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BIOCHEMICAL STUDIES ON AFLATOXICOSIS

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Since the diagnosis of aflatoxicosis was confirmed in Great Britain in poults, ducklings, pigs and calves (Sargeant, Sheridan, O'Kelly & Carnaghan, 1961) similar outbreaks have been encountered in South Africa in a few pigs and dogs and in numerous ducklings and New Hampshire chickens. A striking feature of the outbreaks in chickens was the susceptibility of the New Hampshire breed and the absence of any lesions, mortality or impaired weight gains in other breeds used commercially in South Africa. Cornish Game, White Rock, White Leghorn and Rhode Island Red chickens consuming the same food and in many instances housed together with affected New Hampshire chickens grew normally. Chickens and ducklings were found to be most susceptible in the one to four weeks age group.

Experimentally it has been shown that after the age of 10 to 12 weeks New Hampshire chickens or ducklings are no longer susceptible to aflatoxin in the order of 0·75 ppm in the ration.

In four separate groups of ducklings and two groups of New Hampshire chickens *Salmonella* spp. were isolated from birds showing typical aflatoxicosis. This was also reported by Siller & Ostler (1961) in poults.

A considerable amount of data is available concerning the chemistry, stability and similar aspects of aflatoxin, as well as the nature of the lesions caused by compounds of this type, but very little seems to have been published about its biochemical mode of action. The only reference in the literature that could be found was that of Smith (1963) concerning leucine metabolism in liver preparations.

The paucity of this information prompted the investigation described hereunder. It was hoped that this work would also provide some indication of the basis underlying the various species differences mentioned earlier.

In the initial stages the work was planned to indicate the major metabolic pathways affected by aflatoxin and the toxins of other *Aspergillus* species found to be harmful. Certain key enzymes in the glycolytic and tricarboxylic acid cycles and in the respiratory chain or oxidative phosphorylation mechanisms were accordingly selected for study. As the work progressed the assays of certain enzymes showing no significant difference between test and control birds were omitted. The investigation was then confined to the metabolic systems obviously affected. Plasma proteins and haemoglobin determinations were included in the investigation to give an indication of the overall effects of these toxins on protein or haemoglobin synthesis.

A small number of birds which had been fed rations incorporating yellow maize infected with *Aspergillus amstelodami* was studied. These showed lesions both macroscopically and histophathologically identical to those produced by aflatoxin (Rabie, de Klerk & Terblanche, 1964).

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Until all the toxins produced by the various species of this genus have been adequately characterized by chemical means, it is suggested that all compounds of this nature produced by *Aspergillus* species be referred to collectively as " aspergillins " and that the name " aflatoxins " be restricted to the toxins of *A. flavus*. This practice will be followed in the text of this paper.

Materials and Methods

The toxicity of the groundnut cake meal used throughout this investigation was controlled at regular intervals by thin layer chromatographic techniques developed by Coombs & Sanders (1963). Silica gel was used for the preparation of the plates instead of alumina.

The ground nut cake meal was finely ground and specimens for analysis were taken with a sampling device from at least five different sites in each bag of meal. These samples were then thoroughly mixed and assayed for aflatoxin content. The cake meal used in this work contained 8 ppm of B_1 aflatoxin.

All control groups of Pekin ducklings, New Hampshire, White Leghorn, Cornish Game and Rhode Island Red chickens were given a standard chicken mash free from groundnut cake meal, while the chickens and ducklings placed on toxic rations were given the same basic diet except that groundnut cake meal was added to give a final aflatoxin concentration of 0·5 ppm in the ration.

The chickens used were all housed in electrically heated battery cages. The control groups of the different breeds of chickens were kept together to ensure consumption of identical rations and those used in the toxin trials were kept together in a similar manner. The Pekin ducklings were housed separately under identical conditions.

The investigation commenced with day-old birds and seven to ten days later the first of the control and test birds were killed for biochemical and histopathological investigation. Thereafter birds were slaughtered at weekly intervals until the end of each particular experiment.

Blood specimens were collected immediately prior to slaughter and the liver removed immediately afterwards. All determinations and assays were completed as soon as possible after collection of material. Liver specimens from each group of birds were examined macroscopically and then preserved for subsequent histopathological study.

All enzyme assays performed on liver tissue, excepting that of cytochrome c reductase, were carried out with 10 per cent homogenates in ice-cold 0·25 M sucrose solution. These were made in an all-glass manual Potter type homogenizer. The homogenizing medium used for cytochrome c reductase estimations was as specified in the original method cited below. All homogenates were kept in ice-baths at 0° C during use.

Potassium oxalate was used as the anticoagulant for those specimens of blood on which the activities of certain enzymes present in plasma were determined.

The methods used for the assay of both liver and plasma enzymes and for the determination of the various blood constituents studied are listed in Table 1. The abbreviations used for the names of certain enzymes in the text which follows are also given in this table.

TABLE 1. *Methods used for enzyme assays and for the determination of certain blood constituents*

Enzyme or blood constituent	Abbreviations used in text	Reference
(*a*) Liver enzymes—		
Hexokinase	—	Crane, R. K. & Sols, A. (1953)
Glucose-6-phosphatase	G-6-P-ase	Swanson, Marjorie A. (1950)
α Glycerolphosphate dehydrogenase	Glyc.-P-D	Beisenherz, G. *et al.* (1953)
Glyceraldehyde phosphate dehydrogenase	GAP-D	Sigma Technical Bull. No. 10 (1961)
Glucose-6-phosphate dehydrogenase	G-6-P-D	Kornberg, A. & Horecker, B. L. (1957) cit. Colowick, S. P. & Kaplan, N. O.
6-phosphogluconate dehydrogenase	6-P-G-D	Kornberg, A. & Horecker, B. L. (1957) cit. Colowick, S. P. & Kaplan, N. O.
Isocitric dehydrogenase	ICD	Ochoa, S. (1948).
Succinic dehydrogenase	SD	Sclater, E. C. & Bonner, W. D. (1952)
Uridine-diphospho glucose dehydrogenase	UDPGD	Pontis, H. G. & Leloir, L. F. (1962)
Diaphorase	—	Mahler, H. R. *et al.* (1952)
ATP-ase	—	Umbreit, W. *et al.* (1964)
DPN-ase	—	Zatman, L. J. *et al.* (1953)
Cytochrome c reductase	Cyto-c-red	Mahler, H. R. *et al.* (1952)
Cytochrome b	Cyto-b	Stotz, E. cit. Colowick, S. P. & Kaplan, N. O. (1957)
Glutathione reductase	GR	Racker, E. cit. Colowick, S. P. & Kaplan, N. O. (1957)
(*b*) Plasma enzymes—		
Lactic dehydrogenase	LDH	Wróblewski, F. & la Due, J. S. (1955)
Aldolase	—	Sibley, J. A. & Lehninger, A. L. (1949)
Glutamic oxalacetic transaminase	GOT	King, J. (1958)
Glutamic pyruvic transaminase	GPT	King, J. (1958)
(*c*) Determinations on blood—		
Haemoglobin	Hb	Drabkin, D. cit. King, E. & Wootton, J. D. P. (1956)
Haematocrit	H-crit	Standard methods
Total plasma proteins	TPP	Weichselbaum, T. E. (1946); Kingsley, G. R. (1940)
Plasma protein electrophoresis	—	King, E. & Wootton, J. P. D. (1956)

NOTE (I) For the sake of uniformity the activities of the various liver enzymes listed above have been expressed in terms of the original units, as defined by the authors concerned per 100 mg of fresh liver, with the exceptions noted in (II) below. Plasma enzyme activity is expressed in the case of Aldolase and LDH as units/ml of plasma and GOT and GPT as units/100 ml plasma.

(II) The activities of the enzymes listed below have for convenience been defined as follows:—

(*a*) G-6-P-ase: One unit is that amount of enzyme which will liberate one micromole of phosphorus from the substrate, under the conditions of the original method, within one hour.

(*b*) ATP-ase: One unit is that amount of enzyme which will liberate one micromole of phosphorus from the ATP substrate in one hour, under the conditions of the

original method. This assay includes both Ca^{++} and Mg^{++} activated ATP-ase, the reaction being initiated by the addition of both anions to the substrate at zero time.

(*c*) Diaphorase activity: The method measures reduction of 2, 6-dichlorophenol-indophenol at 600 mμ, activity being expressed as micromoles of diaphorase per given weight of tissue. A calibration curve was constructed using serial dilutions of pig heart diaphorase (Sigma Chem. Co. Type III, 4·5 μM enzyme/mgm of protein) for use in establishing the diaphorase activity of the liver samples.

In all three instances activities were expressed as units of enzyme/100 mgm of fresh liver. DPN-ase is units/20 mg fresh liver.

(III) The cyto-c-red assayed in this work is DPNH-cytochrome c reductase. The Glyc.-P-D assayed is DPN dependent.

(IV) The abbreviations ATP, DPN and DPNH refer to adenosine-5′-triphosphate, β-diphosphopyridine nucleotide and reduced β-diphosphopyridine nucleotide respectively.

All enzyme assays were performed with a Unicam S.P. 500 spectrophotometer and the photometric procedures used for the determination of various blood constituents were carried out with an Evans Electroselenium portable Model " A " photoelectric colorimeter. Plasma protein electrophoresis was performed in an Evans Electroselenium paper electrophoresis apparatus. Strips were run for 16 hours with a current setting of 2 milliamps per 34 $\times$ 5 cm paper strip in a veronal: acetate buffer (pH 8·6).

Chemicals used throughout were of " Analytical Reagent " grade. Materials used for the preparation of substrates or enzymes in pure form used for standardizing enzyme assays, were obtained from the Sigma Chemical Co., St. Louis, Mo., U.S.A. or from C. F. Boehringer und Soehne, Mannheim, West Germany, and were of the highest purity offered by these manufacturers.

Results

(*a*) *Ducklings:* During the initial stages of this investigation a group of ducklings was maintained on a standard type of ration to which infected groundnuts had been added to give a final aflatoxin content of 0·5 ppm on a dry weight basis. The aim of this preliminary experiment was to localize, as far as possible, the probable sites of action of aspergillins on the major metabolic pathways. This facilitated a more detailed study in the subsequent work. The initial study included, therefore, a wide variety of enzymes representative of the glycolytic cycle and pentose monophosphate pathways, the tricarboxylic acid cycle and the electron transfer chains or oxidative phosphorylation mechanisms.

This experiment commenced with one-week old ducklings and was allowed to proceed for four weeks during which time affected birds and healthy controls of the same age were slaughtered at weekly intervals for study.

The enzymes to which attention was paid at this stage are shown in Tables 2 and 3. These tables present values obtained from healthy control birds of various ages, used in this and subsequent experiments. The figures presented in these tables must be considered merely as a guide to the probable " normal " values pertaining to the various enzymes listed, in liver or blood of healthy birds of the different age groups studied. Too few birds have been studied to present these values in absolute statistical terms.

The figures obtained for the activities of some of the hepatic enzymes from birds of the same age group revealed a fair variation from day to day. It was, therefore, decided for purposes of interpretation, to compare only those figures

obtained in any single batch of assays, which generally included livers from equal numbers of control and poisoned birds. The variations mentioned were generally attributable to fluctuations in laboratory temperature or to speed of processing the extirpated liver samples.

TABLE 2.—*Control Ducklings.*—Values established for various liver enzymes in different age groups (given as ranges in units of enzyme/100 mgm of fresh liver)

Age Group	SD	DPN-ase	Cyto-c-red	Diaphorase
9 days..........	194–324	0·5 –1·6	1·58–2·34	0·4–2·2
11 days..........	138–246	0·75–1·8	2·0 –2·6	1·0–1·4
17 days..........	226–324	0·3 –0·6	0·82–2·2	2·4–3·1
21–23 days.......	259–356	0·9 –1·05	1·1 –2·3	1·6–2·8
28–30 days.......	194–470	0·78–1·5	0·92–1·32	1·4–5·8
35 days..........	226–515	1·88–1·90	1·04–1·12	1·5–5·2
49 days..........	420	1·85–1·88	2·20–2·24	2·0–2·15
56 days..........	194–259	0·86–1·4	1·0 –1·16	0·6–1·4

TABLE 3.—*Control Ducklings:* Ranges of values established for various liver enzymes at four weeks of age (The figures represent units of enzyme/100 mgm of fresh liver)

Hexokinase	G-6-P-ase	Glyc.-P-D	G-6-P-D	6-P-G-D	G-A-P-D
73–129	34–58	160–260	100–640	460–1080	45–64
U-D-P-G-D	ATP-ase	Cyto-b	GR	ICD	
100–200	255–465	0·04–0·32	211–363	16–26	

From this work it was apparent that of the enzymes selected for study, only SD and the various members of the electron transfer chain and oxidative phosphorylation mechanisms were affected to any significant degree. Since the variation between affected and control birds, as regards these enzymes, was most striking in birds four weeks old, the relevant figures are reproduced in Table 4. Hexokinase and cyto-b activity appeared to be increased in a small number of affected birds throughout the test period, while in some the values obtained for glyc-P-D appeared to be much lower than in the corresponding controls. The variations between control and affected birds as regards these latter enzymes were, however, not consistent enough to merit further attention at this stage. Many of the affected birds presented a marked anaemia and a grossly abnormal plasma protein pattern as revealed by paper electrophoresis. These aspects were studied in greater detail in the subsequent experiment and are reported fully below.

TABLE 4.—*Ducklings:* Mean values for the activities of certain liver enzymes showing apparent differences between control birds and those on toxic mash at the age of four weeks. (The figures in the column represent units enzyme/100 mgm of fresh liver).

Groups	SD	Cyto-c-red	DPN-ase	Diaphorase	ATP-ase
Control birds............	370	1·76	1·13	7·42	356
Affected birds...........	267	0·67	1·87	2·36	606

From the results of this preliminary work the more detailed experiment described earlier under " Materials and Methods " was planned. Biochemical studies were confined to the enzymes appearing significantly affected and to the apparent anaemia and disturbances in plasma protein levels.

The most significant variations between control and poisoned ducklings were seen in the activities of liver SD and cyto-c-red. Figures 1 and 2 are representative of the results obtained from assays of these two enzymes throughout the test period. The difference between the two groups of birds was again most striking from the fourth week of intoxication onwards.

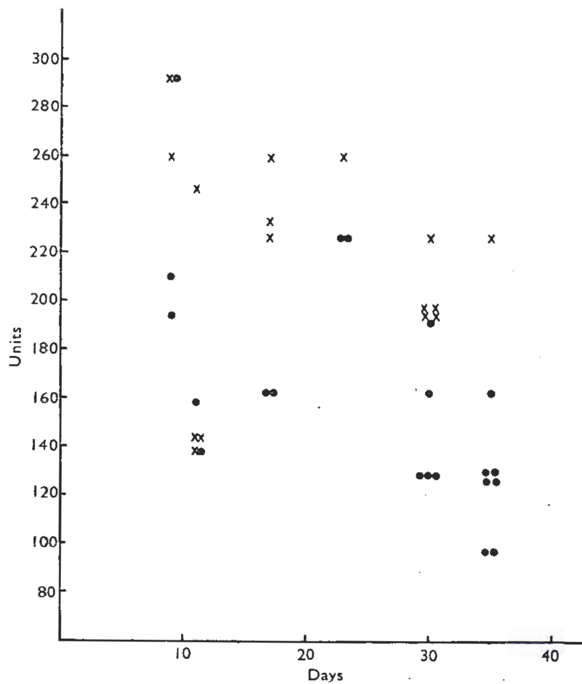

FIG. 1.—Ducklings: succinic dehydrogenase activity in liver. (Enzyme activity is expressed as units/100 mgm of fresh liver).

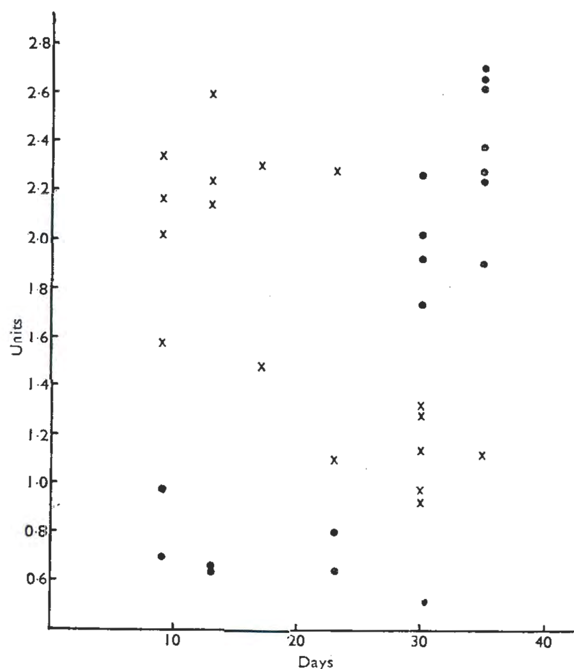

FIG. 2.—Ducklings: cyto-c-red activity in liver. (Enzyme activity is expressed as units/100 mgm of fresh liver).

Liver SD activity in affected birds was particularly low towards the end of the test period while that of cyto-c-red was consistently lower than in the control birds during the first three weeks of intoxication but rose towards the end of the experiment. The activity of this latter enzyme, as illustrated in Fig. 2, appeared to decline somewhat in normal ducks after about three weeks of age.

Little significant difference could be detected between affected and control ducklings throughout the test period with regard to liver diaphorase activity. The values found for DPN-ase were on the whole lower in affected birds than in the controls from about thirty days onwards. The apparent elevations of this enzyme, seen in birds on toxic mash in the previous experiment, were not discerned in this work.

Values for the various blood constituents determined at different times throughout the test period are presented in Table 5.

A moderate anaemia was evident at six weeks of age in ducklings maintained on the toxic test ration. This is readily appreciated by comparison of the figures for Hb and H-crit obtained from the two groups of birds. The affected ducks moreover showed evidence of a marked and sustained hypoproteinaemia throughout the experiment, the difference between these and the control birds being particularly clear at eight weeks of age.

The electrophoretic pattern of plasma proteins revealed a striking difference between control and affected ducklings which became more exaggerated as the intoxication progressed. This difference is readily appreciated by studying Fig. 3, 4 and 5 which depict electrophoretograms representative of the two groups of birds.

TABLE 5.—*Ducklings:* mean values for various blood constituents or determinations performed at different times throughout the test period

Blood constituent or determination	Control birds		Birds on toxic mash	
	Age Group	Mean Value	Age Group	Mean Value
Hb (gm %)	6 weeks	10·14	6 weeks	8·07
H-crit (%)	6 weeks	35	6 weeks	25·9
T.P.P. (gm %)	4 weeks 5 weeks 8 weeks	2·9 2·9 4·7	4 weeks 5 weeks 8 weeks	1·5 1·7 1·6
Plasma Aldolase (units/100 ml)	4 weeks 5 weeks 8 weeks	74·5 22·5 49·5	4 weeks 5 weeks 8 weeks	118·0 76·4 55·3
Plasma LDH (units/100 ml)	4 weeks 5 weeks 8 weeks	45 60 45	4 weeks 5 weeks 8 weeks	130 70 300
Plasma GOT (units/100 ml)	4 weeks 8 weeks	31 64	4 weeks 8 weeks	49 120
Plasma GPT (units/100 ml)	4 weeks 8 weeks	131 119	4 weeks 8 weeks	148 226
Plasma Alk. Phosph. (units/100 ml)	4 weeks 8 weeks	33·1 25·6	4 weeks 8 weeks	39·3 38·0

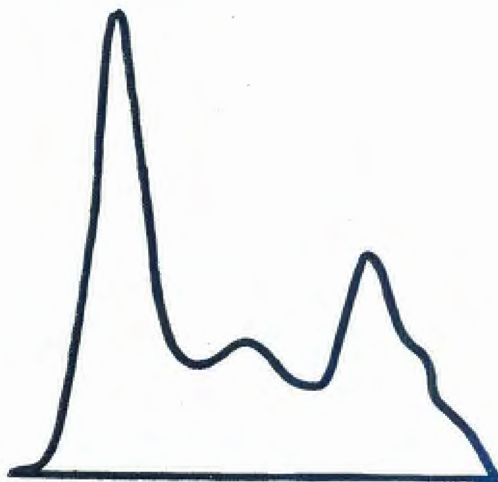

FIG. 3.—Plasma proteins; paper electrophoretogram. Control duckling, 3 weeks of age.

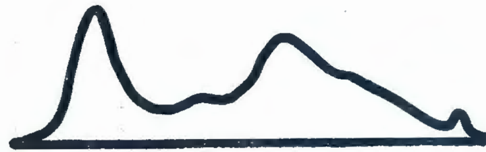

FIG. 4.—Plasma proteins; paper electrophoretogram. Duckling maintained on toxic mash; 3 weeks of age.

FIG. 5.—Plasma proteins; paper electrophoretogram. Duckling maintained on toxic mash; 8 weeks of age.

On considering these figures it is apparent that the hypoproteinaemia noted earlier is due to a marked decrease in all the major plasma proteins, the albumin fraction being particularly reduced towards the end of the experiment.

Values for plasma LDH, GOT and GPT were markedly elevated in affected birds at eight weeks of age. These elevations can be correlated with the severest histopathological lesions (see Table 6). Somewhat higher figures for plasma aldolase and alkaline phosphatase were also found in affected ducklings throughout the test period. The rather high plasma aldolase figures found in these birds at four weeks of age is noteworthy and is associated with similarly elevated LDH values.

In Table 6 the most important biochemical findings are related to the relevant histopathological lesions found in the various age groups as the experiment proceeded.

(*b*) *New Hampshire chickens:* As in the case of the ducklings a preliminary investigation was conducted with a group of New Hampshire chickens receiving toxic meal, and control birds on groundnut free rations to establish which enzyme systems appeared to be significantly affected. This initial study was run concurrently with the pilot experiment on ducklings and included the same enzyme assays. The experiment commenced with day-old chickens and proceeded until they were six weeks old. Tables 7 and 8 represent values obtained from healthy control birds of various ages used in this and the subsequent experiment. The same remarks made in connection with the figures obtained from control ducks apply to these results.

The results obtained from this experiment indicate that in the New Hampshire the enzymes affected were in general the same as those affected in ducks, notably SD, diaphorase, cyto-c-red, DPN-ase, glyc-P-D and cyto-b. In the affected chickens, however, the values found for DPN-ase and cyto-b activity were generally lower in affected birds than in the corresponding controls, while little difference in ATP-ase activity could be discerned between the livers of ducklings in either group throughout the experiment. G-6-P-ase activity, which appeared to be unaffected in ducklings, was in general appreciably higher in affected chickens than in the controls.

Since the differences between the two groups of birds with regard to glyc-P-D, G-6-P-ase and cyto-b activities appeared quite clearcut, these assays were not repeated in the subsequent work and the results obtained are presented in graphic form in Fig. 6, 7 and 8.

TABLE 6.—*Ducklings:* Correlation of salient biochemical features with the relevant histopathology

Age Groups	SD	Cyto-c-red	TPP	Plasma LDH GOT & GPT	Anaemia	Histopathology
1–2 weeks.......	Little difference between groups	Markedly low values in affected birds	Lttle difference between groups	Little difference between groups	Little difference between groups	(1)
3 weeks.......	Generally lower values in affected birds	Low values in affected birds	Lower values in affected birds	do. 1–2 weeks	Affected birds are definitely anaemic	(2)
4 weeks.......	Noticeably lower values in affected birds	Values in affected birds higher than those in controls	Markedly lower values in affected birds	Very high values (LDH) or slightly higher values in affected birds	do. 3 weeks	(3)
5 weeks.......	Markedly low values in affected birds	Values in affected birds much higher than those in controls	do. 4 weeks	Appreciable difference between groups	Anaemia very noticeable in affected birds	(4)
6–8 weeks.......	Markedly low values in affected birds	do. 5 weeks	Extremely low values in aflected birds	All values extremely high in affected birds	Prominent in affected birds	(4)

Note.—The figures in the column for histopathology above refer to the following findings:—

(1) Livers markedly yellow in colour with occasional petechiae. Diffuse fatty changes evident on microscopic examination with very slight bile duct proliferation.

(2) Livers greyish yellow in colour with numerous petechiae. Microscopic examination reveals a tendency towards cirrhotic changes and marked proliferation of the bile ducts. Typical of aflatoxicosis.

(3) Livers are obviously cirrhotic. The histopathology is typical of chronic aflatoxicosis.

(4) Livers show advanced cirrhosis and are enlarged. Many possess a distinct greenish coloured appearance. Gallbladder contains small amounts of light green bile. The histopathology is typical of chronic aflatoxicosis.

TABLE 7.—*New Hampshire Chickens:* Values established for various liver enzymes in different age groups (Given as ranges in units of enzyme/100 mgm of fresh liver)

Age Group	SD	DPN-ase	Cyto-c-red	Diaphorase
7–10 days.......	162–388	0·37–1·05	1·80–2·68	1·80–2·50
14–16 days.......	194–485	0·44–1·2	1·82–3·30	1·64–4·00
21 days.......	194–402	0·60–0·67	1·72–2·78	1·90–2·50
28–30 days.......	162–421	0·30–1·27	1·74–3·18	1·30–6·4
35 days.......	321–485	0·75–1·05	1·70–2·86	2·10–4·80
42 days.......	194–275	0·50–0·68	1·44–3·36	1·60–5·40

TABLE 8.—*New Hampshire Chickens:* Ranges of values established for various liver enzymes at four weeks of age (The figures represent units of enzyme/100 mgm of fresh liver)

Hexokinase	G-6-P-ase	Glyc.-P-D	G-6-P-D	6-P-G-D	G-A-P-D
60·8–104	44–84	160–240	20–60	100–200	23·5–45·4
U-D-P-G-D	ATP-ase	Cyto-b	GR	ICD	
300-450	279–600	0·36–0·68	118–217	6–18	

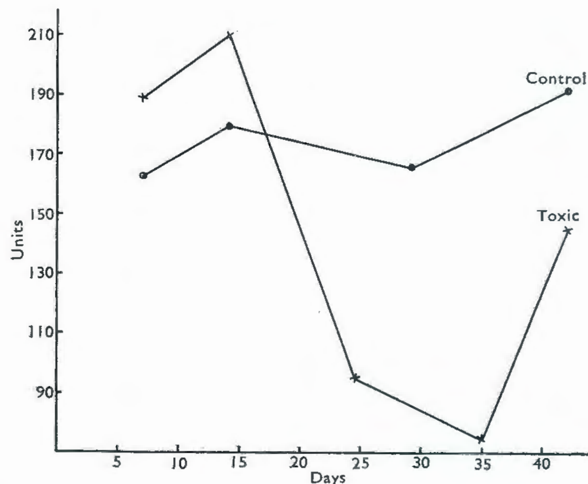

FIG. 6.—New Hampshire chickens: glyc-P-D activity in liver. (Enzyme activity is expressed as units/100 mgm of fresh liver).

On the basis of the results obtained in this preliminary work, a detailed experiment was planned on similar lines to that performed with the latter group of ducklings and to run concurrently with this work. The enzymes selected for study in this experiment were SD, cyto-c-red, DPN-ase and diaphorase.

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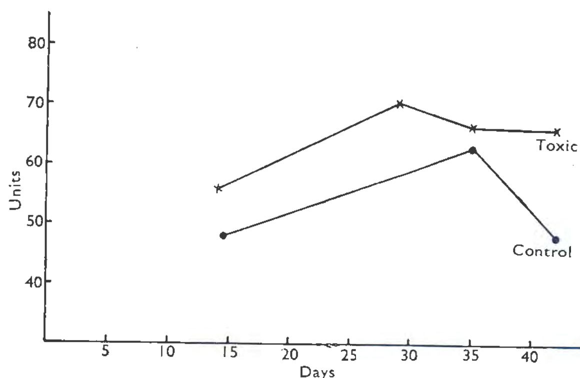

FIG. 7.—New Hampshire chickens: G-6-P-ase activity in liver. (Enzyme activity is expressed as units/100 mgm of fresh liver).

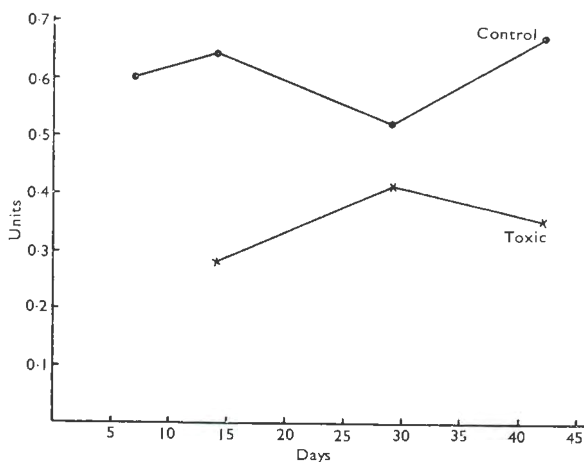

FIG. 8.—New Hampshire chickens: cyto-b activity in liver. (Enzyme activity is expressed as units/100 mgm of fresh liver).

Interpretation of the results was rendered difficult owing to a considerable day to day variation in the figures obtained from the control birds (New Hampshires). The reasons for such fluctuations have been discussed. It was, therefore, decided to express the mean figures obtained from the experimental birds at each individual assay as a percentage of the mean figures obtained from the control birds on that particular day. Before making this calculation the numbers of figures used from experimental and control birds were equalized by random selection from results from the greater group. The results obtained in this manner are presented in Table 9.

A study of this table shows that, as in the case of the affected ducklings, SD activity was markedly depressed in New Hampshire chickens maintained on toxic mash for the first thirty-five days of the test period. Unlike the affected ducklings, however, these chickens revealed markedly lowered liver diaphorase activity particularly during the latter stages of the experiment. The liver cyto-c-red activity was generally lower than in the control birds throughout the test period. Up to thirty-five days DPN-ase activity was also generally lower in the livers of affected birds. It is noteworthy that at forty-two days the values for SD, diaphorase and cyto-c-red appeared to be higher than the values obtained from the control birds of this age group. Whether this is of any significance is difficult to decide at this stage, since the number of birds concerned was small.

TABLE 9.—*New Hampshire Chickens: Liver Enzyme Assays.*—For each particular enzyme the mean values obtained from affected birds of each age group have been expressed as a percentage of the mean value obtained from control birds of the same age group (n = number of birds of each particular age in either affected or control groups. % = percentage of the mean value obtained from control birds of the same age)

Age Group	SD		Cyto-c-red		Diaphorase		DPN-ase	
	n	%	n	%	n	%	n	%
7–10 days.......	7	56	10	98	7	85	7	67
14 days.......	9	91	11	84	11	90	11	83
21 days.......	—	—	2	88	2	90	2	81
28–30 days.......	17	87	14	99	13	64	11	112
35 days.......	7	67	8	75	7	34	2	81
42 days.......	3	110	—	—	3	119	3	143

Note.—A dash in the columns above indicates paucity of information.

Owing to the difficulty encountered in obtaining sufficient blood from these chickens, especially in the early stages of the experiment, the blood studies were limited to Hb, H-crit, TPP and electrophoresis of the plasma proteins, plasma LDH and plasma aldolase. Sufficient figures for comparative purposes were obtained from birds in the three to five weeks age group. These results are presented as mean figures for each group in Table 10.

TABLE 10.—*New Hampshire Chickens:* Mean values for various blood constituents or determinations performed at different times throughout the test period

Blood constituent or determination	Control Birds		Birds on Toxic Mash	
	Age Group	Mean Value	Age Group	Mean Value
Hb (gm %)........................	3 weeks	8·20	3 weeks	7·36
	4 weeks	8·20	4 weeks	7·29
	5 weeks	10·62	5 weeks	8·76
H-crit (%).........................	3 weeks	24	3 weeks	22
	4 weeks	24	4 weeks	19
	5 weeks	24	5 weeks	23
TPP (gm %).......................	3 weeks	3·57	3 weeks	2·52
	4 weeks	3·04	4 weeks	2·53
	5 weeks	3·91	5 weeks	2·49
Plasma Aldolase (units/100 ml).......	3 weeks	76	3 weeks	68
	4 weeks	86	4 weeks	107
	5 weeks	59	5 weeks	56
Plasma LDH (units/100 ml)..........	3 weeks	40	3 weeks	38
	4 weeks	55	4 weeks	85
	5 weeks	100	5 weeks	78

A mild anaemia was again apparent amongst the affected birds. Total plasma protein figures were again lower in the birds on toxic mash than in the corresponding controls. In general little significant difference was found between the plasma aldolase and LDH levels of the two groups except for a moderate rise of both in the plasma of affected birds at four weeks of age.

Paper electrophoretograms of the plasma proteins of affected birds from each age group presented a picture almost identical to that seen in affected ducklings. A marked reduction in all the plasma protein fractions was evident, the albumin fraction being once more particularly affected. Electrophoretograms representative of the different groups of birds are depicted in Fig. 9, 10, 11 and 12.

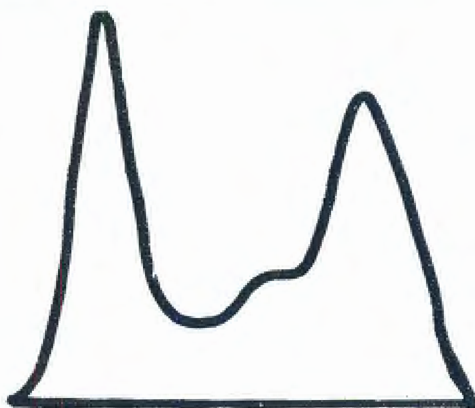

FIG. 9.—Plasma proteins; paper electrophoretogram. Control New Hampshire chicken, 3 weeks of age.

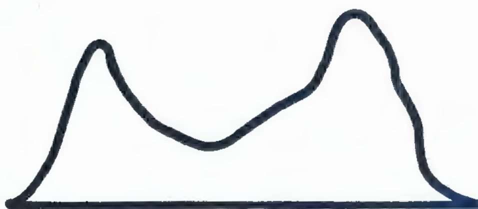

FIG. 10.—Plasma proteins; paper electrophoretogram. New Hampshire chicken maintained on toxic mash; 3 weeks of age.

FIG. 11.—Plasma proteins; paper electrophoretogram. New Hampshire chicken maintained on toxic mash; 4 weeks of age.

FIG. 12.—Plasma proteins; paper electrophoretogram. New Hampshire chicken maintained on toxic mash; 5 weeks of age.

In Table 11 some of the most notable biochemical findings are related to the relevant histopathology found in the various age groups as the experiment proceeded.

TABLE 11.—*New Hampshire Chickens:* Correlation of salient biochemical features with the relevant histopathology

Age Group	SD	Cyto-c-red	Diaphorase	TPP	Anaemia	Histopathology
7–10 days........	Low in affected birds	Little difference between groups	Low in affected birds	—	—	(1)
14 days..........	do. 7–10 days	Low in affected birds	do. 7–10 days	—	—	(2)
21 days..........	—	do. 14 days	do. 7–10 days.	Low in affected birds	Mild in affected birds	(3)
28–30 days.......	do. 7–10 days	Little difference between groups	do. 7–10 days	do. 21 days	do. 21 days	(4)
35 days..........	do. 7–10 days	do. 14 days	Very low in affected birds	do. 21 days	do. 21 days	(4)
42 days..........	Apparently higher in affected birds than in controls	—	Apparently higher in affected birds than in controls	Very low in affected birds	Noticeable in affected birds	(4)

Note.—(*a*) A dash in the columns above indicates paucity of information.

(*b*) The figures in the column for histopathology above refer to the following findings:—

(1) Livers markedly yellow in colour with occasional petechiae. Diffuse fatty changes evident on microscopic examination with very slight bile duct proliferation.

(2) Livers greyish yellow in colour with numerous petechiae. Microscopic examination reveals a tendency towards cirrhotic changes and marked proliferation of the bile ducts. Typical of aflatoxicosis.

(3) Livers are obviously cirrhotic. The histopathology is typical of chronic aflatoxicosis.

(4) Livers show advanced cirrhosis and are enlarged. Many possess a distinct greenish coloured appearance. Gallbladder contains small amounts of light green bile. The histopathology is typical of chronic aflatoxicosis.

(*c*) *Leghorn chickens:* The White Leghorn has been found to be very resistant to the effects of aflatoxin in amounts encountered during natural outbreaks. An experiment to investigate this aspect was planned to run concurrently with the initial work on ducklings and Hampshires. The birds received the same toxic mash as the New Hampshire chickens and an equal number of control birds of the same age was given a groundnut free ration.

No significant differences in enzyme activity were observed between the birds on toxic mash and the controls. Very little day to day variation was encountered with any particular enzyme assay in any of the birds and the levels of enzymes in the liver remained remarkably constant throughout the growth period covered, viz. from hatching to five weeks of age. In Table 12 are presented values found in four-weeks old Leghorn chickens for those liver enzymes which showed significant variations in either ducklings or Hampshires. At this age, as has been demonstrated, the latter birds show the most striking changes.

TABLE 12.—*Leghorn chickens aged four weeks:* Liver enzyme activities in birds on toxic mash and control birds

Enzyme	Range in control birds	Range in birds on toxic mash
SD	226–291	252–324
Cyto-c-red	1·88–2·58	1·3 –2·48
Diaphorase	1·2–2·6	1·2–2·4
DPN-ase	0·52–0·90	0·45–0·90
Glyc-P-D	160–220	140–220

Note.—The figures in the columns above represent units/100 mgm of fresh liver.

Similarly plasma protein electrophoretograms failed to demonstrate any significant variations between the test birds and the controls nor did the relevant blood chemistry and histopathology reveal anything of note.

(*d*) *Cornish Game, Cornish Game × White Rock and Rhode Island Red Chickens:* As already stated, these breeds, together with the Leghorn, appeared to be far more resistant to aflatoxin poisoning than were New Hampshire chickens or ducklings. In an attempt to explain such differences, the liver enzymes of a limited number of normal birds of the above breeds were examined. These birds were kept on aflatoxin-free rations and most were examined at four to five weeks of age. No differences between the susceptible and non-susceptible breeds have been detected.

Ducklings provide the only notable peculiarity in that at four to five weeks of age they present values for G-6-P-D and 6-P-G-D far higher than seen in any of the breeds of chickens. This possibly implies a very active pentose monophosphate pathway in the liver of ducklings. Values for liver ICD are similarly higher in ducks than in other birds.

(*e*) *Experiments to demonstrate recovery of affected enzyme systems:* At various times throughout the experiments described above, individual ducklings or New Hampshire chickens were removed from the toxic mash and placed on the standard groundnut-free rations consumed by the control birds. In most instances a rapid

return to the control level of activity was shown by those enzymes particularly affected by aflatoxin. Table 13 is representative of the results obtained in these experiments. Only the results of SD and cyto-c-red are shown. Such chickens, when replaced on the toxic diet, again showed abnormal activity values for these enzymes within ten days.

TABLE 13.—*Recovery experiments in ducklings and New Hampshire chickens* (The results shown represent assays from individual birds aged four to five weeks, treated as indicated in the table, the figures being units of enzyme/100 mgm of fresh liver)

	SD		Cyto-c-red	
New Hampshires—				
Control birds	260,	291	3·14,	2·38
Birds on toxic mash	162,	194	0·94,	1·08
Birds removed from toxic mash for 1 week	275,	226	2·78,	2·64
Ducklings—				
Control birds	259,	356	1·10,	2·30
Birds on toxic mash	194,	178	0·64,	0·80
Birds removed from toxic mash for 1 week	226,	226	2·50,	2·00

(*f*) *Investigations into the possible toxicity of meat, milk or eggs derived from animals or birds receiving toxic rations:* Allcroft & Carnaghan (1963), and de Iong, Vles & van Pelt (1964) reported that a metabolite of aflatoxin was present in the milk of cows maintained on highly toxic groundnut cake meal or mashes containing 15 per cent of highly toxic cake meal.

The present experiments were planned and executed as follows:—

(i) Friesland cows were given 16 lb of meal per day containing 20 per cent groundnut cake meal which contained 8·0 ppm of aflatoxin. The cows were maintained on this meal for six months. At different intervals during this period, their milk was given to ducklings maintained on a standard groundnut-free ration. Initially each duckling received 50 ml of milk per day. This was increased to 100 ml after the first week and then to 300 ml fourteen days after the commencement of feeding. The ducklings remained on this diet for eight weeks. A control group of ducklings was placed on the identical mash and each duckling received the same amount of milk as the test birds, but in this instance the milk was provided by cows maintained on a groundnut cake-free meal.

At intervals throughout the test period and at the conclusion of the experiment ducklings from both groups were weighed, slaughtered and their livers examined histologically and biochemically as before. No significant differences between the two groups could be established.

(ii) Dried milk powder was prepared from the milk of both groups of cows mentioned above and was fed *ad libitum* for a period of six weeks to two separate groups of ducks. Throughout and at the end of the test periods duck were weighed, slaughtered and their livers examined as above. At no time was any difference discernible between members of the two groups.

(iii) Powdered milk, prepared from milk of the cows on toxic rations as mentioned above, was extracted with methanol and chloroform as described by de Iong, Vles & van Pelt (1964). The dry and powdered extract so obtained was fed to ducklings for four days without producing any visible histological lesions or any of the biochemical disturbances noted earlier. Thin layer chromatograms performed on this concentrated preparation revealed no sign of aflatoxin or its metabolites.

(iv) A group of hens was maintained on a ration with an aflatoxin content of 0·5 ppm for six weeks. After this period eggs were collected from these hens and fed to a further group of test ducklings, each bird receiving two eggs per day for a period of eight weeks. Daily for the same period a similar group of ducklings received two eggs obtained from hens maintained on a groundnut cake-free ration. Besides the eggs, both groups of ducklings were given the standard groundnut cake-free mashes used throughout this work.

Throughout this experiment and at its conclusion test and control ducklings were weighed, slaughtered and their livers examined histologically and biochemically. No differences between the two groups of birds were discernible.

(v) Muscle tissue taken from ducklings with biochemically and histopathologically confirmed chronic aflatoxicosis was finely minced and dried in air in a dark room overnight. Ten per cent of the duckling meat was included in a groundnut-free mash fed to New Hampshire chickens. In addition to this mash the dried minced meat was freely available to the test chickens throughout the experiment. Control birds were maintained on the standard groundnut-free mash. The same procedure regarding slaughter and examination of test and control birds was adopted. At no time were any significant differences between test and control birds observed.

It is clear from the results of these experiments that, from the practical point of view, aflatoxin or its metabolites are not present in any significant amounts in poultry or dairy products obtained from birds or cows fed on highly toxic rations. With regard to the feeding of meat and eggs, our findings confirm the observations of Allcroft & Carnaghan (1963) but are not in accordance with those of Allcroft & Carnaghan (1963), and de Iong *et al.* (1964) as regards milk. A possible reason for this discrepancy lies in the fact that various breeds of cows may react in different ways to the effects of aspergillins as is the case with New Hampshire and White Leghorn chickens.

(*g*) *Selenium metabolism in mycotoxicosis:* During the course of investigations into mycotoxicoses other than syndromes evoked by *Aspergillus* spp., it was found that in all instances the livers from affected birds contained fairly high levels of selenium. This is true for *Alternaria* and *Fusarium* spp. Most birds studied showed liver selenium levels between 10 to 25 ppm whereas in normal birds under field conditions in this country values of between 0·5 to 3 ppm are generally encountered. Selenium levels in the rations fed were generally low, e.g. *circa* 1 to 4·5 ppm.

The significance of this finding is not clear at this stage. The same observations were made in individual birds from the different experiments in which mashes containing aflatoxin were fed. Elevations of liver selenium were encountered in both ducklings and chickens. Since this phenomenon is not associated with an increased dietary intake of selenium, it is possibly linked to some disturbances in the intestinal absorption or metabolic uses of this element leading to increased hepatic storage. Further investigations into this aspect of mycotoxicosis are in progress at present.

(*h*) *Studies on the mitochondria of livers from New Hampshire chickens or ducklings affected by aflatoxin:* The mitochondria in avian liver cells when stained with iron haematoxylin, carbol-acid fuchsin, light green or Mallory's phosphotungstic acid-haematoxylin, normally show up as minute granules surrounded by a clear halo refractory to staining. The nature of this halo was not determined in the present study.

In acutely affected chicken livers the central granule and its surrounding halo are about twice as large as normal. These changes are seen mostly in cells at the periphery of lesions. Some cells within the lesions show only slightly swollen mitochondria, while others appear normal.

In chronic cases of the disease the increase in size of the mitochondria of affected cells is more obvious (generally about a three-fold increase in size) and all liver cells appear to be similarly affected.

No evidence of disruption of the double membrane structure of affected mitochondria could be seen with the oridinary light microscope. As these bodies are just about at the limits of resolution of this instrument, it is obvious that they will have to be studied by electron microscopy.

In the normal cell of the duckling liver the mitochondria are distributed in a regular fashion throughout the cytoplasm and have the same appearance with the different staining techniques used, as those of chicken liver cells.

A two-fold enlargement of the mitochondria is visible in liver cells of affected ducklings but, unlike the affected chicken livers, there appears to be a marked aggregation of these bodies in that area of the affected cells nearest to the bile canaliculi. A distinct increase in the number of mitochondria could also be seen in some of the liver cells bordering on obviously affected cells.

Discussion

Although the chemistry of the aspergillins and their possible role as carcinogens have evoked universal interest, a search through the available literature has brought very little to light regarding their possible mode of action on the metabolic systems of cells in general.

From the results obtained in the experiments described above it may be inferred that the primary site of action of these toxins is on metabolic systems located in the mitochondria and particularly on dehydrogenases like SD and Glyc-P-D, the electron transfer chain and oxidative phosphorylation mechanisms. It is noteworthy that in both ducklings and New Hampshire chickens there is little evidence of disturbances in glycolysis via the direct Embden-Meyerhof pathway. Most, but not all, of the glycolytic enzymes are located in the cytoplasmic sap except in the brain, the mitochondria of which contain a full complement of glycolytic enzymes and co-factors (Gallagher, 1964). Although some of the enzymes and co-factors of cellular respiration occur in other parts of the cell as well as in mitochondria, it is only in the latter structures that all of the respiratory enzymes, co-enzymes, co-factors and associated electron transfer systems occur together and only the mitochondria can perform the vital function of oxidative phosphorylation.

Whether the lowered activities of SD cyto-c-red, DPN-ase, cyto-b, etc., are due to direct physical damage of the mitochondria, uncoupling of oxidative phosphorylation or direct inhibition of certain members of the electron transfer chain is not at all clear at the moment. Sporidesmin, the toxin produced by *Pithomyces chartarum,*

rapidly inactivates mitochondrial dehydrogenase systems requiring pyridine nucleotides as hydrogen acceptors by inducing loss of these through a powerful physical effect on mitochondria, causing them to swell and disrupt. In rats poisoned with sporidesmin there is in addition to the loss of respiratory activity an inhibition of succinoxidase activity (Gallagher, 1964). The mechanism of cytotoxicity of this fungal product is thus probably inactivation of mitochondrial enzymes resulting in respiratory failure in affected cells.

The cyto-c-red studied in this work is DPN dependent, and hence probably mainly mitochondrial (Gallagher, 1964). This enzyme is known to be particularly sensitive to members of a group of highly selective inhibitors of the electron transfer process, e.g. amytal and antimycin A. The latter is the most efficient inhibitor yet described (Dixon & Webb, 1958; Greenberg, 1960).

Whatever the exact mode of action of aflatoxin may be, the net effect observed in this work is severe interference with mitochondrial dehydrongenase activity. Since the systems concerned are responsible for the generation of by far the largest part of the cell's ATP and since this is required to " drive " energy requiring reactions catalyzed by enzyme systems of other cellular components *int. al.* the synthesis of proteins by microsomes, it follows that these reactions will be severely handicapped by the mitochondrial damage induced. An outstanding example of this is the marked interference with protein synthesis observed in the experimental birds used in this work. This is reflected not only in poor growth and failure to gain weight in affected birds (see Plate 1) but also in the electrophoretic patterns of the plasma proteins.

The electrophoretograms presented for study in Figures 3, 4, 5, 9, 10, 11 and 12 are possibly the most interesting results obtained in this investigation, since they reveal pertinently one of the most important consequences of this intoxication. It is significant that the total plasma protein figures given in Tables 5 and 10 do not show a decrease as the severity of the lesions increases, but tend rather to remain at a constant low level. The TPP figures of the control birds, on the other hand, reveal a slow increase as the birds become older. On studying the electrophoretograms from affected birds the impression is gained that the hypoproteinaemia mentioned earlier is due to a simultaneous reduction in all the plasma protein fractions, in particular the albumins. That the latter should be the most severely reduced is consistent with the advanced liver lesions.

Smith (1963) has demonstrated an inhibition of the incorporation of ^{14}C leucine into proteins using low concentrations of purified aspergillins and rat and duckling liver slices incubated in a Kreb's medium. One is tempted, from the results presented in this paper, to entertain the thought that protein synthesis is in general severely depressed with the essential disturbance probably a failure in, or a markedly reduced rate of ATP synthesis.

The anaemia varying from mild to moderate in the affected experimental birds and severe in natural cases of the disease may also be a reflection of this disturbance of protein synthesis. Since it is generally held that there is a preferential use of dietary amino acids for haemoglobin synthesis, one could reasonably expect the lowered level of protein synthesis to be the result of bias in favour of Hb synthesis. This may give rise to the apparent discrepancy in the severity of the anaemia and hypoproteinaemia as seen in experimental birds. It could be argued with equal force that this anaemia is a logical consequence of the severe hepatic lesions seen in these birds. Further studies will have to be made before this question can be satisfactorily answered.

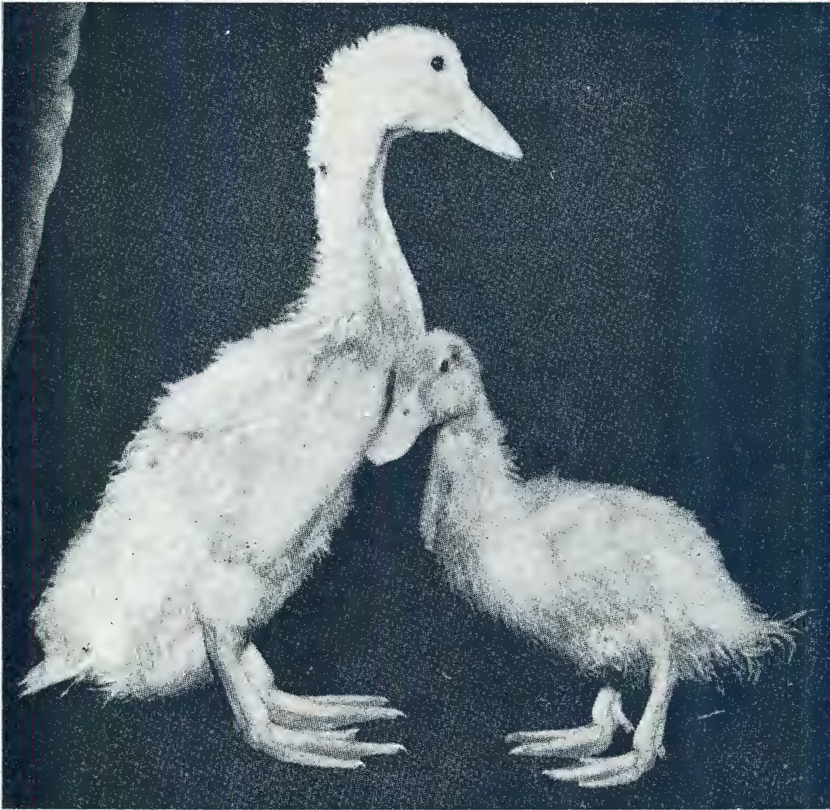

PLATE 1.—Control and affected ducklings (4 weeks of age).

Leveille, Fisher & Feigenbaum (1961) have presented interesting data demonstrating that in chickens the level of serum protein albumin and the A/G ratio bear a direct relationship to the level of protein intake. They have shown that variations in serum protein levels are almost entirely due to changes in the albumin level, " the globulins being rather stable and showing little or no variation ". They concluded that decreased A/G ratios in the chicken indicate a certain degree of depletion of protein stores. The following quotation from the work of these authors is most pertinent to this discussion: " Protein reserves play a vital role in an animal such as the hen, which has such a large protein turnover in the form of eggs ".

That the changes observed in our work are unrelated to a lack of dietary protein may be assumed from the fact that the ration received by both test and control birds was identical with the exception that aflatoxin containing groundnut cake meal was added to the ration of the former group of birds. The mash fed to all control birds was groundnut-free and therefore of lower protein content. Furthermore, no birds, except those that became moribund, ever showed signs of anorexia.

According to the generally accepted tenets of chemical pathology one would expect a markedly decreased A/G ratio with the albumins markedly decreased and a relative or absolute increase in the globulin fractions in animals with severe hepatic lesions of at least one month's standing. It is obvious from these studies that this is not the case in avian aflatoxicosis.

It is believed at this stage that the electrophoretic pattern of the plasma protein in affected birds is possibly unique in avian pathology and one which could have considerable diagnostic significance. Once this finding was found to be consistent in all our experimental birds, plasma from cases of a wide variety of diseases was examined to establish whether this electrophoretic pattern was indeed peculiar to aflatoxicosis. The various birds examined had been submitted to the Poultry Section of this Institute for purposes of diagnosis. Cases selected for study were typical of the various diseases encountered. In none of these diseases has anything been observed so far that even remotely resembles the plasma protein electrophoretic pattern seen in aflatoxicosis. Figures 13 to 17 depict electrophoretograms representative of the findings in the various avian diseases studied and are reproduced here for comparison with those from our experimental birds. The selection of figures presented below includes diseases of mycotic, viral, bacterial or neoplastic origin. Figure 13 is of special interest since it portrays the plasma protein pattern in respiratory tract aspergillosis with mycotic lesions in the turbinate bones and interior nares. A severe secondary bacterial infection of the lesions was present. There appears, on inspection of this figure, to be some decrease in the albumin fraction. This may be relative to the increased globulins, or absolute.

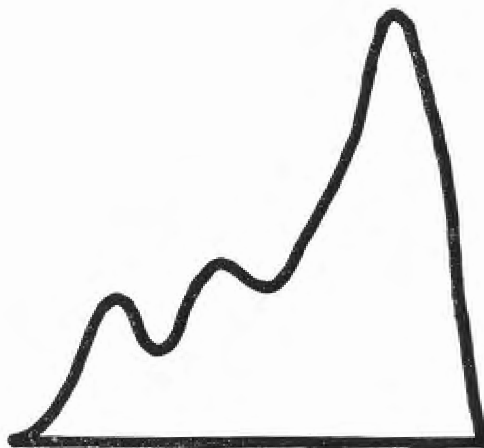

FIG. 13.—Plasma proteins; paper electrophoretogram. Cornish Game x White Rock chicken. *Aspergillus* infection of the turbinate bones and interior nares, with secondary bacterial infection of the lesions.

A possible important sequel to the suppression of globulin synthesis apparent in aflatoxicosis is that affected birds may be rendered more susceptible to paratyphoid and other infectious diseases. Mention of *Salmonella* infections being present in typical cases of aflatoxicosis has been made in the introduction to this paper. *Salmonella* spp. have been isolated by one of us (L.A.) from typical cases of aflatoxicosis amongst ducklings and chickens.

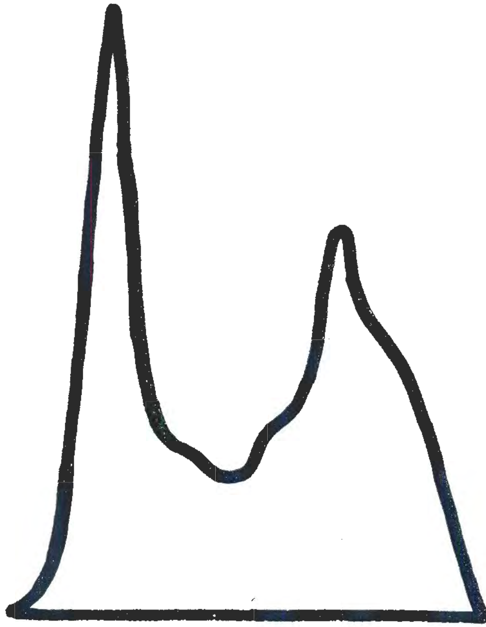

FIG. 14.—Plasma proteins; paper electrophoretogram. Leghorn chicken. Infectious bronchitis.

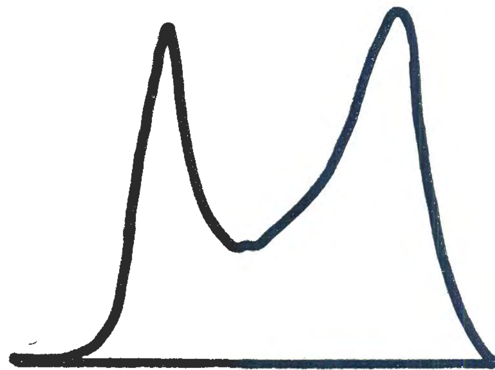

FIG. 15.—Plasma proteins; paper electrophoretogram. Leghorn chicken. Chronic respiratory disease (avian mycoplasmosis).

It is suggested that, until proved otherwise, determinations of TPP, paper electrophoresis of plasma proteins and assays of liver SD and cyto-c-red activity may prove valuable diagnostic aids in instances where aflatoxicosis is suspected, especially prior to structural changes being evident in the liver. It is also possible that this work could serve as the basis for a useful biological assay for the aspergillins. Such a use for this work has been demonstrated in the experiments described earlier in connection with the feeding of dairy or poultry products to test birds.

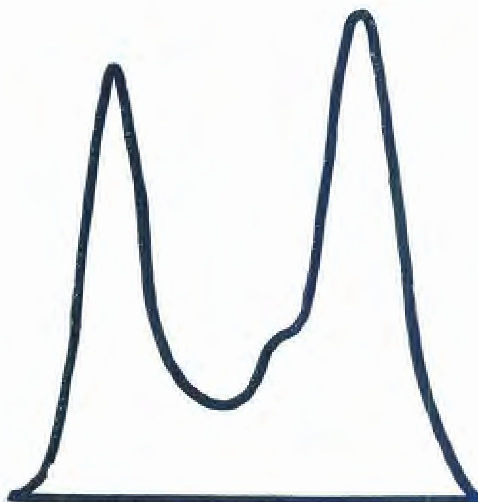

FIG. 16.—Plasma proteins; paper electrophoretogram. Leghorn chicken. Typhoid.

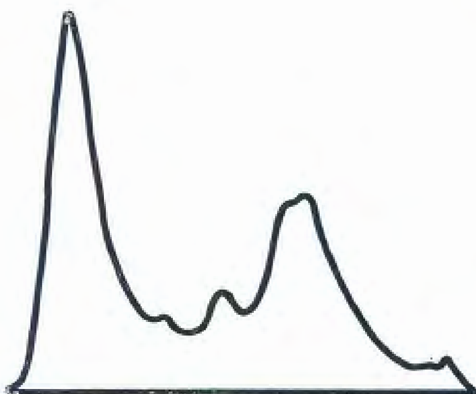

FIG. 17.—Plasma protein; paper electrcphoretogram. Leghorn chicken. Abdominal lipomatosis.

The marked elevation of the plasma enzymes LDH, GOT and GPT in affected ducklings, particularly at eight weeks of age, is noteworthy, especially in view of the alleged role of aflatoxin in carcinogenesis. While this can be correlated with maximum hepatic injury, it is of interest to study some recent findings of Wróblewski (1958) with regard to LDH. In humans and dogs, conditions involving tissue necrosis, e.g. myocardial infarction, muscle trauma, necrotizing pancreatitis and fulminant haemolytic states are associated with markedly elevated plasma LDH activity. However, in inflammatory-necrotic diseases of organs rich in LDH, plasma values are increased minimally or not at all, e.g. homologous serum hepatitis (which may be associated with huge increments in plasma GOT activity). Elevations of plasma LDH activity have been noted in experimentally induced leukaemia and in rodents suffering from transplantable carcinoma or sarcoma. These alterations occur in the absence of demonstrable tissue necrosis and appear in mice affected

by some malignant mouse tumours within 6 to 48 hours after transplantation of tumour tissue. The LDH content of the tumours themselves was too low to account for the plasma elevations noted. Rapid growth of non-malignant tissue in mice, e.g. regenerating liver tissue following subtotal hepatectomy, does not result in increased plasma LDH values.

Regarding the elevations noted in cases of tissue necrosis Wróblewski (1958) states: " It would appear that serum LDH activity alterations associated with tissue necrosis are influenced by the amount of enzyme present in the necrotic tissue, the extent of the necrosis and the rapidity with which it occurs, the rate of loss of enzyme from the tissue and the accessibility of lost enzyme to the circulation and the normal range of serum LDH activity ".

The elevation of plasma aldolase activity in the first 3 to 4 weeks of the intoxication in both ducklings and Hampshire chickens is similarly of interest. Sibley (1958), in a discussion of the significance of plasma aldolase levels, makes the following points clear: Increased levels of aldolase activity are found in the plasma of tumour-bearing rats and in that of some human patients suffering from malignancy. The values usually return to normal after excision of the tumour. Markedly increased plasma levels are found in a wide variety of conditions associated with tissue necrosis, e.g. early acute hepatitis, massive pulmonary infarction, extensive peripheral gangrene, acute haemorrhagic pancreatitis, progressive muscular dystrophy and liver necrosis in rats following inhalation of carbon tetrachloride. The following passage from Sibley's paper is most pertinent: " An abnormally high serum aldolase content results from the rather sudden injury of many cells. It is probably more exact to speak of tissue injury, which would include relatively mild and sometimes reversible changes in the cell membrane, rather than to use the term necrosis, which implies a destruction of cells demonstrable by histological examination. This concept would explain those instances of elevation in serum enzyme values without finding gross or miscroscopic evidence of necrosis of some tissue ". With the rapidly increasing use of electron microscopy in work of this nature, it will soon be possible to define more accurately Sibley's concept of tissue injury and shed more light on some of the intriguing aspects of aflatoxicosis revealed by this work.

While the role of aflatoxin as a carcinogen in the amounts encountered in practice must await further elucidation, it seems reasonably certain from the studies described in this paper that the elevated plasma levels of LDH and aldolase activity encountered in our affected birds resulted directly from injury to hepatic cells rather than from any incipient neoplasia.

A number of unanswered questions arise from these investigations, e.g. why the cyto-c-red activity apparently increases in the livers of affected ducklings from about four weeks of intoxication onwards whilst the activity levels of other affected enzymes remain consistently low; the apparent disturbances in selenium metabolism which have been seen and the precise nature of the essential biochemical differences between susceptible and non-susceptible breeds or species. This investigation has so far shed little light on this latter intriguing aspect of the problem.

From the practical point of view, the ability of birds affected by rations in the order of toxicity used in this work to recover rapidly when placed on toxin free rations, is interesting and important. Equally interesting is the rapid reappearance of the typical biochemical disturbances following reintroduction to toxic mashes. The lesions are reversible provided massive hepatic injury is not present and impairment of growth is minimal. Aflatoxin appears to have little or no cumulative effect and no resistance to the effects of this toxin appears to be built up during sustained exposure to it over the period of greatest susceptibility.

Summary

Biochemical studies have been made on liver tissue and blood from ducklings and various breeds of chickens maintained on standard rations containing 0·5 ppm of aflatoxin B_1. The results obtained from these birds were compared with those from birds of the same age maintained on groundnut-free mashes. Of the various breeds of chickens studied, only New Hampshires have been found to be susceptible to the effects of this level of aflatoxin.

In the livers of affected chickens and ducklings a marked decrease in the activity of certain mitochondrial dehydrogenases and enzymes of the electron transfer chains or oxidative phosphorylation mechanisms is apparent. Mild to moderate anaemia, severe hypo-proteinaemia and grossly abnormal plasma protein electrophoretograms are presented by affected birds. It is believed that the suppression of protein synthesis observed and affecting the albumin fraction in particular, is due to a lowered rate of ATP synthesis consequent to mitochondrial injury. An increase in the activity of certain plasma enzymes, notably those of lactic dehydrogenase, aldolase and glutamic-oxalacetic- or glutamic-pyruvic transaminases, is also seen in these birds. This increase in the activity of plasma enzymes can be correlated with the severe hepatic lesions. The use of these various studies in the diagnosis of this condition, before hepatic lesions are evident, has been suggested.

The ability of affected birds on rations containing 0·5 ppm of aflatoxin to recover rapidly when placed on toxin free rations is noted. Aflatoxin at these levels appears to have no cumulative effects, but no resistance is built up to its actions by tissues of birds exposed to it for long periods.

Susceptible birds revealed no evidence of aflatoxicosis after prolonged feeding of dairy or poultry products obtained from cows or fowls kept on highly toxic rations. From the practical point of view there seems to be little danger inherent in the dietary use of such essential foods.

Acknowledgements

We owe a special debt of gratitude to our professional assistants Adriana Wagner, Anna Brink and Martha Potgieter on whom fell the huge task of doing the hundreds of enzyme assays involved in this work, the plasma protein electrophoreses and the blood studies. Dr. R. C. Tustin performed the histopathological studies and Mr. W. H. Gernecke the histological work on liver mitochondria. We have incorporated his report almost verbatim in this paper. To these officers of this Institute we are most grateful, including also Mr. J. H. Minné of the Department of Toxicology of this Institute who carried out assays of aflatoxin on the materials used.

In conclusion we recall with thanks the valuable parts played by our technicians P. J. de Wet and E. Herne in this investigation, the former in the large number of selenium analyses done and the latter in the organisation and routine work inherent in all our experiments.

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