Studies on the Effect of Aflatoxin B_1 on the Development of the Chick Embryo*† (32816)

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The acute and carcinogenic effects of aflatoxin B_1 in numerous species of adult animals have been well documented (1). In the case of fertile hens' eggs, studies have been concerned with the use of the mortality of the embryo as an index of toxicity of the aflatoxins (2). It was reported that examination of the nonsurviving embryos revealed severe growth retardation. In addition, edema, hemorrhage, underdevelopment of the mesencephalon, mottled and granular liver surface, short legs, and slight clubbing of the down were observed in many of these embryos.

This study is concerned with the effect of sublethal doses of aflatoxin on the development of the embryo and on certain enzymes; namely, acid phosphatase, alkaline phosphatase, glutamic-oxalacetate transaminase (GOT). Considerable information is available describing and analyzing changes in enzymatic activities during development of the chick embryo (3, 4), and showing how these changes can be correlated with the development of cellular function and anatomical development. Changes in the pattern of enzyme development in the embryo may be demonstrative of subtle effects arising from the administration of a toxic substance. The study of enzymes characteristic of organs or specific subcellular entities can localize these effects.

Materials and Methods. Chicken eggs were obtained from a commercial supplier and incubated at 100.5°F and 84.5% humidity for days 1–19, and 100.5°F and 95% humidity for days 19–21. The viability of the untreated eggs was consistently over 90%, and their age was uniform as judged by hatching within a 12-hour period on day 21. The ages given for the embryos refer to the days of incubation.

Five concentrations of aflatoxin in propylene glycol, ranging down from 3 μg/ml in 0.4 log intervals, and the propylene glycol alone were injected. The injected solutions were autoclaved; and injections were made with sterile precautions into the air space of the egg through a hole drilled with a dental drill and sealed with scotch tape. The viability of the embryos was determined by daily candling. For enzyme assays, living embryos, livers, or gonads dissected from living embryos were weighed and kept in an ice-cold 0.25 *M* sucrose solution until homogenization. Pools of five embryos or five organs were used for the preparation of homogentates. Homogenization was carried out using the Potter Elvejhem Homogenizer. The homogenates were strained through cheese cloth and diluted to a suitable concentration for enzyme assay (about 1 mg of protein/ml). Acid phosphatase (pH 5.0) and alkaline phosphatase (pH 10.0) were measured with *p*-nitrophenol phosphate as substrate. The enzyme action was stopped by the addition of 2% (w/v) phosphotungstic acid in 0.1 *N* HCl. After removal of the precipitated protein the *p*-nitrophenol released was determined by addition of NaOH to the supernatant. Glutamic-oxalacetate transaminase was determined by the Karmen method (5). Protein concentration was determined by the Miller method (6).

Results and Discussion. In the first experiment, the toxicity of the aflatoxin B_1 injected into the air cell of the chick embryo was determined as shown in Table I. An examination was made of all surviving embryos on day 19 for gross external morphological effects. The surviving embryo from Treatment I showed severe growth retardation and a bloated stomach. Under Treatment II, 13 surviving embryos showed growth retardation; and of these, 11 had bloated stomachs. For 43 survivors of Treatment III, there was no evidence of growth retardation, and only 4 embryos had bloated stomachs. All embryos

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TABLE I. The Effect of Aflatoxin B_1 on the Viability of the Chicken Embryo. Fertile hens' eggs were injected into the air sac with 0.04 ml of a solution of aflatoxin B_1 in propylene glycol. Viability was determined by candling.

Treatment no.	Aflatoxin (μg/ml)		Days of incubation 3	4	5	6	7	10	13	17	18	19	Total	Percent
I	3.000	alive	45	22	9	5	3	3	3	1	1	1	1	2
		dead	2	23	13	4	2	0	0	2	0	0	46	98
II	1.190	alive	39	38	36	34	32	32	30	20	15	13	13	32
		dead	1	1	2	2	2	0	2	10	5	2	27	68
III	0.476	alive	45	44	44	44	43	43	43	43	43	43	43	96
		dead	1	0	0	0	1	0	0	0	0	0	0	4
IV	0.189	alive	49	49	49	49	49	49	49	49	49	49	49	98
		dead	1	0	0	0	0	0	0	0	0	0	1	2
V	0.075	alive	48	47	47	47	47	45	45	45	45	45	45	90
		dead	2	1	0	0	0	2	0	0	0	0	5	10
VI	Propylene glycol	alive	43	42	42	42	42	41	41	41	41	41	41	95
		dead	0	1	0	0	0	1	0	0	0	0	2	5

from Treatments IV, V, and VI appeared normal.

In the second experiment, eggs were treated with aflatoxin according to Treatment II, Table I (1.190 μg/ml or 0.0476 μg/egg). Each point in Figs. 1–4 represents at least five replications, and each replication is from a pool of five embryos or five organs. The differences discussed below are highly significant statistically.

Figure 1 shows the effect of this dose of aflatoxin (0.0476 μg/egg) on the development of the embryo, liver, and gonads. The embryos of the aflatoxin-treated eggs showed the same rate of increase in weight as the controls up to day 12 of incubation, but the subsequent weight gain of the treated embryos was less than that of the controls. On day 19, embryos from the aflatoxin-treated eggs were only 60% of the weight of embryos from control eggs. Livers from embryos of aflatoxin-treated eggs showed a similar decrease in weight gain from day 12 in comparison to the controls (Fig. 1). At day 19, the weight of the livers from embryos of aflatoxin-treated eggs was 50% of the controls' weight (Fig. 1). Gonads from the test embryos did not differ greatly in weight from controls at any time during the period of development. Propylene glycol alone did not have any effect on rate of weight gain of the embryo, liver, and gonads (Fig. 1).

Only minor changes in the pattern of development of the enzymes studied (acid phosphatase, alkaline phosphatase, and GOT) in the whole embryo, liver, and gonads were noted. In the case of acid phosphatase (Fig. 2), the enzyme activity of the whole embryo was slightly elevated between days 10–12 and also from days 13–16. Gonadal acid phosphatase was elevated on days 12–13 and also on days 14–18. No changes were observed in the acid phosphatase activity of the liver.

Alkaline phosphatase activity (Fig. 3) was slightly increased in the embryo from days 12–14 and 14–16; in the liver from days 12–14 and 18–19; and in the gonads from days 12–14. Glutamic-oxalacetate transaminase activity (Fig. 4) showed an increase in the whole embryo from days 3–17, but was slightly depressed in the liver from days 8–11 and 11–14. There does not appear to be any direct correlation between the slight changes in the pattern of enzyme development in the embryos of treated eggs and the more marked changes in the growth of the embryos. The minor enzymatic changes did not appear to follow any definite time course.

The main finding of this study is that treatment of fertile hens' eggs with a sublethal

dose of aflatoxin B_1 causes a depression in the growth of the embryo. This effect appears at day 12 of development and continues until hatching. This fact, coupled with the lack of gross morphological abnormalities, suggests that the effect is primarily on the growth of the embryo rather than on the early stages of differentiation. The decreased weight of the embryo is paralleled by a decreased liver weight, although the ratios of liver weight to body weight of both treated and control animals were not markedly different. There were

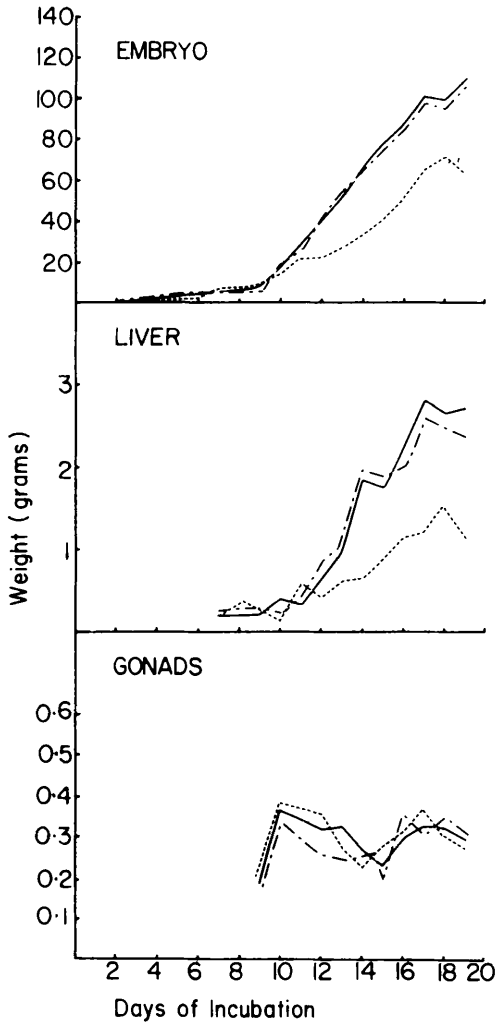

FIG. 1. Effect of aflatoxin B_1 (.0476 μg/egg) on the weight gain of the whole embryo, liver, and of protein. ——— control; -•-• propylene glycol; and - - - aflatoxin B_1.

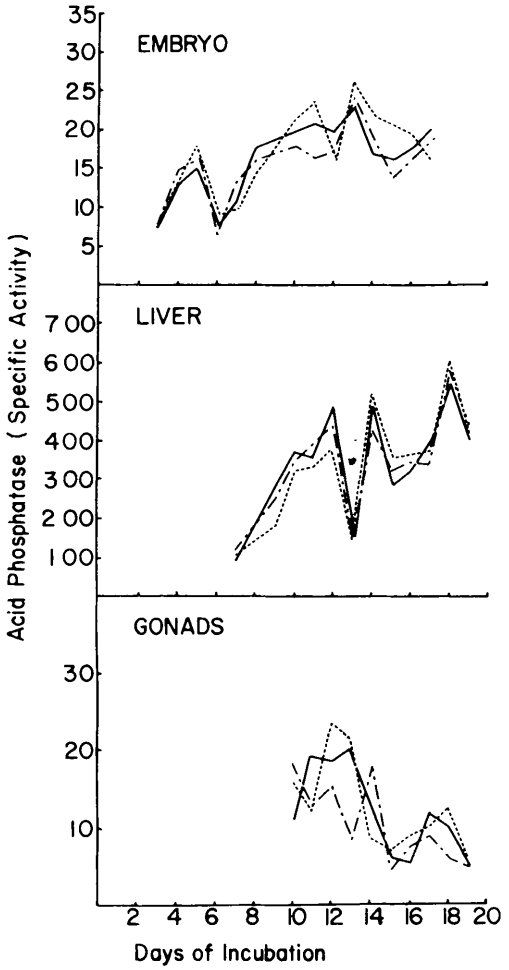

FIG. 2. Effect of aflatoxin B_1 (.0476 μg/egg) on the development of acid phosphatase activity in the whole embryo, liver, and gonads. Units of specific activity is mμmoles of substrate hydrolyzed per 30 min per mg of protein. ——— control. -•-• propylene glycol; and - - - aflatoxin B_1.

no histological abnormalities of the liver on day 13. However, the increased alkaline phosphatase noted during this period (Fig. 3) may be indicative of minor damage in liver function.

The depressed or stunted growth of embryos may be characteristic of the action of aflatoxin on embryonic development. Similar results have been reported with rats, where a sublethal dose administered in early pregnancy produced no effects on the fetus, but fetal growth was retarded when the aflatoxin

was given on day 16 of pregnancy (7). No histological changes were observed in the livers of these fetal rats.

Summary. Surviving embryos of fertile hens' eggs injected with an effective dose (approximately LD_{70} of aflatoxin B_1 showed a marked depression in growth only after day 12 of development. Surviving embryos did not show any gross morphological abnormalities. The pattern of development of several enzymes (acid phosphatase, alkaline phosphatase, and GOT) in the aflatoxin-treated eggs showed only minor differences from that of the controls. An effective dose (approximately LD_{70} of aflatoxin B_1 does not affect the early stages of differentiation and growth, but does depress growth after day 12 of development.

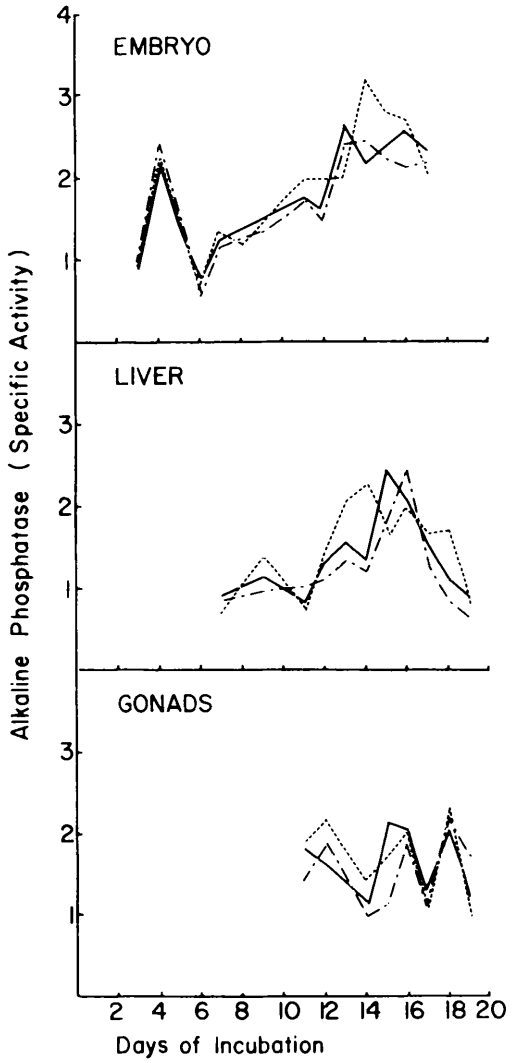

FIG. 3. Effect of aflatoxin B_1 (.0476 μg/egg) on the development of alkaline phosphatase activity in the whole embryo, liver, and gonads. Unit of specific activity is mμmoles of substrate hydrolyzed per 30 min per mg of protein. ——— control. –•–• propylene glycol; and – – – aflatoxin B_1.

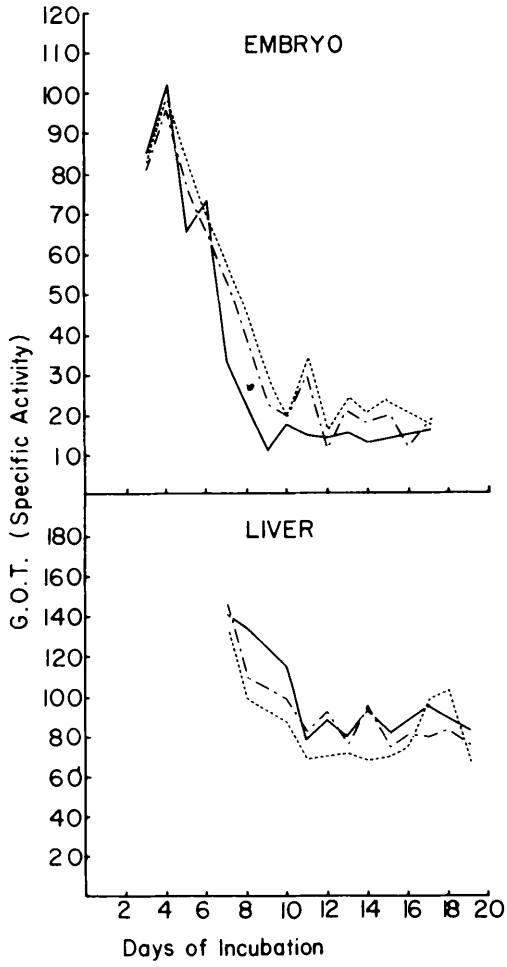

FIG. 4. Effect of aflatoxin B_1 (.0476 μg/egg) on the development of GOT activity in the whole embryo and liver. Unit of specific activity is mμmoles of substrate utilized per 30 min per mg of protein. ——— control; –•–• propylene glycol. and - - - - aflatoxin B_1.

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1. Wogan, G. N., Bacteriol. Rev. 30, 460 (1966).
2. Verrett, M. J., Marliac, J. P., and McLaughlin, J. Jr., J. Assoc. Offic. Agr. Chemists 37, 1003 (1964).
3. Moog, F., in "Embryonic Nutrition," D. Rudniek, ed., p. 87. Univ. of Chicago Press, Chicago, Illinois, 1958.
4. Solomons, J. B., Develop. Biol. 1, 182 (1959).
5. Karmen, A., J. Clin. Invest. 34, 131 (1955).
6. Miller, G. L., Anal. Chem. 31, 364 (1959).
7. Butler, W. H., and Wigglesworth, J. S., Brit. J. Pathol. 47, 242 (1966).

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Effect of Proteolytic Enzymes and Protein Denaturing Agents on Normal and Dystrophic Myosins* (32817)

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The presence of increased amounts of proteolytic enzymes within the muscle cell in dystrophic animals is well established (1–4). Although a decrease in the concentration of contractile proteins in experimental muscular dystrophy is equally well documented (5–8), the role of the increased proteolytic enzyme activity in the mechanism of muscular dystrophy is not clear.

The aim of this work was to look for probable structural and functional differences between the normal and dystrophic proteins. Evidence was sought for the possibility that dystrophic contractile proteins are more sensitive to the action of proteolytic enzymes than normal contractile proteins. Also the reactivity of dystrophic and normal contractile proteins to denaturing agents was compared. Moreover the effect of denaturing agents on the sensitivity of dystrophic and normal contractile proteins to proteolysis and the action of these agents on the superprecipitation of normal and dystrophic muscle proteins was also studied. The results of this study are described below.

Materials and Methods. Adenosine triphosphate (ATP) and pronase (protease of B Subtilis type VIII) were purchased from Sigma Co. and trypsin was obtained from Worthington Biochemicals Co. All other reagents used were of reagent grade. Myosin A (MyA) and myosin B (MyB) were prepared as described by Mommaerts (9).

Rats were used 2 weeks after the bilateral dissection of the ischiadic nerves essentially as described by Kohn (7). The 2-year dystrophic hens (leghorn) were a generous gift of D. L. J. Pierro, University of Connecticut. Dystrophic mice (129/Re-dy) were purchased from The Jackson Laboratory, Bar Harbor, Maine.

The extent of trypsin and pronase digestion was followed by pH stat measurements essentially as described by Mihalyi and Harrington (10). A Radiometer TTT1 + SBU + SBR2 combination was used. Experiments were carried out in a water-jacketed cell under nitrogen gas in a total volume of 2 ml. Usually, 7.0 mg of myosin and 0.07 mg of trypsin or pronase were incubated and 10.0 m*M* NaOH was used for titration. The proteolytic enzymes were dissolved in 0.005 *M*/1 HCl and stored in the frozen state. The concentration was estimated by measuring their optical density at 280 mμ (10).

Superprecipitation experiments were measured with the turbidity increase method of Ebashi (11) in a Brice-Phoenix light scattering photometer connected to a 10-inch Beckman recorder. Usually, 0.7 mg of MyB was used for an experiment in the 25-mm quadratic turbidity cell. All experiments were performed in 15-ml total volume under constant mechanical stirring. The ATP was added to the mixtures from a microburette inserted in a hole on top of the photometer above the cell.

Results and Discussion. The results of a

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