DUCKLING PLASMA AND LIVER TISSUE PARAMETERS AT DIFFERENTIAL AFLATOXICOSIS LEVELS

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PREFACE

The author is sincerely grateful to his wife, Frankie, for her patience and encouragement during the course of this study. Also a special thanks to my parents, Mr. and Mrs. Keith Armstrong, for their assistance and encouragement throughout my graduate and undergraduate work.

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CHAPTER I

INTRODUCTION

More than 100,000 turkey poults and numbers of other species of domestic animals in the South and East of England died within a few months in 1960 from an unknown disease that was then termed "turkey X disease." Investigations were undertaken and the cause traced to a shipment of peanut meal from Brazil. Ultimately, a metabolite produced by the common soil fungus, Asperillus flavus, named aflatoxin, was determined as the toxin responsible for these losses.

The aflatoxins are established to be among the most potent of the known chemical carcinogens. These toxins are acutely toxic to most animal species with sensitivity apparently decreasing with age. White Pekin ducklings were chosen as the bioassay animal for this study because of widespread occurrence and acute sensitivity to aflatoxin injury with an almost immediate induction of bile duct proliferation. This induced hepatic aberration is now considered to be the confirmational test for aflatoxins.

The purposes of this study were to assay selected blood plasma and liver indicator enzyme components and related conjugates of Pekin ducklings at induced differential aflatoxicosis levels, and to correlate the aflatoxin dose intake and day age response with gross organ, histological sections of liver tissue, and plasma enzyme abnormalities.

CHAPTER II

LITERATURE REVIEW

Aflatoxin is the generic name of a series of highly toxic carcinogens produced by Aspergillus flavus and at least four other species of Aspergilli (10,17). Aflatoxin is also reported to be produced by four different species of Penicillium (14,17).

Although many mycotoxins with closely related configurations are termed aflatoxins, the B and G groups dominate in grain and oilseed products. The structures of these compounds are shown in Figure 1 (6). The coumarin-lactone structure, common to all aflatoxins, is characteristic of many natural, physiologically active compounds. The bifuran structure, however, is known to occur in only one other natural compound, sterigmatocystin, a metabolite of A. versicolor (12). A. flavus can utilize essentially all food and feed products as growth media and has been reported to have produced aflatoxin in barley, corn, wheat, cocoa beans, copra, palm kernels, soya flour, cottonseed, and locust beans in addition to peanuts (11).

The known pathology and toxicology of aflatoxin poisoning in animals has been extensively reviewed (1,2,13,28,29). Hepatomas have been induced with low aflatoxin dose levels in poultry, cattle, swine, trout, and rodents. Although there is some species variation in susceptibility, the LD_{50} for a single dose of aflatoxin B_1 is in the range of 0.5-1.0 mg/kg body weight for most experimental animals.

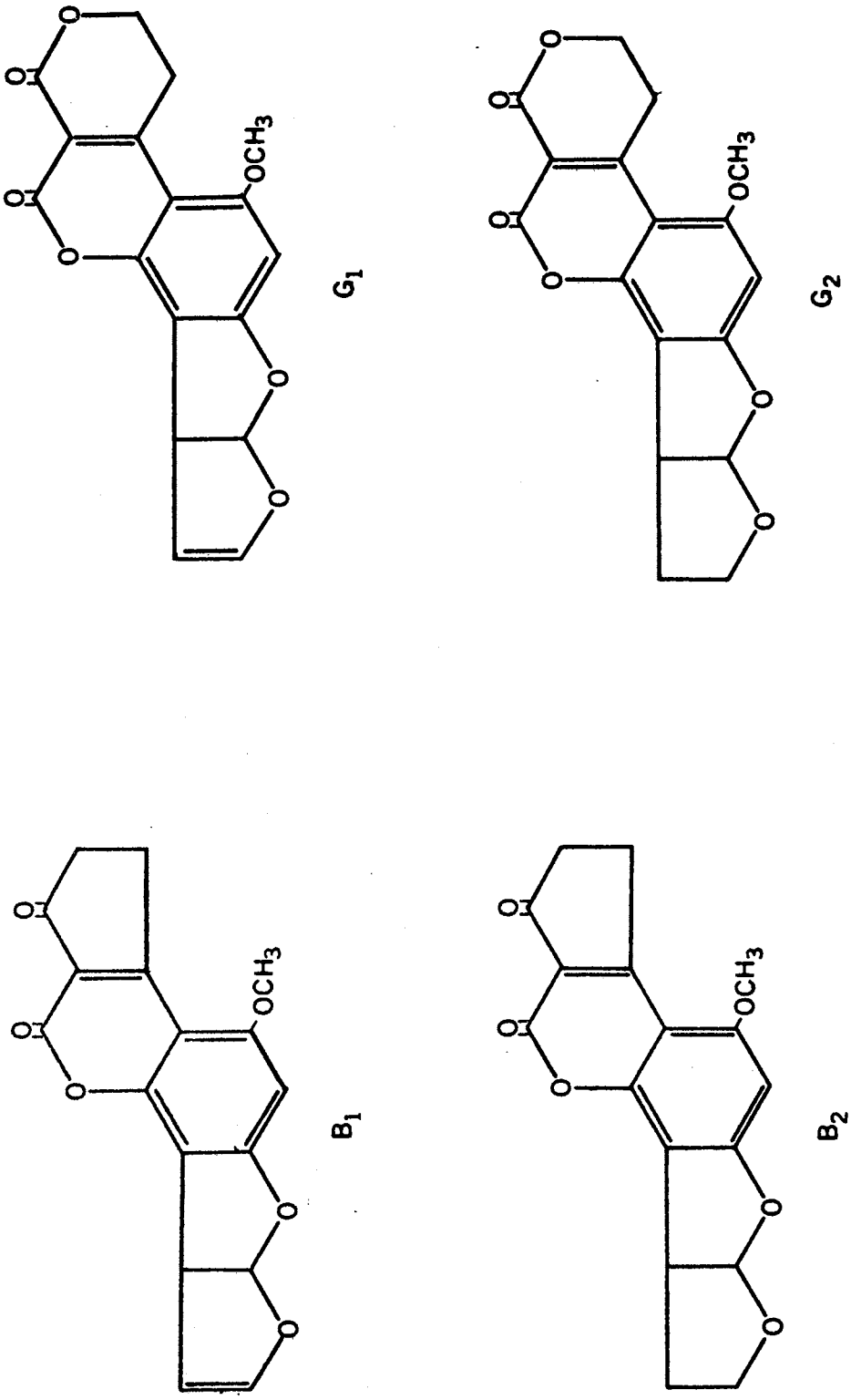

Figure 1. The Structures of Aflatoxins B_1, B_2, G_1, and G_2.

Carnaghan et al. (8) reported the 7-day LD_{50} values of the four aflatoxins in the 1-day old duckling as 18.2 ug aflatoxin B_1, 84.8 ug B_2, 39.2 ug G_1, and 172.5 ug G_2 on a 50 g body weight basis.

The toxic properties of the aflatoxins manifest differently depending on the test system, dose and duration of exposure (15,26). Proliferation of the bile ductular cells is the most characteristic and easily identifiable early pathological effect in most animal species. Allcroft (1) found that aflatoxin caused enlarged hepatic cells and enlarged nuclei in cattle, pigs, ducklings, and turkey poults.

The toxic effects of aflatoxin B_1 have also been investigated in animal cells in vitro. In cell cultures, lethality has been reported at concentrations of 1-5 ug/ml of medium. Inhibition of growth and mitotic rate has been reported at concentrations of 0.03 ug/ml (22).

Wogan (27) reported a marked decrease in liver glycogen, an accumulation of liver lipid with no remarkable change in liver protein content of 7-day old ducklings fed 0.6 ug aflatoxin B_1 per 100 g body weight daily for five days. Kohler and Schumacher (16) detected necrobiotic and regressive changes in the mitochondria of duckling liver cells as revealed by histological thin sections and electron micrographs. These workers also noted reticular fibres arising from mesenchymal cells in the livers of ducklings fed 0.03 mg and 0.01 mg of aflatoxin per day for 40 days.

Allcroft and Lewis (2) reported an increase in serum alkaline phosphatase activity up to the twelfth week, followed by a decline

to normal values during the terminal phase at twenty weeks in calves fed a diet containing approximately 2.4 ppm aflatoxin. There was no significant change in fat content of the liver or in serum glutamic oxalacetic transaminase values. Carnaghan et al. (7) found erratic liver and serum alkaline phosphatase values with no consistent differences between groups of chicks fed a control diet and one containing about 1.5 ppm aflatoxin B_1. Brown and Abrams (5) reported somewhat higher plasma alkaline phosphatase values in poisoned ducklings throughout the test period of eight weeks. There was little difference in plasma glutamic oxalacetic transaminase or glutamic pyruvic transaminase values between the two groups for the first three weeks of the test. After this period until termination of the study at eight weeks the values for these enzymes increased in the affected birds. The diet was an unspecified standard chicken mash containing toxic peanut meal to give a final aflatoxin concentration of 0.5 ppm.

CHAPTER III

MATERIALS AND METHODS

Duckling bioassay rations were basically the standard formulation for aflatoxin evaluations: 60% peanut meal which included the aflatoxin meal addition, 10% casein, 21% sucrose, 5% corn oil, 2% Phillips-Hart salt mixture and 2% vitamin mixture. Day-old ducklings were obtained from the Hile Duckling Hatchery, Carey, Ohio, by overnight parcel post.

The experiment commenced with day-old ducklings and the control and test birds were killed at two day intervals for the duration of the 10-day test period. Initial weight, final weight, gain, feed intake, and aflatoxin dose level were determined for treatment response confirmations and statistical evaluations. Ducklings were sacrificed by severing the throat arteries to allow total blood collection for hematocrit determinations and quantitation of plasma components. Livers were post-mortum extracted intact and total bile content removed with a hypodermic. A subjective icteric rating was given each liver, total fresh weight determined and weighed fresh samples removed for lipid component extraction.

High aflatoxin peanut meal was attained with A. flavus spore inoculated, high moisture, ground peanuts incubated in open trays for 96 hours at 30 C at saturated humidity. The aflatoxin content was determined using a modification of the extraction method of

Pons et al. (23,24) and UV polaroid recordings for quantitation (9). The toxic ration contained 1.578 ug/g total aflatoxins (.948 ug B_1 and .630 ug G_1).

Total lipids in the liver were determined by the sulfophospho-vanillin reaction photometrically quantitated at 530 nm with a Baush and Lomb "Spectronic 20" colorimeter. The cholesterol concentrations in the plasma and liver lipid fraction were determined fluorometrically by reaction with acetic anhydride and sulfuric acid utilizing a Perkin-Elmer 203 fluorescence spectrophotometer.

The plasma $_L$-alanine:2-oxoglutarate aminotransferase (glutamic pyruvic transaminase, GPT) assay procedure utilized the substrates 2-oxoglutarate and $_L$-alanine in buffer solution. The resultant pyruvate is converted to lactate by dihydronicotinamide dinucleotide ($NADH_2$) in the presence of lactate:NAD-oxidoreductase (lactate dehydrogenase, LDH). The decrease in $NADH_2$ concentration is measured by the decrease in absorbance at 340 nm and is proportional to the GPT concentration. The $_L$-aspartate:2-oxoglutarate aminotransferase (glutamic oxalacetic tranaminase, GOT) quantitation procedure utilized 2-oxoglutarate and $_L$-aspartate in buffer solution with plasma enzyme reaction resulting in glutamate and oxalacetate. The latter is converted to malate by $NADH_2$ in the presence of malate:NAD-oxidoreductase (malate dehydrogenase, MDH). The rate of reaction is measured by the decrease in $NADH_2$ absorbance at 340 nm and is proportional to the GOT concentration.

Plasma orthophosphoric monoester phosphohydrolase (alkaline phosphatase) was assayed by the method of Bessey, Lowry, and Brock (4). p-Nitrophenylphosphate is split by the enzyme into

p-nitrophenol and phosphoric acid. The reaction is stopped by the addition of sodium hydroxide, which also converts the p-nitrophenol to its anion which is quantitated photometrically at 400 nm.

Enzyme assays were performed with a Beckman DK-B spectrophotometer.

CHAPTER IV

RESULTS AND DISCUSSION

The most obvious result of aflatoxicosis in ducklings is the rate of weight gain. Day-old White Pekin ducklings fed an aflatoxin-free diet gain four to six times their original weight during the 10-day bioassay period. Ducklings that received an aflatoxin containing diet seldom doubled their original weight. The effects of aflatoxin diet and day-age are shown in Table I and Figure 2. Differences in weight as a function of treatment were statistically highly significant with coefficient variation 25.63%. Characterisic of the syndrome are lethargy and general loss of appetite. Palatability of the feed is evidently not a factor as ducklings transferred from the control diet to the aflatoxin diet avidly ingest normal amounts of feed for 1 to 2 days. Intubation of 6 mg of aflatoxin B_1 per 100 g body weight daily for five days also causes the characteristic lethargy, loss of appetite, and gross reduction in weight gain (27).

The effects of aflatoxin diet and day-age on lipid retention in the liver is presented in Table II and Figure 3. Lipid retention can be clearly seen in the livers of aflatoxin fed ducklings by staining histological thin sections with sudan IV and is indicated by the yellow to orange-red color of the gross organ. The presence of aflatoxin in the feed affected lipid retention in the liver and

TABLE I

EFFECT OF AFLATOXIN DIET AND DAY-AGE ON THE RATE OF WEIGHT GAIN OF WHITE PEKIN DUCKLINGS

Diet	Day-age	Weight gain (g) I	II	III	IV	Sum
Toxin-free Meal	2	21.8	18.9	32.8	26.9	100.4
	4	44.3	65.1	42.3	54.2	205.9
	6	131.0	80.9	107.6	122.3	441.8
	8	158.5	158.3	226.2	201.5	744.5
	10	273.8	271.1	192.0	246.0	982.9
Aflatoxin Meal	2	9.0	1.2	13.5	12.2	35.9
	4	20.6	12.3	19.7	15.5	68.1
	6	28.5	24.1	30.8	30.3	113.7
	8	32.1	36.8	56.1	43.4	168.4
	10	81.6	32.2	53.9	57.6	225.3
	Sum	801.2	700.9	774.9	809.9	3086.9

Treatment F values

Diet	222.0	p = 0.005
Day-age	62.8	p = 0.005
Interaction	27.3	p = 0.005

C.V. 25.63%

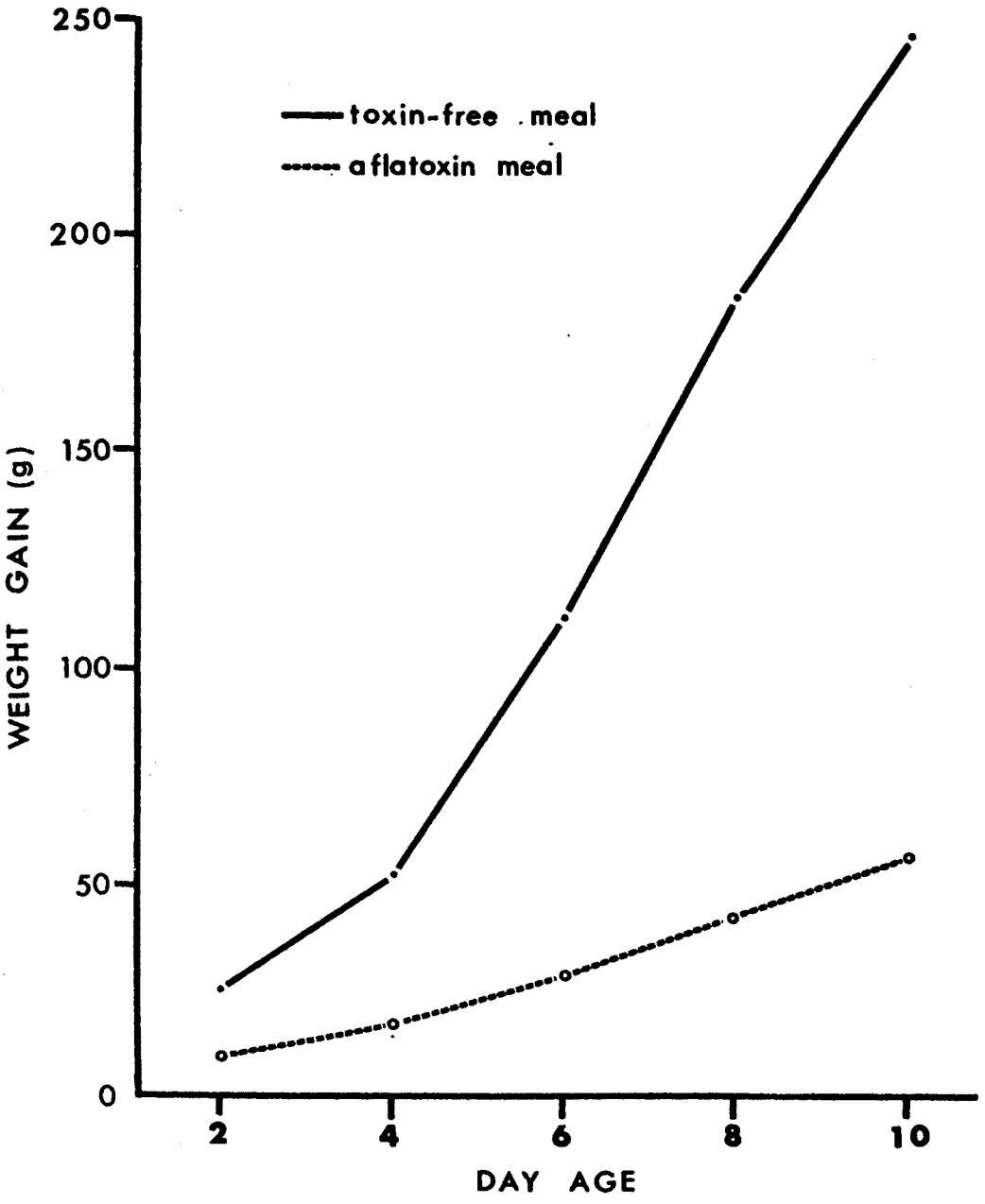

Figure 2. Effect of Aflatoxin Diet and Day-age on the Rate of Weight Gain of White Pekin Ducklings.

was significant at the 0.5% level of probability. The day-age and interaction of diet and day-age were not significantly different. The coefficient of variation was 27.46%. The livers of 1-day old ducklings contained an average of 231.5 mg lipids per g of liver. After ten days on the toxin-free diet, the control birds had a mean of 77.3 mg/g as contrasted to 143.3 mg/g of the poisoned ducklings.

Lynd and Lynd (19) reported reversal of the normal triglyceride changes in livers of ducklings ingesting 14.99 to 16.70 ug of aflatoxin per day. The relative proportions of palmitic and stearic acids decreased, oleic and linoleic increased with linoleic levels much higher in the livers of the ducklings on the aflatoxin diet. These data indicate a blockage of the normal oxidative degradation of fatty acids in the liver. Intracellularly, fatty acid oxidation occurs principally in the mitochondria (21). Electron-microscopic studies on the livers of ducklings ingesting 0.03 and 0.01 mg of aflatoxin per day showed necrobiotic and regressive changes in the mitochondria.

The abnormal metabolism of lipids is also reflected in the liver and plasma cholesterol levels. The effects of aflatoxin diet and day-age on cholesterol concentrations in the liver and blood plasma is presented in Tables III and IV, respectively, and is illustrated in Figure 4. Highly significant F values resulted from treatments of aflatoxin diet and day-age with interaction of these factors apparently absent. Cholesterol is a key intermediate in the biosynthesis of the general class of steriods which includes the bile acids. The salts of the bile acids act as emulsifying and solubilizing agents of neutral fats in the intestine and also

TABLE II

EFFECT OF AFLATOXIN DIET AND DAY-AGE ON THE RETENTION OF LIVER LIPIDS BY WHITE PEKIN DUCKLINGS

Diet	Day-age	Liver Lipids (mg/g) I	II	III	IV	Sum
Toxin-free Meal	2	133	70	125	154	482
	4	124	60	77	111	372
	6	64	62	78	101	305
	8	76	69	85	72	302
	10	83	67	75	84	309
Aflatoxin Meal	2	199	259	148	134	740
	4	166	179	116	149	610
	6	132	159	127	129	547
	8	128	144	108	200	580
	10	80	199	131	163	573
	Sum	1185	1286	1070	1297	4820

Treatment F values

Diet	37.4	p = 0.005
Day-age	N.S.	
Interaction	N.S.	
	C.V. 27.46%	

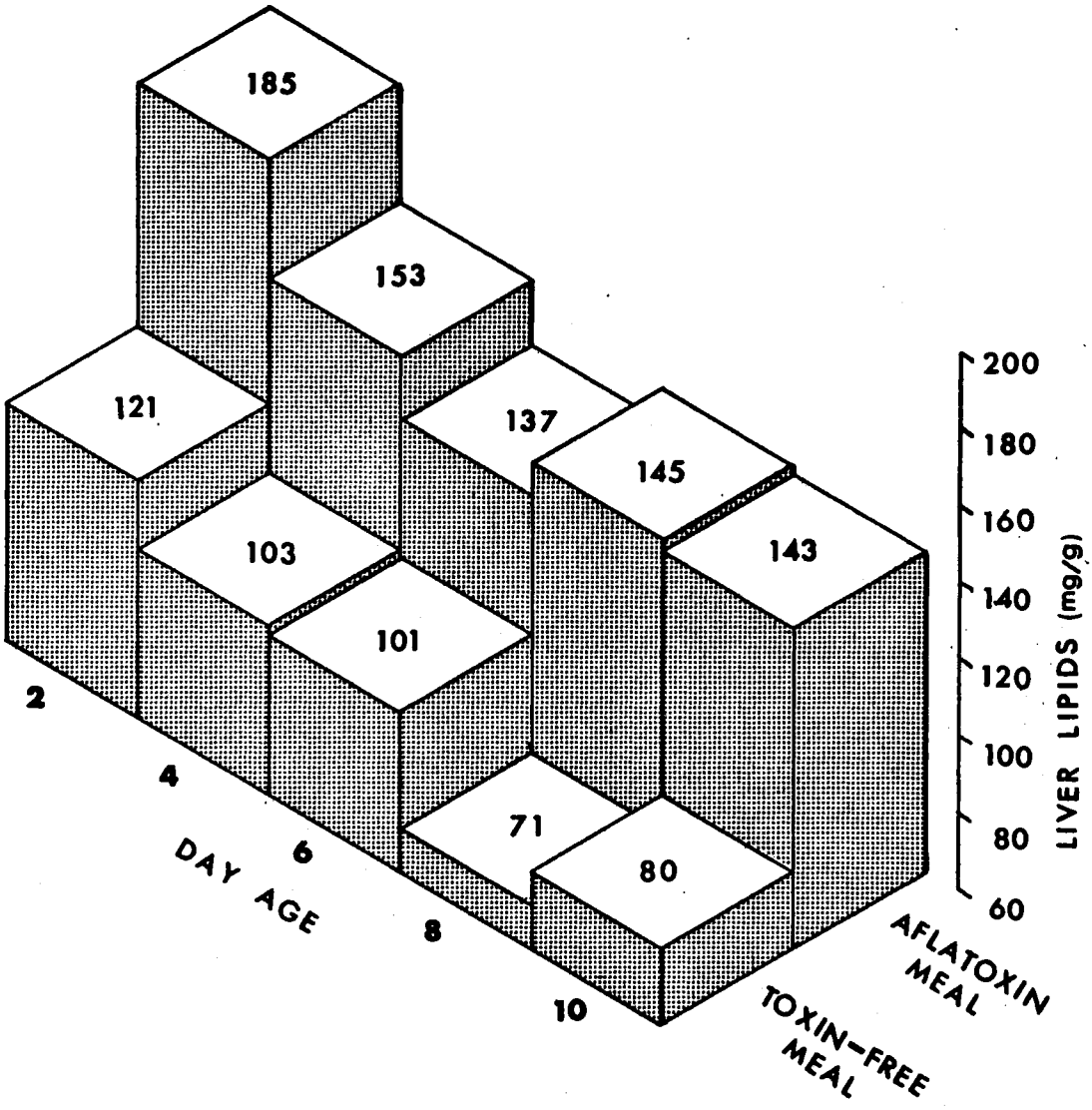

Figure 3. Effect of Aflatoxin Diet and Day-age on the Retention of Liver Lipids by White Pekin Ducklings.

activate lipases. Tsay and Lynd (25) found gross abnormalities in the thin layer chromatography R_f values and fluorescence of bile components from aflatoxin poisoned ducklings as compared with non-poisoned birds. The retention of cholesterol in the liver and the lower concentration of plasma cholesterol in poisoned ducklings indicates a possibility of interference with the normal metabolism of triglycerides and steroids.

Wroblewski and LaDue (30) concluded that serum glutamic oxaloacetic transaminase activity is a fairly sensitive index of liver cell injury from cancer in rats and humans. Liver parenchymal cell damage should be reflected as increased plasma transaminase activity from constituent enzyme leakage. Brown and Abrams (5) found little difference in plasma GOT and GPT activity between ducklings on feed containing 0.5 ppm aflatoxin and controls at 1-2 weeks of age. Results of the plasma GOT assays of ducklings on 1.578 ppm aflatoxin feed are presented in Table V and Figure 5. Treatment effects significant at the 0.5% level of probability were diet and day-age with interaction significant at the 5% level with coefficient of variation of 11.77%. Increases in plasma GOT activity appear the second day of the 10-day bioassay period with maximum differences occurring on the eighth day. The significant increase with low coefficient of variation of plasma GOT activity can be correlated with other biochemical abnormalities and histological aspects for use in diagnosis of aflatoxicosis.

Plasma GPT activity is presented in Table VI. F values for treatment response were not satistically significant. GOT of rat liver occurs in two forms: one occurring in the soluble portion

TABLE III

EFFECT OF AFLATOXIN DIET AND DAY-AGE ON THE CONCENTRATION OF LIVER CHOLESTEROL OF WHITE PEKIN DUCKLINGS

Diet	Day-Age	Liver Cholesterol (mg/g) I	II	III	IV	Sum
Toxin-free Meal	2	51.2	33.0	46.1	31.0	161.3
	4	35.1	12.4	28.4	25.1	101.0
	6	5.8	23.0	13.7	16.8	59.3
	8	8.0	9.5	17.5	10.1	45.1
	10	14.6	10.1	14.2	12.1	51.0
Aflatoxin Meal	2	44.3	56.4	38.2	35.1	174.0
	4	44.3	44.3	30.3	38.4	157.3
	6	35.1	42.3	33.7	30.4	141.5
	8	33.7	32.6	28.6	27.2	122.1
	10	21.3	24.2	22.0	23.8	91.3
	Sum	293.4	287.8	272.7	250.0	1103.9

Treatment F values

Diet	41.2	p = 0.005
Day-age	16.2	p = 0.005
Interaction	N.S.	

C.V. 23.95%

TABLE IV

EFFECT OF AFLATOXIN DIET AND DAY-AGE ON THE CONCENTRATION OF PLASMA CHOLESTEROL OF WHITE PEKIN DUCKLINGS

Diet	Day-age	Plasma Cholesterol (mg/100 ml) I	II	III	IV	Sum
Toxin-free Meal	2	420	315	574	636	1945
	4	423	542	787	652	2404
	6	611	456	678	524	2269
	8	483	400	540	596	2019
	10	249	345	424	498	1516
Aflatoxin Meal	2	413	363	388	434	1598
	4	369	437	635	527	1968
	6	345	303	227	278	1153
	8	342	313	396	262	1313
	10	218	234	162	338	952
	Sum	3878	3708	4811	4745	17,137

Treatment F values

Diet	34.5	p = 0.005
Day-age	7.9	p = 0.005
Interaction	N.S.	

C.V. 19.90%

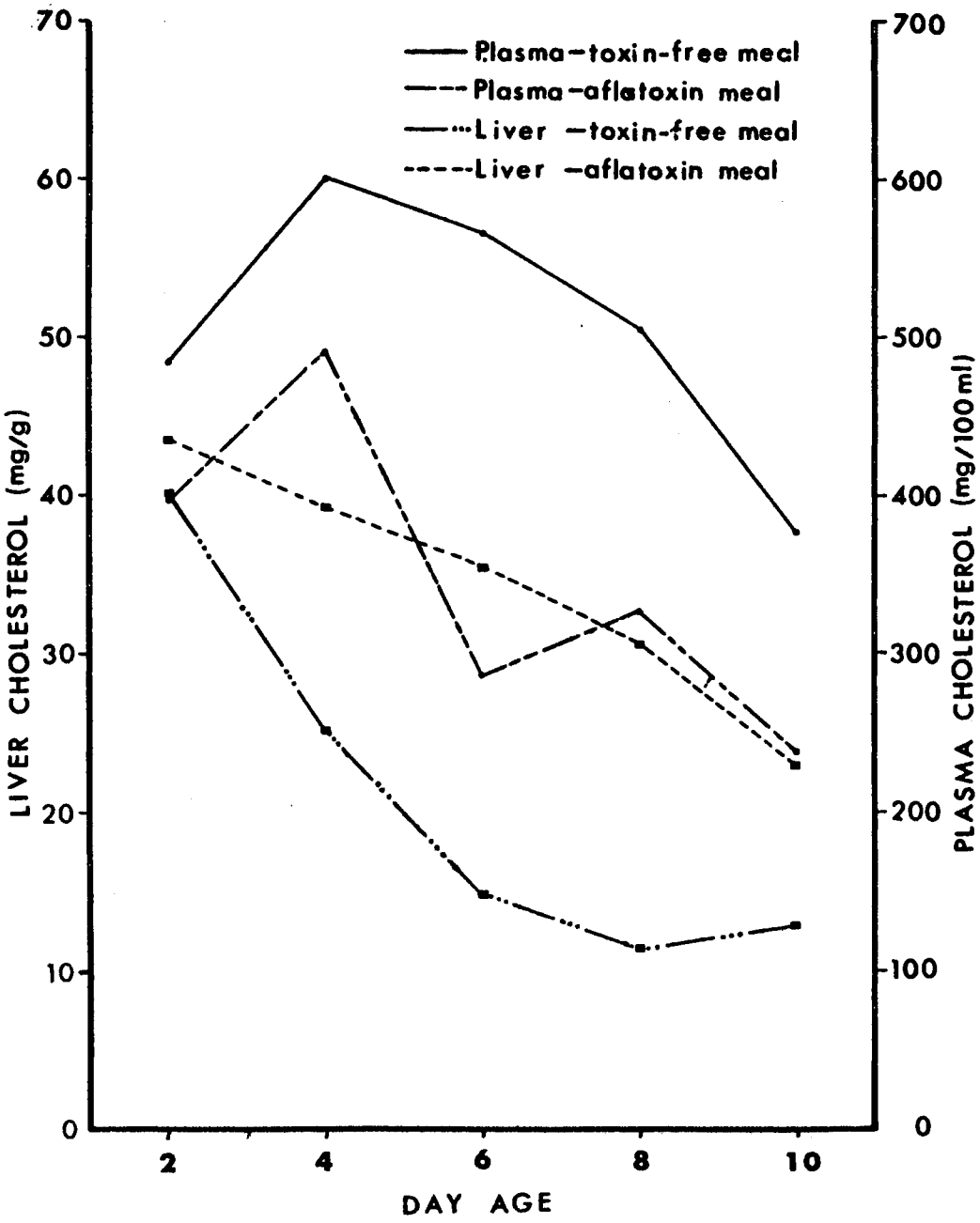

Figure 4. Effect of Aflatoxin Diet and Day-age on the Concentrations of Liver and Plasma Cholesterol of White Pekin Ducklings.

TABLE V

EFFECT OF AFLATOXIN DIET AND DAY-AGE ON PLASMA GLUTAMIC OXALACETIC TRANSAMINASE ACTIVITY OF WHITE PEKIN DUCKLINGS

Diet	Day-age	GOT Activity (mU/ml) I	II	III	IV	Sum
Toxin-free Meal	2	3.48	3.29	3.60	3.57	13.94
	4	3.60	5.09	5.38	3.68	17.75
	6	3.61	4.97	3.72	4.20	16.50
	8	4.10	4.35	4.97	4.46	17.88
	10	5.59	4.99	4.97	4.47	20.02
Aflatoxin Meal	2	3.78	3.98	3.57	4.02	15.35
	4	4.47	5.22	4.97	5.48	20.14
	6	3.98	4.22	4.97	5.22	18.39
	8	7.70	6.46	5.97	6.74	26.87
	10	5.47	6.71	6.96	6.46	25.60
	Sum	45.78	49.28	49.08	48.30	192.44

Treatment F values

Diet	32.0	p = 0.005
Day-age	18.3	p = 0.005
Interaction	4.0	p = 0.05

C.V. 11.70%

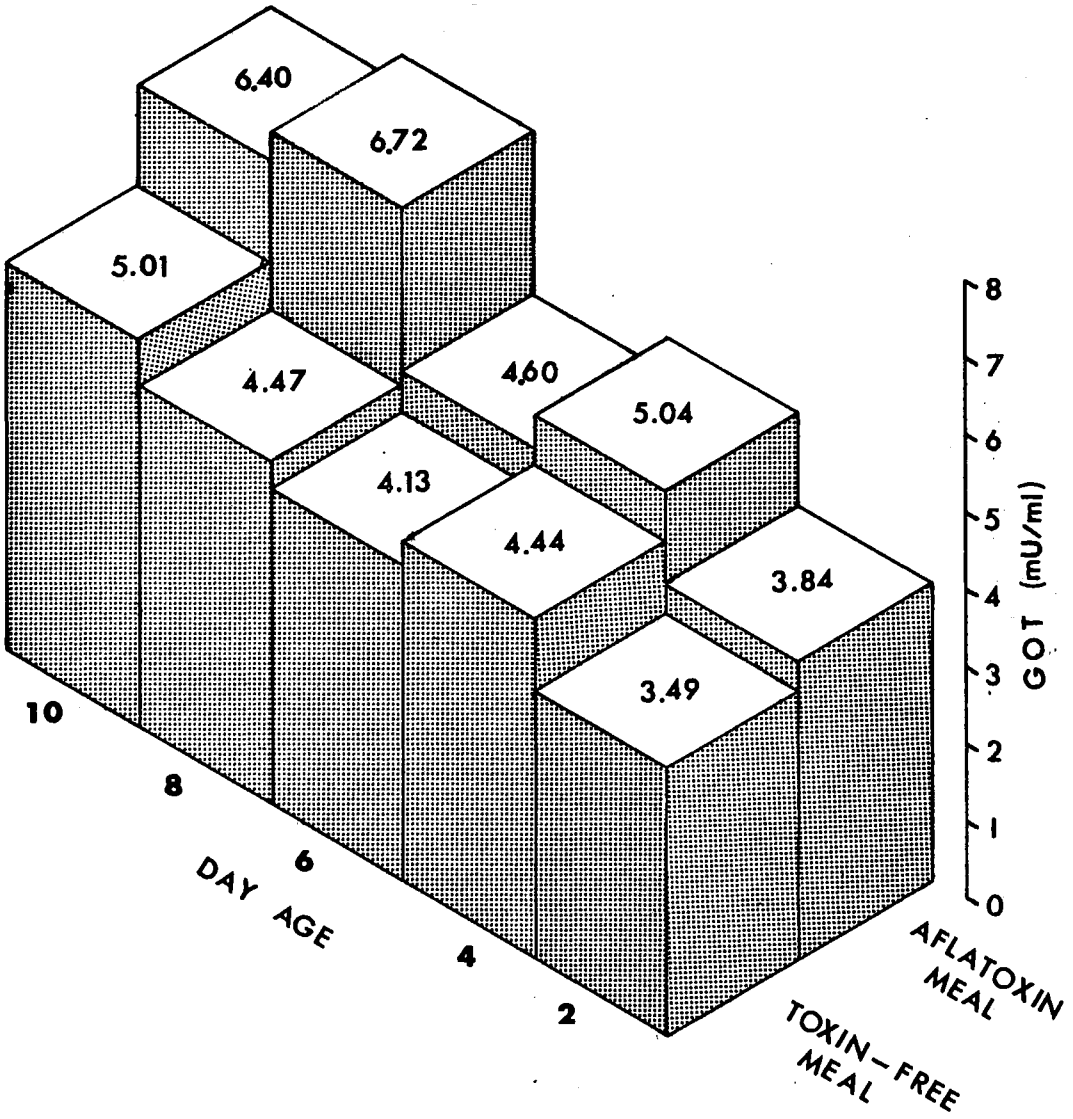

Figure 5. Effect of Aflatoxin Diet and Day-age on Plasma Glutamic Oxalacetic Transaminase Activity of White Pekin Ducklings.

of the cell and the other associated with the mitochondria, while GPT occurs only in the soluble portion (20). Brown and Abrams (5) reported a two-fold enlargement of the mitochondria in liver cells of affected ducklings and a marked aggregation of mitochondria in the area of the affected cells near the bile canaliculi. These workers also reported an increase in the number of mitochondria in liver cells adjacent to obviously affected cells. The significant increase in plasma GOT activity and nonsignificant difference in plasma GPT activity could then be explained as the result of the increase in number, aggregation near the bile canaliculi and necrobiosis of the mitochondria in the livers of poisoned ducklings.

Values for plasma alkaline phosphatase activity are given in Table VII and Figure 6. Increased alkaline phosphatase activity in plasma or sera has been reported in ducklings, pigs, and calves (2,3,5,18). Treatment differences due to diet and interaction of diet and day-age were significantly different at the 5% level of probability with day-age apparently having no effect. Plasma alkaline phosphatase activity is generally elevated in cases of severe liver damage. The 10-day bioassay period is apparently too short a time for damage to occur to the plasma membrane with consequent leakage of enzymes located in the cytoplasm of liver cells.

TABLE VI

EFFECT OF AFLATOXIN DIET AND DAY-AGE ON PLASMA GLUTAMIC PYRUVIC TRANSAMINASE ACTIVITY OF WHITE PEKIN DUCKLINGS

Diet	Day-age	GPT Activity (mU/ml) I	II	III	IV	Sum
Toxin-free Meal	2	4.10	5.62	4.98	4.22	18.92
	4	3.48	5.46	6.71	3.73	19.38
	6	4.23	4.47	3.98	4.97	17.65
	8	4.23	4.85	6.21	6.21	21.50
	10	5.47	4.87	5.42	4.22	19.98
Aflatoxin Meal	2	4.88	4.22	5.96	2.73	17.79
	4	3.48	5.47	7.95	4.22	21.12
	6	6.17	2.98	2.73	3.23	15.11
	8	5.71	2.98	4.22	3.23	16.14
	10	3.98	3.23	3.73	4.47	15.41
	Sum	45.73	44.15	51.89	41.23	183.00

Treatment F values

Diet	N.S.
Day-age	N.S.
Interaction	N.S.

TABLE VII

EFFECT OF AFLATOXIN DIET AND DAY-AGE ON PLASMA ALKALINE PHOSPHATE ACTIVITY OF WHITE PEKIN DUCKLINGS

Diet	Day-age	Alkaline Phosphatase Activity (mU/ml) I	II	III	IV	Sum
Toxin-free Meal	2	17.0	16.0	13.4	18.4	64.8
	4	10.3	13.7	21.8	18.4	64.2
	6	26.9	18.8	33.8	35.7	115.2
	8	10.9	24.0	25.0	24.4	85.3
	10	24.5	15.1	20.5	15.2	75.3
Aflatoxin Meal	2	17.9	15.5	16.7	19.8	69.9
	4	19.7	12.2	23.8	17.6	73.3
	6	15.6	13.4	17.0	12.5	58.5
	8	22.6	17.6	12.6	16.8	69.6
	10	17.8	19.3	15.4	10.6	63.1
	Sum	183.2	165.6	200.0	190.4	739.2

Treatment F values

Diet	5.6	p = 0.05
Day-age	N.S.	
Interaction	3.6	p = 0.05

C.V. 25.47%

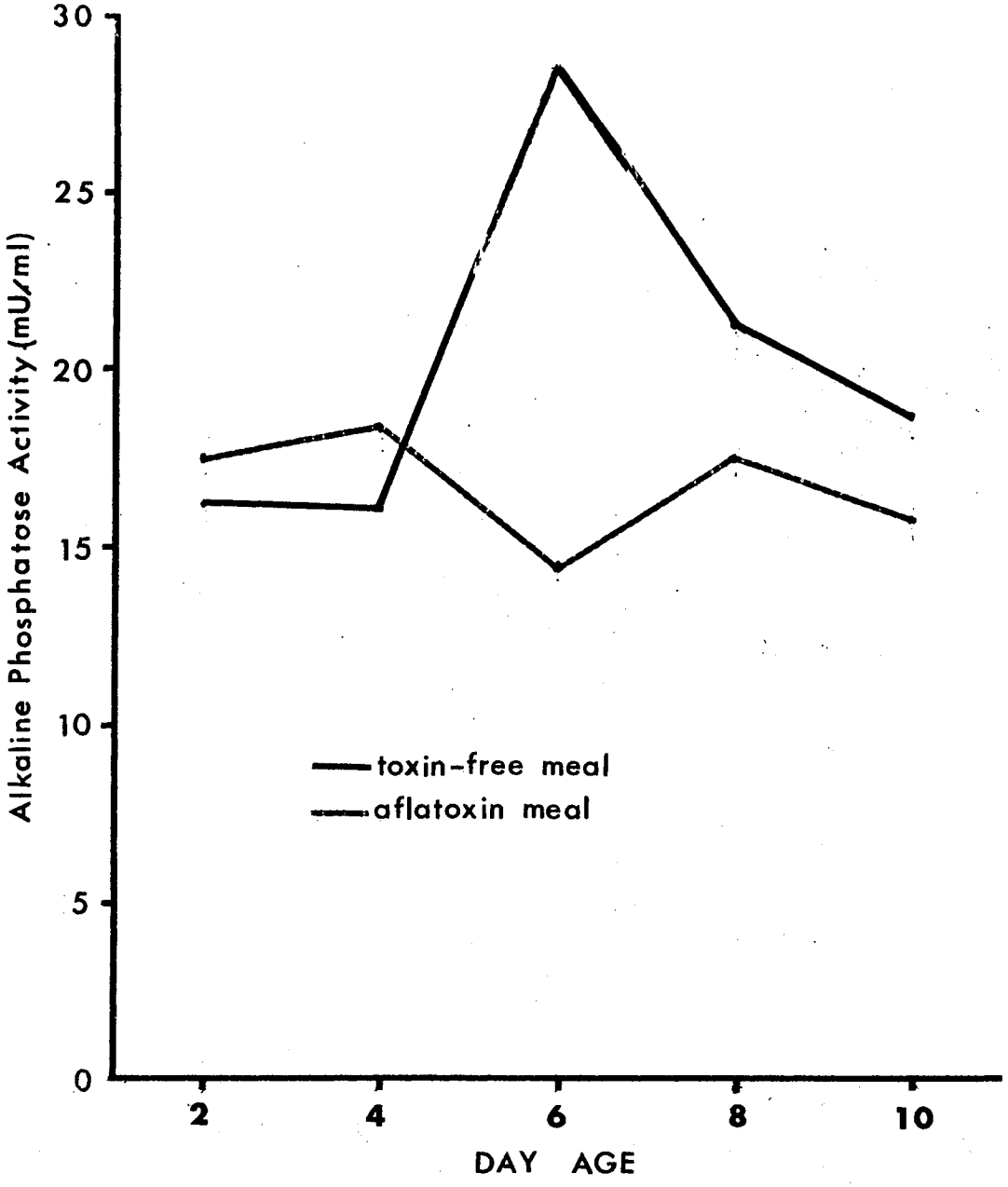

Figure 6. Effect of Aflatoxin Diet and Day-age on Plasma Alkaline Phosphatase Activity of White Pekin Ducklings.

CHAPTER V

SUMMARY AND CONCLUSIONS

The objectives of this study were to assay selected blood plasma and liver indicator enzyme components and related conjugates of White Pekin ducklings at induced differential aflatoxicosis levels, correlate the aflatoxin dose intake and day-age response with gross organ, histological sections of liver tissue and plasma enzyme abnormalities, and indicate the metabolic pathways and cell organelles affected by aflatoxin. White Pekin ducklings were used as the bioassay organism because of the acute sensitivity of this species to aflatoxin injury and its use as the standard confirmation test for aflatoxins.

Plasma enzymes or related conjugates investigated in this study included $_{L}$-alanine:2-oxoglutarate aminotransferase (GPT), $_{L}$-aspartate: 2-oxoglutarate aminotransferase (GOT), orthophosphoric monoester phosphohydrolase (alkaline phosphatase), and plasma cholesterol. Liver components studied for response to aflatoxin ingestion were lipids and cholesterol.

The rate of weight gain, color of the liver, and bile duct proliferation in the livers were typical responses of ducklings on the aflatoxin containing diet.

The marked increase in plasma GOT activity with low coefficient of variation (11.77%) suggests the use of this test as a quantitative

assay for aflatoxicosis if confirmed by additional diagnostic biochemical aberrations and chemical evaluations of the contaminated feed. Plasma and liver cholesterol levels, lipid retention in the liver, and plasma alkaline phosphatase activity can be diagnosed as indicative of liver injury due to aflatoxin poisoning but cannot be used as a reliable estimate of the magnitude of that injury because of the high variation between ducklings.

Results from this and other studies indicates that aflatoxin poisoning results in enlargement, increase in number, and aggregation of the liver cell mitochondria with interferences in the metabolic pathways associated with this organelle.

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