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EFFECT OF AFLATOXIN B_1 ON NET SYNTHESIS OF ALBUMIN, FIBRINOGEN, AND α_1-ACID GLYCOPROTEIN BY THE ISOLATED PERFUSED RAT LIVER*

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Abstract—The isolated perfused rat liver has been used to study the influence of aflatoxin B_1 on net synthesis of the plasma proteins albumin, fibrinogen, α_1-acid glycoprotein and α_2-(acute phase) globulin. Aflatoxin B_1 was used because it may inhibit messenger RNA synthesis from DNA in a manner similar to that generally accepted for actinomycin D, thereby allowing estimates of half-lives of individual species of messenger RNA to be made by observation of changes in the rate of synthesis of individual proteins after administration of the drug. Livers of adult male Sprague–Dawley rats were perfused for 12 hr with defibrinated rabbit blood with L-lysine-1-^{14}C and 500 mg glucose continually infused. Net changes in the specific plasma proteins were measured serologically. Aflatoxin B_1 was added to the perfusate (half the total dose at the outset and the other half infused); although a dose of 62·5 μg aflatoxin B_1 did not affect the net synthesis of albumin, fibrinogen, α_1-acid glycoprotein or α_2-(acute phase) globulin, doses of 125, 250, 500 or 1000 μg were associated with increasingly severe inhibition of synthesis manifest after 2–4 hr of perfusion. These data are consistent with short half-lives for messenger RNA for these plasma proteins; however, histological evidence of progressively more widespread parenchymal cell degeneration, necrosis and interstitial hemorrhage associated with doses of aflatoxin B_1 above 62·5 μg strongly suggests that the impaired protein synthesis may in part have been secondary to cytotoxic effects.

L-Lysine-1-^{14}C incorporation into hepatic protein was inhibited progressively by increasing doses of aflatoxin B_1. Significant elevations in rate of $^{14}CO_2$ production from L-lysine-1-^{14}C, in urea synthesis and in α-amino acid nitrogen accumulation were observed in perfusions with more than 125 μg aflatoxin B_1.

RECENT experiments with rat liver[1–5] have indicated that aflatoxin B_1, a toxic metabolite of certain strains of *Aspergillus flavus*, binds to DNA and interferes with the transcription of messenger RNA in a manner similar to that proposed for actinomycin D.[6–9] Actinomycin D has been found to cause gradual cessation of net synthesis of rat serum albumin (RSA) and fibrinogen by the isolated perfused rat liver, indicating that the half-lives for the messenger RNA for these proteins are less than 3 hr.[10] Because of the possibility that aflatoxin B_1 acts like actinomycin D and could thereby help to produce further evidence as to the half-lives of specific messenger

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RNA's, its effects on net synthesis of RSA, fibrinogen and α_1-acid glycoprotein[11, 12] by the isolated perfused rat liver have been investigated and are herein reported.

Effects of aflatoxin B_1 on hepatic synthesis of α_2-(acute phase) globulin,[13–16] on incorporation of L-lysine-1-^{14}C into plasma proteins and hepatic protein, on the oxidation of L-lysine-1-^{14}C to $^{14}CO_2$, on net urea synthesis, on net changes in α-amino acid nitrogen and on the histological appearance of the perfused livers are also described.

METHODS

The perfusion system and experimental conditions were essentially as previously described[10, 17] except for certain changes: (a) perfusions were 12 hr in duration; (b) the total infusion volume was 18·0 ml with a continuous rate of 1·5 ml/hr; (c) each infusion contained 500 mg glucose; (d) each perfusate contained 10,000 units of heparin (heparin sodium USP, Upjohn); (e) at the start of perfusion, 3000 units of penicillin (buffered potassium penicillin G for injection, USP, Squibb) and 3·0 mg streptomycin (streptomycin sulfate for injection, USP, Lilly) were added to each infusion and to the perfusate. (Preliminary experiments showed that these doses had no effect on net synthesis of RSA, fibrinogen and α_1-acid glycoprotein by isolated normal rat livers perfused with defibrinated rabbit blood[10] for 12 hr.) In all perfusions, the infusion contained 15 μc (0·62 mg) L-lysine-1-^{14}C as well as glucose and antibiotics. Infusion, blood sampling, collection of "respiratory" $^{14}CO_2$, and ^{14}C assay procedures were carried out as previously described.[10]

Liver donors, adult male rats of Sprague–Dawley strain, weighed between 326 and 373 g; all were allowed drinking water at all times and in all experiments food was withdrawn 18 hr before perfusion. Perfusion medium was prepared as before from defibrinated rabbit blood,[10] so that initial concentrations of the rat plasma proteins, RSA, fibrinogen and α_1-acid glycoprotein would be low enough for accurate serological measurement of net changes during perfusion.

Aflatoxin B_1* was dissolved in dimethylsulfoxide and added in equal amounts to the infusions and perfusates at the start of the experiments. The total volume of dimethylsulfoxide was either 0·05 or 0·1 ml in all experiments with aflatoxin B_1. In control perfusions with dimethylsulfoxide, 0·1 ml was used (0·05 ml in infusion and 0·05 ml added to perfusate). Total doses of aflatoxin B_1 ranged from 62·5 to 1000 μg. Control perfusions were also carried out with no dimethylsulfoxide or aflatoxin B_1 in either the infusion or the perfusate.

Procedures for serological quantitation of RSA, fibrinogen, α_1-acid glycoprotein and α_2-(acute phase) globulin, for analysis for fibrinogen by clotting with thrombin (chemically determined fibrinogen), for measuring ^{14}C incorporation into RSA, fibrinogen, plasma total protein and hepatic protein, and for determining net changes in urea and α-amino acid nitrogen were as previously described.[10, 17]

At the end of the perfusions representative specimens of the livers were fixed with formalin and all were stained simultaneously with haematoxylin and eosin for histological examination.

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RESULTS

Histological findings. These findings are presented first to facilitate understanding of the other data from these experiments. In general they are consistent with a previously reported account[18] of the histological effects of aflatoxin B_1 on rat liver in intact animals. Livers from control perfusions with dimethylsulfoxide appeared normal, grossly and histologically. Livers from perfusions with 62·5 μg aflatoxin B_1 also appeared grossly and microscopically normal, except for a slight loss of cytoplasmic basophilia. Loss of cytoplasmic basophilia was more pronounced with increasing doses of aflatoxin B_1, and was quite severe with a dose of 500 μg. No histological sections were available from the liver perfused with 1000 μg aflatoxin B_1. Focal areas of periportal and midlobular interstitial hemorrhage and hepatic parenchymal cell degeneration and necrosis were noted in sections from perfusions with 125 μg aflatoxin B_1. Injured cells showed vacuoles in cytoplasm, partial loss of nuclear chromatin, and pyknosis. Such changes were more widespread and more marked with 250 μg aflatoxin, and 500 μg produced interstitial hemorrhage throughout all areas of the hepatic lobules with large areas of parenchymal cell necrosis. At all dose levels of aflatoxin B_1, littoral cells and bile duct epithelial cells appeared normal. The interstitial hemorrhage associated with aflatoxin B_1 appeared to correlate with surface mottling first noted grossly between 6 and 10 hr of perfusion. It is noteworthy that none of the doses of aflatoxin employed significantly altered perfusate flow through the liver from the high rates (1–3 ml/g liver/min) observed in control experiments.

Net biosynthesis of rat serum albumin. The addition of more than 62·5 μg aflatoxin to perfusions was associated with increasingly severe suppression of RSA synthesis. Net changes in RSA synthesis are shown in Fig. 1. Because of variable retention of preformed RSA and its appearance in the perfusate in the first 2 hr, it is more meaningful to compare the changes observed from 2 through 12 hr. On this basis a roughly linear increase in RSA production is seen in control perfusions with and without dimethylsulfoxide from 2 to 8 hr; thereafter the rate of RSA production decreases somewhat. In this and in all other figures the control data of experiments with and without dimethylsulfoxide are so closely similar that they are plotted together as a shaded control area. Increases of RSA in perfusions with 62·5 μg aflatoxin do not differ from control values; however, 125 μg reduces synthesis from 2 to 12 hr to approximately 50 per cent of controls in two of three perfusions. In a third perfusion with 125 μg, the suppression of synthesis from 2 to 12 hr is approximately similar to that seen with doses of 250, 500 or 1000 μg.

Incorporation of L-lysine-1-^{14}C into the plasma albumin fraction was measured (Fig. 2) in most of the experiments of Fig. 1. The very low incorporation into the albumin for the first 2 hr supports the view that albumin appearing in the perfusate before that time was largely preformed. The suppression of lysine-^{14}C incorporation with total doses of aflatoxin in excess of 62·5 μg is increasingly marked with increasing dose, and is approximately 7 per cent of controls with 1000 μg.

Net biosynthesis of fibrinogen. In most of the experiments of Fig. 3, crude fibrin precipitates were prepared as previously described[10, 17] from appropriate aliquots of perfusate plasma by treatment with bovine thrombin; the protein content of these fibrin precipitates was measured and was grossly at variance with the results of the highly specific serological method for fibrinogen. Our unpublished data lead us to conclude that fibrin precipitates obtained by bovine thrombin treatment of plasma

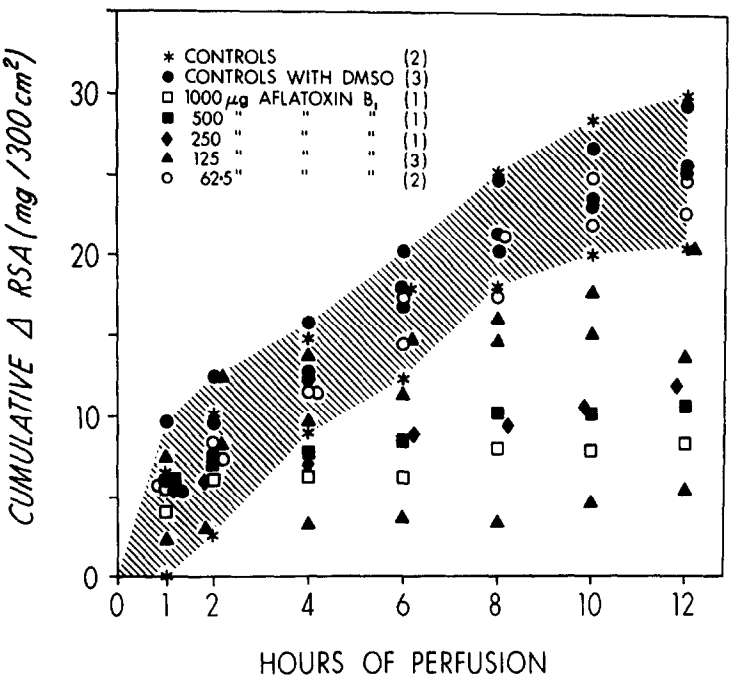

FIG. 1. Cumulative net change in RSA per 300 cm^2 of body surface area of liver donor rat. In this and in all subsequent figures, numbers in parentheses indicate number of experiments performed and shaded areas indicate extremes of values for the five control experiments (three with dimethylsulfoxide and two without).

samples, particularly those with very low fibrinogen levels (0·1–0·45 mg/ml), contain several other plasma proteins which are not removed by 4-fold washing with saline. Accordingly, we have presented here (Fig. 3) only the results of the serological estimation of fibrinogen content.

Shown in Fig. 3 are the effects of aflatoxin on the serologically measured net synthesis of fibrinogen. Although a total dose of 62·5 μg aflatoxin had no significant effect on fibrinogen synthesis, 125 μg sharply suppressed synthesis of fibrinogen from 4 to 8 hr with virtually no further increase in net synthesis from 8 to 12 hr. Doses of 250, 500 and 1000 μg lead to only slightly greater suppression of fibrinogen synthesis.

Net biosynthesis of α_1*-acid glycoprotein.* Figure 4 shows net changes in serologically measured α_1-acid glycoprotein. Although experiments with 62·5 μg aflatoxin B_1 do not differ from control values, with 250, 500 and 1000 μg aflatoxin B_1 all synthesis is essentially stopped after 4 hr. In three experiments with 125 μg, suppression of synthesis reckoned from 2 hr on is almost as severe as that caused by higher doses.

Concentration of α_2*-(acute phase) globulin in perfusates.* By the twelfth hour, α_2-(acute phase) globulin was present at very low levels (6·0 to 13·5 units/ml[17]) in all perfusions except those in which 250, 500 or 1000 μg aflatoxin B_1 was administered. In the latter experiments, none of this protein was detected in any perfusate sample.

Incorporation of L-lysine-1-^{14}*C into plasma proteins other than albumin and fibrinogen and into hepatic protein.* Detailed ^{14}C assays were carried out on this fraction from almost all experiments; the results are, relatively speaking, similar to those presented

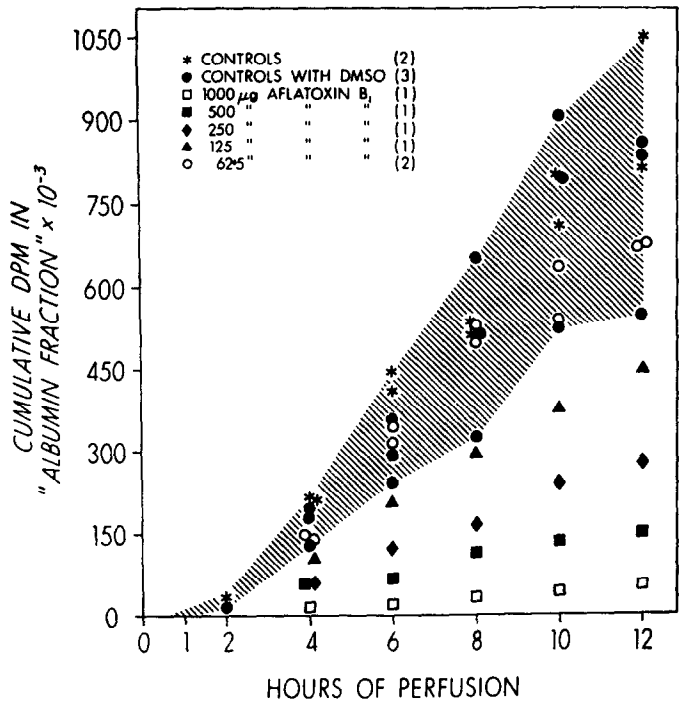

FIG. 2. Cumulative disintegrations per minute of L-lysine-1-^{14}C in RSA.

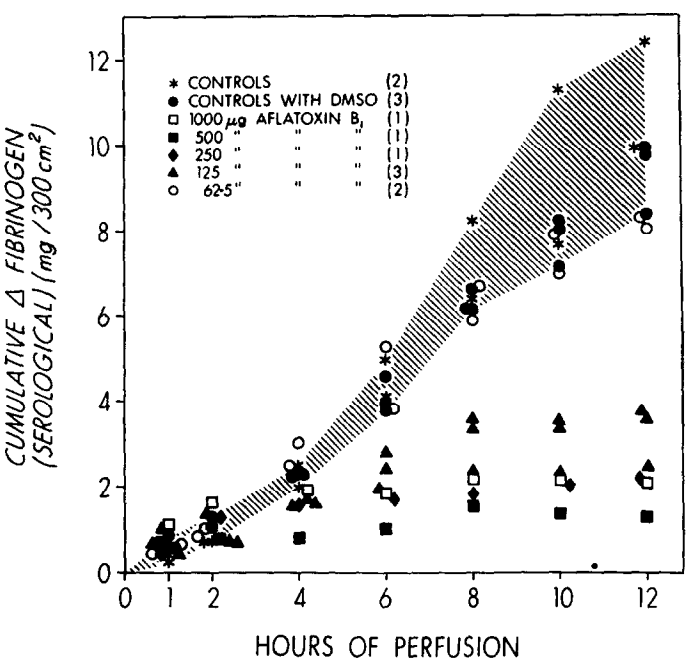

FIG. 3. Cumulative net change in fibrinogen measured serologically per 300 cm^2 of body surface area of liver donor rat.

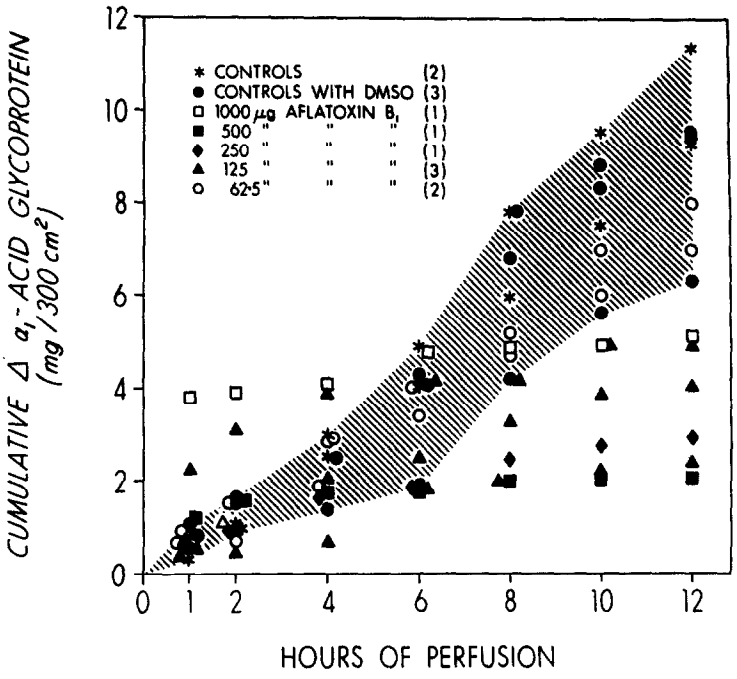

FIG. 4. Cumulative net change in α_1-acid glycoprotein per 300 cm^2 of body surface area of liver donor rat.

above for L-lysine-1-^{14}C incorporation in albumin (Fig. 2) and are depicted in Fig. 5. Notable are the lack of substantial incorporation into plasma proteins in the first 2 hr and the graded decrease in incorporation with increasing aflatoxin dose.

Incorporation of L-lysine-1-^{14}C into liver proteins is presented in Fig. 6; in contrast to the lack of inhibition of albumin and fibrinogen synthesis with 62·5 μg aflatoxin, this dose suppresses incorporation of L-lysine-1-^{14}C into liver proteins to a significant extent, which becomes more pronounced with larger doses of aflatoxin.

Effect of aflatoxin B_1 on oxidation of L-lysine-1-^{14}C. Figure 7 shows essentially linear increase in $^{14}CO_2$ production from the second to the twelfth hour in control experiments. Increased rates of $^{14}CO_2$ production are evident by 4 hr in perfusions of livers with aflatoxin B_1; in general, the greater the suppression of protein synthesis, the higher is the observed oxidative destruction of the L-lysine-1-^{14}C to $^{14}CO_2$.

Net changes in blood urea nitrogen and α-amino acid nitrogen. Control experiments show essentially linear synthesis of urea nitrogen throughout the 12-hr perfusions (Fig. 8). As in the case of $^{14}CO_2$ production (Fig. 7), there is marked elevation in the rate of urea synthesis (Fig. 8) and α-amino acid nitrogen accumulation (Fig. 9) by 2 hr in experiments with 500 or 1000 μg aflatoxin B_1.

Effects of aflatoxin B_1 on bile secretion. Bile was secreted constantly, but at a gradually decreasing rate in control perfusions, reaching total volumes of 8·1 and 9·0 ml by 12 hr. In control perfusions with dimethylsulfoxide, bile was also produced continuously at a gradually decreasing rate but reached lower total volumes (3·5 to

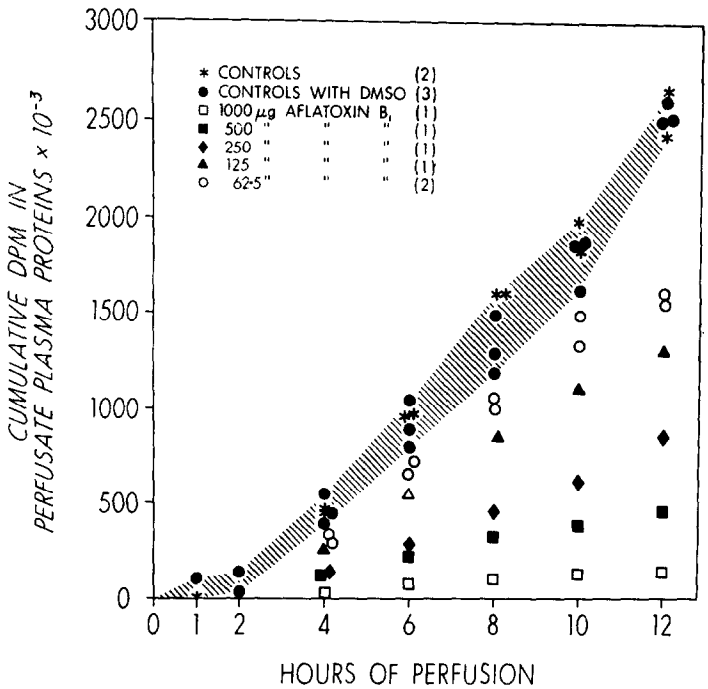

FIG. 5. Cumulative disintegrations per minute of L-lysine-1-^{14}C in plasma total protein minus albumin and fibrinogen.

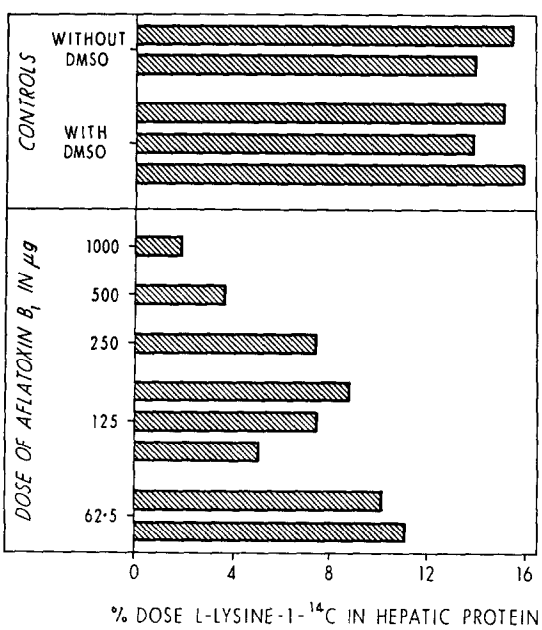

FIG. 6. Percentage of dose of L-lysine-1-^{14}C in hepatic protein at the end of 12-hr perfusion.

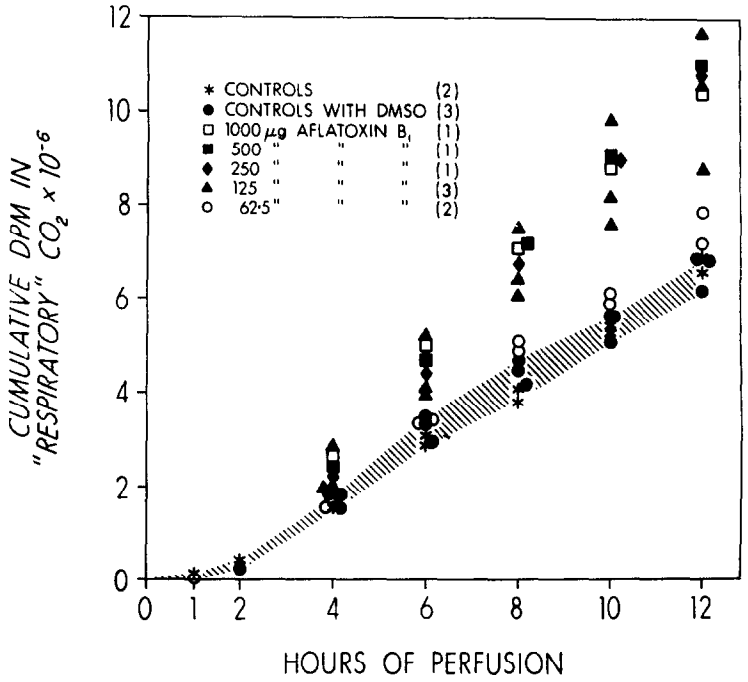

FIG. 7. Cumulative disintegrations per minute (from L-lysine-1-^{14}C) in respiratory CO_2.

7·6 ml). Bile flow from perfusions treated with 62·5 μg aflatoxin B_1 ceased at 8 and 11 hr with 3·2 and 5·3 ml bile respectively; all higher doses of aflatoxin B_1 were associated with cessation of bile flow between 4 and 8 hr with the formation of a total of 2·7 to 3·3 ml bile.

DISCUSSION

Although the effects of aflatoxin B_1 on protein synthesis in the isolated perfused rat liver are unequivocal, our understanding of the mechanisms of its action is conjectural. A number of reports indicate that the earliest and most fundamental action of aflatoxin on cells may be to bind to DNA and inhibit synthesis of RNA[1–5] much in the same way as has been proposed for actinomycin D.[6–9] The inhibition in net synthesis of specific plasma proteins by aflatoxin reported here resembles that which we have described for actinomycin D[10] and is consistent with the previously proposed short half-lives for messenger RNA for certain plasma proteins.[10, 17] However, the histological evidence of damage to livers from perfusions with aflatoxin B_1 strongly suggests that the decreased or arrested protein synthesis may in part have been secondary to cytotoxic effects rather than solely to effects mediated by changes in messenger RNA. In a similar vein Pong and Wogan[19] concluded that inhibition of induction of zoxazolamine hydroxylase by aflatoxin B_1 (given in a dose of 3 mg/kg) in rats treated with 3,4-benzpyrene might be due to cytotoxic subcellular disorganization. Aflatoxin B_1 may not always inhibit protein synthesis simply because of its general cytotoxicity; Clifford and Rees[4] have reported that a dose of 7 mg/kg of aflatoxin B_1 did not block substrate induction of tryptophan pyrrolase, although under the same

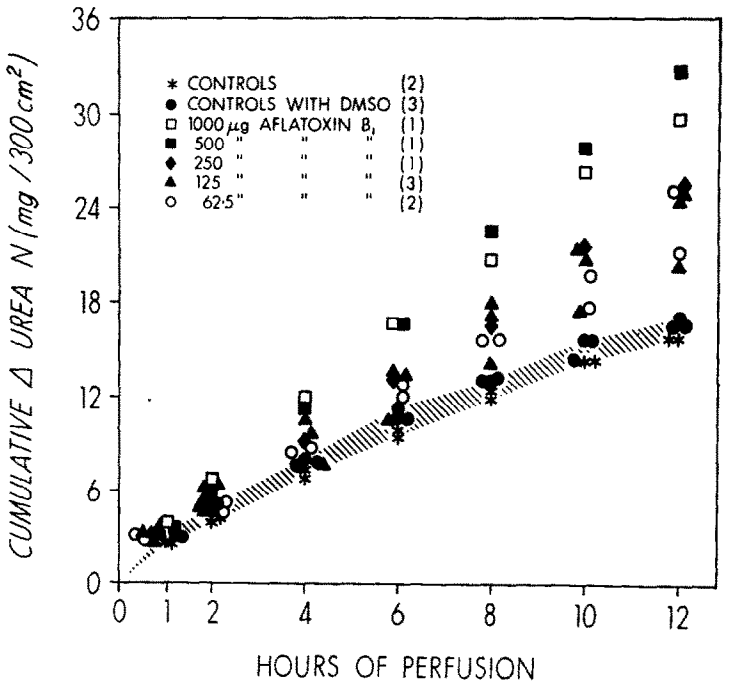

FIG. 8. Cumulative net change in urea nitrogen per 300 cm^2 of body surface area of liver donor rat

conditions induction of this enzyme by hydrocortisone was severely inhibited. To the extent that substrate induction of tryptophan pyrrolase is due to new protein synthesis,[20] the latter inhibition may be regarded as referable to inhibition of messenger RNA synthesis similar to that proposed for actinomycin D.[6–9] However, it must be admitted that inconsistent with the latter view are absence of interference with DNA-dependent RNA polymerase systems *in vitro*[5, 21] and the rapidly reversible and weak binding *in vitro* of aflatoxin B_1 to DNA.[21] The distribution of aflatoxin in hepatic cells is actually consistent with a more general cytotoxic mechanism. At no time during a 24-hr period after administration of ^{14}C-aflatoxin B_1 i.p. to rats was more than 9–17 per cent of the hepatic activity found in the nuclear fraction; while microsomal activity ranged from 20 to 50 per cent, 60 to 28 per cent of hepatic activity was in the microsomal supernatant.[22] It has been proposed that aflatoxin B_1 exerts a toxic effect on cellular membranes.[4, 23] Damage to membranes of hepatic vascular or sinusoidal endothelial cells might have led to the interstitial hemorrhage reported by Butler[18] and described in the present report.

Our experiments appear to be the first reported in which aflatoxin B_1 has been studied in the isolated perfused liver and it is relevant to compare doses and effects in this system with results *in vivo*. A single i.p. dose of 6 mg aflatoxin B_1 per kg has been found to be the LD_{50} for male rats;[18] for our liver donors weighing 350 g, an LD_{50} would have been 2·1 mg. Since 5–8 per cent of an i.p. dose of ^{14}C-aflatoxin B_1 is found in the liver 8–24 hr after injection,[22] the hepatic dose would approximate

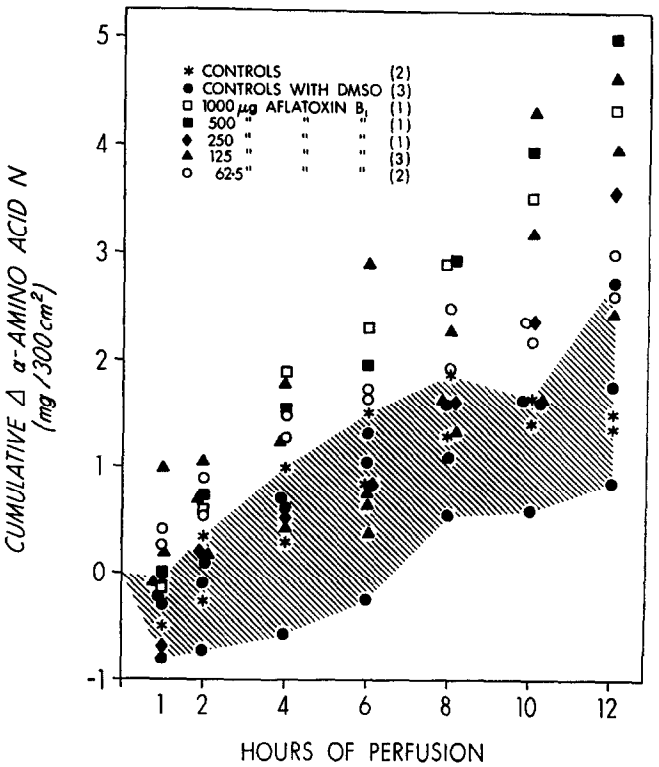

FIG. 9. Cumulative net change in α-amino acid nitrogen per 300 cm^2 of body surface area of liver donor rat.

105–168 μg. Accordingly it is of interest that our histological observations on perfused livers exposed to 125 μg aflatoxin B_1 are similar to those reported by Butler[18] for livers of rats that received an LD_{50} of aflatoxin B_1 16–24 hr before sacrifice. Also, the degree of inhibition we have found in incorporation of L-lysine-1-^{14}C into hepatic protein in experiments with 125 μg aflatoxin B_1 (Fig. 6) is similar to the reported inhibition of incorporation of DL-leucine-1-^{14}C into protein by liver slices from rats given an LD_{50} dose of aflatoxin B_1 1–18 hr before sacrifice.[4] However, our data (Fig. 6) contrast with the failure of aflatoxin B_1 *in vivo* to cause inhibition of incorporation of *DL*-leucine-1-^{14}C into hepatic protein.[4] Since experiments *in vivo* with doses of 5 or 7 mg per kg of aflatoxin B_1 have shown severe inhibition of hepatic nuclear RNA synthesis,[3, 4] it is likely that similar inhibition occurred in our perfusions with 125 μg or more of aflatoxin B_1.

Incorporation of L-lysine-1-^{14}C into RSA (Fig. 2) appears less effectively inhibited by aflatoxin B_1 than net synthesis of this protein (Fig. 1). This may be related to the fact that the RSA fraction assayed for radioactivity was contaminated with small amounts of other plasma proteins,[10] the synthesis of which may have been less severely inhibited by aflatoxin, as seen in Fig. 5.

Several comparisons are noteworthy between our current data on the effects of aflatoxin B_1 on the isolated perfused rat liver and those of our previous report on the action of actinomycin D.[10] Both antimetabolites cause an increase in the rate of oxidation of L-lysine-1-^{14}C to $^{14}CO_2$, probably secondary to inhibition of utilization of amino acids for protein synthesis. Aflatoxin B_1 caused elevations in the production of urea and the rate of accumulation of α-amino acids in perfusate. Such elevations were not seen with actinomycin D,[10] possibly because it may have caused less cell damage and catabolic effect than aflatoxin B_1. Both antimetabolites caused severe suppression of synthesis of plasma and liver proteins as judged by serological measurement and L-lysine-1-^{14}C incorporation. In contrast to the immediate and almost total suppression of protein synthesis noted with puromycin,[10] both actinomycin D and aflatoxin B_1 became maximally effective only after 2–6 hr.

The low levels of α_2-(acute phase) globulin in the blood samples of all liver donor rats taken at the time of perfusion (2·0–7·1 units/ml) and the low but gradually increasing concentrations of α_2-(acute phase) globulin observed in the control perfusions demonstrate that this protein is present in the blood of normal adult rats and suggest that it is being slowly synthesized by the liver. The absence of this protein from perfusate samples of experiments with 250 μg or more of aflatoxin B_1 indicates that the low levels observed in control experiments were the result of net synthesis rather than of simple leakage of performed or stored α_2-(acute phase) globulin.

In the 12-hr control perfusions presented in this paper, preservation of normal histology, protracted production of bile, continued synthesis of RSA, fibrinogen, α_1-acid glycoprotein and urea, and constant rate of production of $^{14}CO_2$ indicate that hepatic functions can be maintained for at least 10–12 hr in the isolated perfused rat liver. Such data strongly support the view that the isolated perfused rat liver can be an effective tool for studies *in vitro* of more than a few hours' duration.

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