	23.8	12.8	0.0	0.0	0.0
B	100	84.1	72.7	54.6	29.6	17.7	10.2	7.3	5.7
C	100	98.7	69.1	69.1	65.8	34.2	21.4	23.4	12.8
D	100	100.0	95.1	85.1	67.9	54.4	42.4	32.1	19.3

µg BSP in 10 µl of bile

EXP	0 min	15 min	30 min	45 min	60 min	75 min	90 min
A	0.0	1.5	62.5	92.0	80.0	48.5	38.5
B	0.0	4.0	62.5	167.0	139.0	111.0	90.0
C	0.0	3.0	28.0	36.0	34.5	31.0	27.0
D	0.0	0.3	0.5	3.5	65.0	104.0	104.0

Bile volume (µl)

EXP	Liver wt (g)	15 min	30 min	45 min	60 min	75 min	90 min
A	9	46	44	42	39	18	32
B	14	32	32	34	26	18	20
C	8	68	78	74	58	60	54
D	12	10	10	22	15	16	26

Cumulative BSP (µg) excreted in bile

EXP	BSP added	15 min	30 min	45 min	60 min	75 min	90 min
A	2460	6.9	281.9	668.3	980.3	1067.6	1171.6
B	3300	12.8	212.8	780.6	1142.0	1341.8	1521.8
C	2280	20.4	238.8	505.2	705.3	891.3	1037.1
D	2780	0.3	0.6	8.3	105.8	272.2	542.6

Cumulative % excretion of BSP in bile

EXP	0 min	15 min	30 min	45 min	60 min	75 min	90 min
A	0.0	0.2	11.5	27.2	39.8	43.4	47.6
B	0.0	0.3	6.4	23.7	34.6	40.7	46.0
C	0.0	0.8	10.4	22.2	30.9	39.1	45.5
D	0.0	0.0	0.0	0.3	3.8	9.8	19.6

100% - % BSP excreted in bile

EXP	0 min	15 min	30 min	45 min	60 min	75 min	90 min
A	100.0	99.8	88.5	72.8	60.2	56.6	52.4
B	100.0	99.7	93.6	76.3	65.4	59.3	53.9
C	100.0	99.2	89.6	77.8	69.1	60.9	54.5
D	100.0	99.9	99.9	99.7	96.2	90.2	80.4

EXP NO	HALF LIFE ($t_{1/2}$)	% RETENTION OF BSP AFTER 60 MINUTES	CUMULATIVE BILE VOLUME µl/g LIVER WT AFTER 60 MINUTES
A	14.50	60.2	19
B	17.80	55.2	9
C	25.14	47.7	35
D	44.70	53.8	5
$\bar{X}$	25.5	54.2	17

APPENDIX IV

WORKING DATA FOR EXPERIMENT 2 - GROUP II

μg BSP in 0.2 ml of perfusate

EXP	0 min	5 min	10 min	15 min	30 min	45 min	60 min	75 min	90 min
AFB_1.1		18.0	16.0	13.0	7.5	3.6	2.0	1.0	0.6
AFB_1.2		13.0	11.5	7.5	6.9	2.0	1.3	0.6	0.6
AFB_1.3		17.5	16.0	13.0	10.0	5.6	3.6	2.3	2.3
AFB_1.4		14.0	12.5	10.0	5.2	2.6	1.6	0.6	0.6
AFB_1.5		13.0	12.5	12.5	12.5	7.2	5.9	3.9	2.6
AFB_1.6		15.0	13.0	10.5	5.6	3.9	2.0	0.6	0.0
AFB_1.7		20.0	15.5	14.5	6.7	2.9	2.0	0.0	0.0

μg BSP in 30 ml of perfusate

EXP	BSP added	5 min	10 min	15 min	30 min	45 min	60 min	75 min	90 min
AFB_1.1	2580	2700	2400	1950	1125	540	293	143	90
AFB_1.2	2910	1950	1725	1125	1028	293	188	90	90
AFB_1.3	2910	2625	2400	1950	1500	834	540	338	338
AFB_1.4	2640	2100	1875	1500	780	390	240	90	90
AFB_1.5	2640	1950	1875	1875	1875	1080	885	585	390
AFB_1.6	2400	2250	1950	1575	833	585	293	90	0
AFB_1.7	3300	3000	2325	2175	1005	435	293	38	0

% BSP remaining in perfusate

EXP	0 min	5 min	10 min	15 min	30 min	45 min	60 min	75 min	90 min
AFB_1.1		100.0	93.0	76.0	44.0	21.0	11.0	6.0	3.5
AFB_1.2		67.0	59.3	38.7	35.3	10.0	6.4	3.0	3.0
AFB_1.3		90.2	82.5	67.0	51.5	28.6	18.6	11.6	11.6
AFB_1.4		79.6	71.0	56.8	29.6	14.8	9.1	3.4	3.4
AFB_1.5		73.9	71.0	71.0	71.0	40.9	33.5	22.2	14.8
AFB_1.6		93.8	81.3	65.9	34.7	24.4	12.2	3.8	0.0
AFB_1.7		90.9	70.5	65.9	30.5	13.2	8.9	1.1	0.0

µg BSP in 10 µl of bile

EXP	0 min	15 min	30 min	45 min	60 min	75 min	90 min
AFB_1.1	0.0	1.0	53.0	92.0	84.0	64.0	44.5
AFB_1.2	0.0	14.5	25.0	46.5	69.5	90.0	10.5
AFB_1.3	0.0	1.0	47.0	73.0	59.0	48.0	34.0
AFB_1.4	0.0	1.0	59.0	104.0	78.0	53.0	32.5
AFB_1.5	0.0	1.0	41.5	83.0	97.0	97.0	85.0
AFB_1.6	0.0	2.0	59.0	78.0	48.0	38.0	19.5
AFB_1.7	0.0	25.0	97.0	86.0	52.0	32.5	16.5

Bile volume (µl)

EXP	Liver wt(g)	15 min	30 min	45 min	60 min	75 min	90 min
AFB_1.1	7.5	52	50	45	39	70	25
AFB_1.2	11.0	80	103	85	75	80	60
AFB_1.3	10.0	51	70	90	58	60	50
AFB_1.4	7.0	56	56	58	47	48	50
AFB_1.5	6.0	35	30	30	21	15	26
AFB_1.6	6.0	65	76	77	68	64	65
AFB_1.7	8.0	90	80	72	65	70	70

Cumulative BSP (µg) excreted in bile

EXP	0 min	15 min	30 min	45 min	60 min	75 min	90 min
AFB_1.1	0.0	5.2	270.2	684.2	1011.8	1459.8	1571.1
AFB_1.2	0.0	116.0	373.5	768.8	1290.0	2010.0	2073.0
AFB_1.3	0.0	5.1	334.1	991.1	1333.3	1621.3	1791.3
AFB_1.4	0.0	5.6	336.0	939.2	1305.8	1560.2	1722.7
AFB_1.5	0.0	3.5	128.0	377.0	580.7	726.2	947.2
AFB_1.6	0.0	13.0	461.4	1062.0	1391.8	1635.0	1761.8
AFB_1.7	0.0	225.0	1001.0	1620.2	1958.2	2185.7	2301.2

Cumulative % excretion of BSP in bile

EXP	0 min	15 min	30 min	45 min	60 min	75 min	90 min
AFB_1.1	0.0	0.0	10.5	26.5	39.2	56.6	60.9
AFB_1.2	0.0	3.9	12.8	26.4	44.3	69.1	71.2
AFB_1.3	0.0	0.1	11.4	34.1	45.8	55.7	61.6
AFB_1.4	0.0	0.2	12.7	35.6	49.5	59.1	65.2
AFB_1.5	0.0	0.1	4.8	14.3	22.0	27.5	35.9
AFB_1.6	0.0	0.5	19.2	44.2	58.0	68.1	73.4
AFB_1.7	0.0	6.8	30.3	49.1	59.3	66.2	69.7

100% - % BSP excreted in bile

EXP	0 min	15 min	30 min	45 min	60 min	75 min	90 min
AFB_1.1	100.0	100.0	89.5	73.5	60.8	43.4	39.1
AFB_1.2	100.0	96.1	87.2	73.6	55.7	30.9	28.8
AFB_1.3	100.0	99.9	88.6	65.9	54.2	44.3	38.4
AFB_1.4	100.0	99.8	87.3	64.4	50.5	40.9	34.8
AFB_1.5	100.0	99.1	95.2	85.7	78.0	72.5	64.0
AFB_1.6	100.0	99.5	80.8	55.8	42.0	31.9	26.6
AFB_1.7	100.0	93.2	69.7	50.9	40.7	33.8	30.3

EXP NO	HALF LIFE ($t_{½}$)	% RETENTION OF BSP AFTER 60 MINUTES	CUMULATIVE BILE VOLUME (µl/g liver wt) AFTER 60 MINUTES.
AFB_1.1	16.7	49.8	25
AFB_1.2	15.1	49.3	31
AFB_1.3	23.7	42.6	27
AFB_1.4	16.8	41.4	31
AFB_1.5	27.2	44.5	19
AFB_1.6	18.8	29.8	48
AFB_1.7	15.5	31.8	38
$\bar{X}$	19.2	41.3	31

APPENDIX V

WORKING DATA FOR EXPERIMENT 3 - GROUP III

μg BSP in 0.2 ml of perfusate

EXP	0 min	5 min	10 min	15 min	30 min	45 min	60 min	75 min	90 min
D.1		21.0	11.0	6.5	2.1	2.0	2.0	0.0	0.0
D.2		15.0	7.9	4.6	2.0	1.0	1.0	0.7	0.0
D.3		18.0	13.0	8.5	4.3	2.5	2.5	2.0	2.0
D.4		12.5	7.5	5.2	2.3	0.6	0.6	0.6	0.6
D.5		11.0	7.9	4.6	2.3	1.3	0.6	0.0	0.0
D.6		17.5	14.0	13.0	7.2	3.9	2.6	1.3	0.6

μg BSP in 30 mls of perfusate

EXP	BSP added	5 min	10 min	15 min	30 min	45 min	60 min	75 min	90 min
D.1	3000	3150	1650	975	315	293	293	0	0
D. 2	2640	2250	1177	683	293	143	143	105	0
D.3	2700	2700	1950	1275	638	375	375	308	308
D.4	2280	1875	1125	780	338	90	90	90	90
D.5	2880	1650	1177	683	338	195	90	0	0
D.6	2340	2625	2100	1950	1080	585	390	195	90

% BSP remaining in perfusate

EXP	0 min	5 min	10 min	15 min	30 min	45 min	60 min	75 min	90 min
D.1		100.0	55.0	33.0	11.0	10.0	10.0	0.0	0.0
D.2		85.0	45.0	26.0	11.0	5.0	5.0	4.0	0.0
D.3		100.0	72.0	47.0	24.0	14.0	14.0	11.0	11.0
D.4		82.0	49.0	34.0	15.0	4.0	4.0	4.0	0.0
D.5		57.0	41.0	24.0	12.0	7.0	3.0	0.0	0.0
D.6		112.0	90.0	83.0	46.0	25.0	17.0	8.0	4.0

µg BSP in 10 µl of bile

EXP	0 min	15 min	30 min	45 min	60 min	75 min	90 min
D.1		2.0	41.5	76.5	73.0	55.5	34.0
D.2		11.0	83.5	83.5	41.0	14.0	5.0
D.3		6.0	59.0	59.0	55.5	31.0	16.0
D.4		8.5	69.0	76.5	43.0	20.0	8.0
D.5		17.0	90.0	76.5	44.5	17.0	6.5
D.6		1.0	36.5	52.0	43.0	44.5	36.0

Bile volume (μl)

EXP	Liver wt(g)	15 min	30 min	45 min	60 min	75 min	90 min
D.1	9.0	67	65	69	80	63	58
D.2	7.5	80	96	95	86	86	84
D.3	7.0	80	82	88	78	74	60
D.4	7.5	100	81	98	82	75	65
D.5	7.0	75	89	80	74	62	80
D.6	7.0	52	50	66	83	54	90

Cumulative BSP (μg) excreted in bile

EXP	BSP added	15 min	30 min	45 min	60 min	75 min	90 min
D.1	3000	134.0	403.8	931.6	1515.6	1865.3	2062.5
D.2	2640	88.0	889.6	1682.9	2035.5	2155.9	2197.9
D.3	2700	48.0	531.8	1051.0	1483.9	1713.3	1809.3
D.4	2280	85.0	647.9	1397.7	1750.3	1900.3	1952.3
D.5	2880	127.5	928.5	1540.5	1869.8	1975.2	2027.2
D.6	2340	52.0	187.7	530.9	887.8	1128.1	1452.1

Cumulative % excretion of BSP in bile

EXP	0 min	15 min	30 min	45 min	60 min	75 min	90 min
D.1	0.0	4.4	13.5	31.0	50.5	62.2	68.7
D.2	0.0	3.3	33.7	63.7	71.7	81.7	83.3
D.3	0.0	1.7	19.7	38.9	54.9	63.5	67.0
D.4	0.0	3.7	28.4	61.3	76.8	83.5	85.6
D.5	0.0	4.4	32.2	53.5	64.9	68.6	70.4
D.6	0.0	0.2	8.0	22.7	37.9	48.2	62.0

100% - % BSP excretion in bile

EXP	0 min	15 min	30 min	45 min	60 min	75 min	90 min
D.1	100.0	95.6	86.5	69.0	49.5	37.8	31.3
D.2	100.0	96.4	66.3	36.3	29.3	18.3	16.7
D.3	100.0	98.3	80.3	61.1	45.1	36.5	33.0
D.4	100.0	96.3	71.6	38.7	23.2	16.5	14.4
D.5	100.0	95.6	67.8	46.5	35.1	31.4	29.6
D.6	100.0	99.8	92.0	87.3	62.1	51.8	38.0

EXPT NO	HALF LIFE ($t_{1/2}$)	% RETENTION OF BSP AFTER 60 MINUTES	CUMULATIVE BILE VOLUME µl/g LIVER WT AFTER 60 MINUTES
D.1	10.5	39.5	31
D.2	9.9	23.3	48
D.3	14.0	31.1	47
D.4	9.6	19.2	48
D.5	12.9	32.1	45
D.6	18.8	45.1	36
$\bar{X}$	12.6	31.7	43

APPENDIX VI

WORKING DATA FOR EXPERIMENT 4 AND 5 - GROUP IV

µg of BSP in 0.2 ml of perfusate

EXP	0 min	5 min	10 min	15 min	30 min	45 min	60 min	75 min	90 min
AFB.D.1		17.0	17.0	17.0	14.0	12.5	11.0	8.5	10.0
AFB.D.2		18.0	19.0	17.5	12.5	10.5	10.5	10.5	10.5
AFB.D.3		17.0	13.5	13.0	12.0	10.0	8.2	5.9	5.2
AFB.D.4		16.5	15.5	15.0	12.0	11.0	7.2	6.5	5.9
AFB.D.5		14.0	12.5	12.0	8.5	6.5	5.9	5.2	4.6

µg of BSP in 30 mls of perfusate

EXP	BSP added	5 min	10 min	15 min	30 min	45 min	60 min	75 min	90 min
AFB.D.1	2700	2550	2550	2550	2100	1875	1650	1275	1500
AFB.D.2	2850	2700	2850	2625	1875	1575	1575	1575	1575
AFB.D.3	2580	2550	2025	1950	1800	1500	1222	885	780
AFB.D.4	2220	2475	2325	2250	1800	1650	1080	975	885
AFB.D.5	2040	2100	1875	1800	1275	975	885	780	683

% BSP remaining in perfusate

EXP	0 min	5 min	10 min	15 min	30 min	45 min	60 min	75 min	90 min
AFB.D.1		94.4	94.4	94.4	77.8	69.4	61.1	47.2	55.6
AFB.D.2		94.0	100.0	92.1	65.8	55.3	55.3	55.3	55.3
AFB.D.3		98.8	78.5	75.6	69.8	58.1	47.4	34.3	31.0
AFB.D.4		100.0	100.0	100.0	81.1	74.3	48.7	43.9	39.9
AFB.D.5		100.0	91.9	88.2	62.5	47.9	43.4	38.2	33.5

µg of BSP in 10 µl of bile

EXP	0 min	15 min	30 min	45 min	60 min	75 min	90 min
AFB.D.1	0.0	0.0	5.0	12.5	13.5	14.0	17.5
AFB.D.2	0.0	0.0	0.0	-	3.0	-	7.0
AFB.D.3	0.0	0.0	34.5	76.5	72.0	66.0	59.0
AFB.D.4	0.0	0.0	6.5	34.0	40.0	35.0	34.0
AFB.D.5	0.0	3.5	16.0	41.5	43.0	30.5	33.0

Bile volume (μl)

EXP	Liver wt(g)	15 min	30 min	45 min	60 min	75 min	90 min
AFB.D.1	6	30	32	31	28	25	20
AFB.D.2	11	10	9	-	11	-	5
AFB.D.3	5	29	20	15	13	13	20
AFB.D.4	5	20	28	30	30	27	26
AFB.D.5	5.5	38	28	12	24	20	10

Cumulative BSP (μg) excreted in bile

EXP	0 min	15 min	30 min	45 min	60 min	75 min	90 min
AFB.D.1	0.0	0.0	16.0	54.8	92.6	127.6	162.6
AFB.D.2	0.0	0.0	0.0	0.0	3.3	3.3	6.8
AFB.D.3	0.0	0.0	69.0	183.8	277.4	363.2	481.2
AFB.D.4	0.0	0.0	18.2	120.2	240.2	334.7	424.4
AFB.D.5	0.0	13.3	58.1	107.9	211.1	272.1	305.1

Cumulative % excretion of BSP in bile

EXP	0 min	15 min	30 min	45 min	60 min	75 min	90 min
AFB.D.1	0.0	0.0	0.6	2.0	3.4	4.7	6.0
AFB.D.2	0.0	0.0	0.0	0.0	0.1	0.1	0.2
AFB.D.3	0.0	0.0	2.7	7.1	10.7	14.1	18.6
AFB.D.4	0.0	0.0	0.8	5.4	10.8	15.1	19.1
AFB.D.5	0.0	0.6	2.8	5.3	10.3	13.3	14.9

100% - % BSP excreted in bile

EXP	0 min	15 min	30 min	45 min	60 min	75 min	90 min
AFB.D.1	100.0	100.0	99.4	98.0	96.6	95.6	94.0
AFB.D.2	100.0	100.0	100.0	100.0	99.9	99.9	99.8
AFB.D.3	100.0	100.0	97.3	92.9	89.3	85.9	81.4
AFB.D.4	100.0	100.0	99.2	94.6	89.2	84.9	80.9
AFB.D.5	100.0	99.4	97.2	94.7	89.7	86.7	85.1

EXP NO	HALF LIFE ($t\frac{1}{2}$)	% RETENTION OF BSP AFTER 60 MINUTES	CUMULATIVE BILE VOLUME µl/g LIVER WT AFTER 60 MINUTES
AFB.D.1	68.7	35.5	20
AFB.D.2	42.4	44.7	-
AFB.D.3	57.0	41.9	15
AFB.D.4	50.7	40.5	22
AFB.D.5	50.1	46.3	19
$\overline{X}$	53.8	41.8	19

APPENDIX VII

Statistical analysis

Student's 't' test

$$t\ cal = \frac{\bar{X}_1 - \bar{X}_2}{\text{standard error of pooled variance}}$$

$$= \frac{\bar{X}_1 - \bar{X}_2}{\sqrt{\frac{(n_1-1)S_1^2 + (n_2-1)S_2^2}{n_1 + n_2 - 2} \times \frac{1}{n_1} + \frac{1}{n_2}}}$$

$\bar{X}$ = Mean value

n = Number of replications

S = Standard error