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A Comparative Study of the Effect of Aflatoxin B_1 and Actinomycin D on HeLa Cells

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1. The cytotoxic effects of aflatoxin B_1 on HeLa cells were examined and effects of short exposures of the cells to the toxin were found to be reversible. 2. Aflatoxin B_1 inhibited the synthesis of both ribosomal and heterodisperse RNA. It is proposed that the toxin's mechanism of action on ribosomal RNA synthesis is related to its inhibitory effect on the maturation of the 45s-ribosomal-RNA precursor. 3. Protein synthesis is inhibited to a greater extent by aflatoxin B_1 than by actinomycin D. In contrast with actinomycin D, aflatoxin B_1 was shown to disaggregate polyribosomes directly.

Aflatoxin B_1 is one of a group of closely related pentacyclic substances produced by certain strains of *Aspergillus flavus-oryzae* (Asao *et al.* 1963; Van Dorp *et al.* 1963). These substances are toxic to some micro-organisms (Burmeister & Hesseltine, 1966), exert marked toxic and carcinogenic activities on the liver and other tissues of experimental animals (Lancaster, Jenkins & Philp, 1961; Butler, 1964; Barnes & Butler, 1964; Dickens, Jones & Waynforth, 1966) and cause chromosome breakage in root seedlings (Lilly, 1965). Aflatoxin B_1 inhibits nucleolar RNA synthesis, the induction by cortisone of tryptophan pyrrolase in the livers of rats *in vivo* and synthesis of both RNA and protein in rat liver slices (Clifford & Rees, 1966, 1967; Sporn, Dingman, Phelps & Wogan, 1966). These properties, and our finding that aflatoxin B_1 interacts with DNA *in vitro*, suggested a mechanism of action similar to that of actinomycin D, namely inhibition of DNA transcription leading to a secondary inhibition of protein synthesis (Clifford & Rees, 1966). But, unlike actinomycin D, aflatoxin B_1 inhibits DNA and RNA synthesis to the same extent (De Recondo, Frayssinet, Lafarge & Le Breton, 1965, 1966). Both toxins produce similar nuclear and nucleolar ultrastructural lesions in cells in culture (Simard, 1966; Simard & Bernhard, 1966).

In the present investigation a more detailed comparison between the effects of aflatoxin B_1 and actinomycin D on cellular and protein synthesis was made by using HeLa cells. This cell line is particularly useful for the purpose since the site and detailed processes of RNA formation in this cell have been extensively studied (Scherrer & Darnell, 1962; Scherrer, Latham & Darnell, 1963; Warner, Soeiro, Birnboim, Girard & Darnell, 1966; Penman, 1966; Penman, Vesco & Penman, 1968; for further references see Darnell, 1968). Our results show that the effect of aflatoxin B_1 on nucleic acid and protein synthesis in HeLa cells differs from that of actinomycin D.

MATERIALS

HeLa cells

A rapidly growing line of HeLa cells developed at the Wellcome Laboratory of Tropical Medicine, Beckenham, Kent, and kindly made available by Dr K. Apostolov, was used throughout.

Media

Growth Medium. Medium 199 was supplemented with 10% (v/v) calf serum and buffered with 2% (w/v) $NaHCO_3$. Penicillin and streptomycin were added in final concentrations of 100 i.u./ml. and 100μg./ml. respectively.

Maintenance medium. Medium 199 buffered with 4% (w/v) $NaHCO_3$ and with added penicillin (100 i.u./ml.) and streptomycin (100μg./ml.) was used.

Earle's saline. This had the composition given by Earle (1943).

Hypo-osmotic saline buffer, pH 7·4. This contained (final concentrations): 10mM-NaCl, 10mM-tris–HCl and 1·5mM-$MgCl_2$.

Radioactive materials

All radioactive materials were obtained from The Radiochemical Centre, Amersham, Bucks. They were: [G-^{3}H]uridine (2·65c/m-mole); [6-^{3}H]thymidine (5·0c/

m-mole); DL-[1-^{14}C]leucine (36·6mc/m-mole); L-[*Me*-^{14}C]-methionine (53·7mc/m-mole); and U-^{14}C-labelled *Chlorella* protein hydrolysate (54mc/m-mole of C).

Metabolic inhibitors

Aflatoxin B_1. A crude mixture of aflatoxins was a gift from the Medical Research Council and aflatoxin B_1 was separated from this mixture by a t.l.c. technique devised by W. Lijinsky (unpublished work). When required, 1mg. of aflatoxin B_1 was dissolved in 0·05ml. of *NN*-dimethylformamide and made up to 1ml. with sterile distilled water. In all experiments in which aflatoxin B_1 was used an equivalent quantity of dimethylformamide–water mixture was added to the control cultures.

Actinomycin D. Actinomycin D was obtained from Merck, Sharp and Dohme Ltd., Westpoint, Pa., U.S.A., as a powder in 0·5mg. ampoules containing 20mg. of mannitol as an inactive ingredient. When required for use it was dissolved in sterile distilled water to the required concentration.

Nucleic acid preparation

Calf thymus DNA and yeast RNA were obtained commercially from the Sigma Chemical Co., St Louis, Mo., U.S.A.

METHODS

Cell propagation

For use in incorporation experiments $1·5 \times 10^6$ cells from stock cultures in 10ml. of growth medium were seeded into 8oz. Pyrex babies' feeding bottles. These were kept stationary at 37° and used after 2 days incubation.

For morphological studies, 10^5 cells from stock cultures in 1ml. of growth medium were seeded into 6in. × §in. test tubes and incubated for 3 days at 37° before use. If photomicrography was to be performed a sterile cover slip (22mm. × 7mm.) was introduced at the time of seeding, which was easily removed for staining and examination after incubation and the formation of a monolayer.

Subcellular fractionation

At the end of appropriate experiments, media were poured off the cell monolayers and replaced with 10ml. of ice-cold Earle's saline, which was immediately frozen on the side of the feeding bottle opposite to that of the monolayer, care being taken to avoid freezing the monolayer. The frozen medium was then allowed to melt slowly so that vigorous shaking of the frozen medium over the monolayer removed the cells from the glass.

Nucleoli, nucleoplasm and total cytoplasmic fractions were separated from approx. 10^7 cells by methods described by Penman (1966) and Penman, Smith & Holtzman (1966), all manipulations being carried out at 4°.

Polyribosomes from about 3×10^7 cells were separated by a method modified from that described by Penman, Scherrer, Becker & Darnell (1963). Centrifuged cell pellets were resuspended in 1·5ml. of hypo-osmotic saline buffer. The cells were allowed to swell for 10min. and were then homogenized with 12 strokes in a tight-fitting Dounce homogenizer. Then 0·02ml. of 10% (w/v) sodium deoxycholate was added and the suspension was mixed with a Pasteur pipette. After centrifugation at 1800*g* for 10min. to remove nuclei, 1ml. of the opalescent supernatant was layered immediately on to 19ml. of 5–25% (w/v) sucrose gradient in hypo-osmotic saline buffer, and was centrifuged at 4° for 40min. at 28000 rev./min. (85000$g_{av.}$) in the 20ml. swing-out bucket head (no. 2418) of the MSE Superspeed 50 ultracentrifuge. The polyribosome fractions were analysed by spectrophotometry at 254nm. and by radioactivity-counting methods.

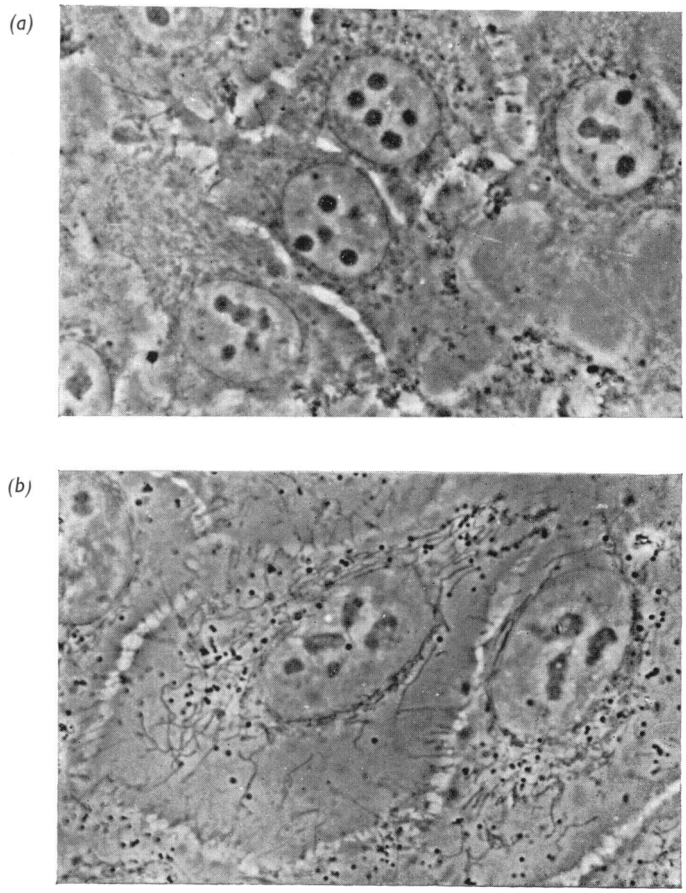
Extraction of macromolecular components

DNA. DNA from 4×10^6–15×10^6 cells was extracted by a method modified from that of Marmur (1961). Centrifuged cell pellets were washed with 5ml. of Earle's saline and resuspended in 2ml. of 1M-$NaClO_4$ containing 1% (w/v) of sodium dodecyl sulphate. After the addition of 2ml. of chloroform containing 1% (v/v) of 3-methylbutan-1-ol, the mixture was shaken for 5min. in a stoppered tube and then centrifuged at 1250*g* for 10min. before the chloroform phase was removed and discarded. After two more extractions with 2ml. of the chloroform reagent, the aqueous phase was removed, mixed thoroughly with 4ml. of ethanol and left overnight at −15°. Next day, the mixture was centrifuged at 10000*g* for 10min. and the DNA pellet dissolved in 1·5ml. of dilute saline citrate solution (Marmur, 1961). After adjustment of the salt concentration to standard saline citrate solution strength, the E_{260} of each sample was measured in a Unicam SP.500 spectrophotometer and radioactivity was determined as described below.

RNA. RNA from subcellular fractions was extracted by the method of Penman (1966), which gives a high yield of RNA. When RNA was extracted from whole cells the shorter method of Scherrer & Darnell (1962) was used. The final RNA precipitates from these procedures were dissolved in 20mM-sodium acetate buffer, pH5, containing 0·1M-NaCl and 1mM-EDTA, and the E_{260} was measured in the Unicam SP.500 spectrophotometer and radioactivity was determined as described below.

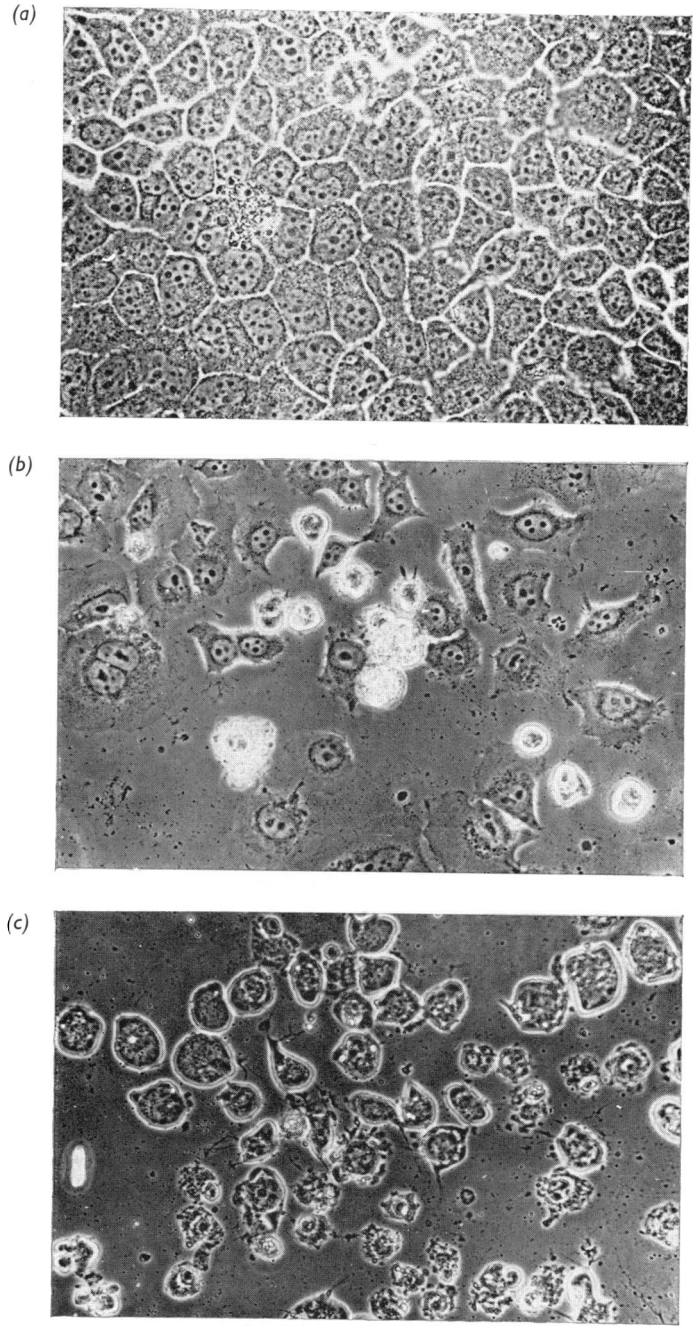
In certain experiments, RNA was fractionated by rate-zonal centrifugation in sucrose density gradients. A 0·5ml. portion of the RNA sample was layered on to 19·5ml. of a 5–40% (w/v) sucrose gradient prepared in 20mM-sodium acetate buffer, pH5·0, containing 0·1M-NaCl and 1mM-EDTA, and centrifuged at 4° in the swing-out bucket head (no. 2418) of the MSE Superspeed 50 ultracentrifuge at 22000 rev./min. for 16hr.

Protein. Washed-cell pellets from approx. 4×10^6 cells were resuspended in 1ml. of water and the protein was precipitated by adding 2ml. of 20% (w/v) trichloroacetic acid. After centrifugation at 2500*g* for 10min. the precipitate was extracted with 2·5ml. of 5% (w/v) trichloroacetic acid at 90° for 20min. The residue was then washed twice with 2·5ml. of 5% (w/v) trichloroacetic acid and dissolved in 1·5ml. of 1M-NaOH at 37° overnight. Samples of this solution were analysed by radioactivity counting and the protein content was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).



EXPLANATION OF PLATE 1

Phase-contrast photomicrographs of HeLa-cell monolayers (*a*) untreated and (*b*) treated with aflatoxin B_1 (10μg./ml.) for 24hr. Magnification: $\times$2000.



EXPLANATION OF PLATE 2

Phase-contrast photomicrographs of HeLa-cell monolayers (*a*) untreated, (*b*) treated with aflatoxin B_1 (20μg./ml.) for 24hr. and (*c*) treated with aflatoxin B_1 (40μg./ml.) for 24hr. Magnification $\times$450.

Incorporation of radioactively labelled precursors

[^{3}H]*Uridine and* [^{3}H]*thymidine.* These isotopes were usually used in concentrations of 0·2 μc/ml. of medium and the incubation time is given in the figures and text. When RNA was to be fractionated on a sucrose density gradient, [^{3}H]uridine concentrations of 1 μc/ml. were used.

[^{14}C]*Methionine.* [^{14}C]Methionine was added to methionine-free maintenance medium to give a specific radioactivity of 1 μc/ml.; this gave a final concentration of methionine of 2·6 μg./ml., which is above the minimum required for normal RNA synthesis (Zimmerman & Holler, 1967).

^{14}C-*labelled amino acids.* In some experiments polyribosomes were labelled with radioactive amino acids by exposing cells to ^{14}C-labelled protein hydrolysate at a concentration of 2 μc/ml. After incorporation for 1 min. the medium was quickly poured off and replaced with ice-cold Earle's saline.

Measurement of radioactivity

Up to 0·5 ml. of radioactively labelled samples was added to vials containing 15 ml. of the thixotropic scintillation mixture described by Bray (1960) and Gordon & Wolfe (1960). Vials were then loaded into a Packard Tri-Carb scintillation spectrometer model 3375 and held at 4° for 1 hr. to allow for temperature equilibration and the decay of light-stimulated photon emission in the samples. Each sample was counted for 10 min. or until 10000 counts had been registered, whichever was the shorter period. All counts were corrected for the appropriate background level and quench, and are expressed as c.p.m. or c.p.m./μg. of DNA or RNA. Factors for converting extinction readings into μg. of DNA or RNA/ml. were 52 and 58 respectively. The former was based on measurements with calf thymus DNA and the latter on measurements with yeast RNA (Sigma Chemical Co.), both performed in our own laboratory.

Estimations of total nucleotide pool

[^{3}H]Uridine or [^{3}H]thymidine (1·0 μc/ml.) was incorporated into monolayers for 30 min., after which the medium was replaced with 10 ml. of ice-cold Earle's saline. Cells, twice washed in 3 ml. of ice-cold Earle's saline, were homogenized in ice-cold 0·6 M-$HClO_4$ in a Dounce homogenizer and centrifuged at 0° and 2000g for 2 min. The residue was re-extracted with 0·2 M-$HClO_4$ and the pooled supernatant fluids were neutralized with KOH. Then $KClO_4$ was removed by centrifugation and the E_{260} of portions of the supernatant was measured in a Unicam SP.500 spectrophotometer, and the radioactivity was determined as described above.

RESULTS

Cytotoxicity of aflatoxin B_1. The cytotoxicity of aflatoxin B_1 was assessed by adding different concentrations of aflatoxin B_1 to cell cultures for various times. Continuous exposure of HeLa cells to 10 μg. of aflatoxin B_1/ml. led, after 24 hr., to the development of filamentous structures in the cytoplasm (Plate 1); at a concentration of 20 μg./ml., cytoplasmic granularity and cellular degeneration became evident, and with 40 μg./ml. rounding-up and necrosis of the cells was complete (Plate 2). On the other hand exposure of the cells to 20 or 40 μg. of aflatoxin B_1/ml. for 2 hr. or less produced a marked degree of cytoplasmic granularity when cells were examined after 48 hr., but their capacity to reach confluence after subculture, although delayed, and their return to normal morphology indicated that the changes were fully reversible. In contrast, exposure to 40 μg./ml. for 4 hr. produced irreversible lesions leading to cell death by 14 days.

Effect of aflatoxin B_1 on protein and nucleic acid synthesis. The effect of 20 or 40 μg. of aflatoxin B_1/ml. was measured by comparing the incorporation of [^{3}H]uridine, [^{3}H]thymidine and [^{14}C]leucine into RNA, DNA and protein respectively. Table 1 shows that incorporation of all these precursors was inhibited to a degree related to the concentration of aflatoxin B_1. Because the cytotoxic effect of aflatoxin B_1 administered for short periods was reversible, it was decided to examine the reversibility of the toxin's inhibitory effect on RNA and protein synthesis. Fig. 1 illustrates the results of experiments in which the inhibitory effect of the toxin (20 μg./ml.) administered for 30 min. was investigated. The results show that after withdrawal of aflatoxin B_1 the inhibition of [^{3}H]uridine

Table 1. *Inhibitory effect of aflatoxin B_1 on the incorporation of* [^{3}H]*uridine,* [^{3}H]*thymidine and* [^{14}C]*leucine into RNA, DNA and protein respectively*

These results are from a typical experiment in which monolayers were exposed to aflatoxin B_1 and the appropriate radioactively labelled precursor for 30 min. and the radioactivity incorporated is expressed as a percentage of that incorporated in the untreated monolayers.

Concn. of aflatoxin (μg./ml.)	Inhibition (%)		
	RNA	DNA	Protein
20	34·5	11·4	11·0
40	48·3	13·8	25·8

and [^{14}C]leucine incorporation was almost completely reversed within 24hr.

The effects of aflatoxin B_1 on the incorporation of [^{3}H]uridine and [^{14}C]leucine by HeLa cells were compared with those of actinomycin D. Experiments in which continuous incorporation of labelled precursors was determined at regular intervals up to 90min. showed that both toxins at the concentrations employed inhibited RNA synthesis to the same extent, but that only aflatoxin B_1 inhibited protein synthesis (Fig. 2). Another experiment confirmed that aflatoxin B_1 inhibited protein synthesis to a greater degree than did actinomycin D over a period of 8hr. (Fig. 3), although both drugs, at the concentration employed, inhibited RNA synthesis to about the same extent.

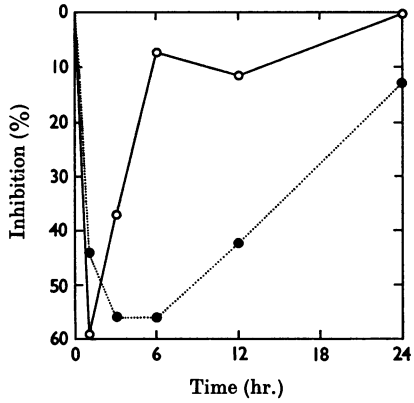


Fig. 1. Recovery of protein and RNA synthesis after exposure of HeLa cells to aflatoxin B_1: ten bottles containing HeLa-cell monolayers were treated with 20μg. of aflatoxin B_1/ml., and after 30min. the toxin-containing medium was replaced with growth medium. At the same time ten control bottles were similarly treated but with the aflatoxin B_1 omitted. At suitable times two control and two aflatoxin-treated monolayers were labelled for 30min. simultaneously with [^{3}H]uridine and [^{14}C]leucine. At each time the incorporation of radioactive label is expressed as a percentage of the control value. ○, Incorporation of [^{14}C]leucine into protein; ●, incorporation of [^{3}H]uridine into RNA.

Because inhibition of uridine incorporation into RNA may be due to a failure of uridine to enter the nucleotide pool, the effects of aflatoxin B_1 (40μg./ml.) and actinomycin D (2μg./ml.) were tested on this latter step. Neither drug in appropriate experiments inhibited entry of uridine into the nucleotide pool. It was therefore concluded that their inhibitory effect on uridine incorporation into RNA was due to a failure of nucleotide precursors to be incorporated into RNA.

Effect of aflatoxin B_1 and actinomycin D on ribosomal-RNA synthesis. Our preliminary experiments showed that ribosomal-RNA-precursor profiles of HeLa-cell monolayers were similar to those found in HeLa cells grown in suspension (Fig. 4) (Penman, 1966; Warner, Soeiro *et al.*, 1966). The effect of 40μg. of aflatoxin B_1/ml. or 0·2μg. of actinomycin D/ml. on these ribosomal-RNA-precursor profiles

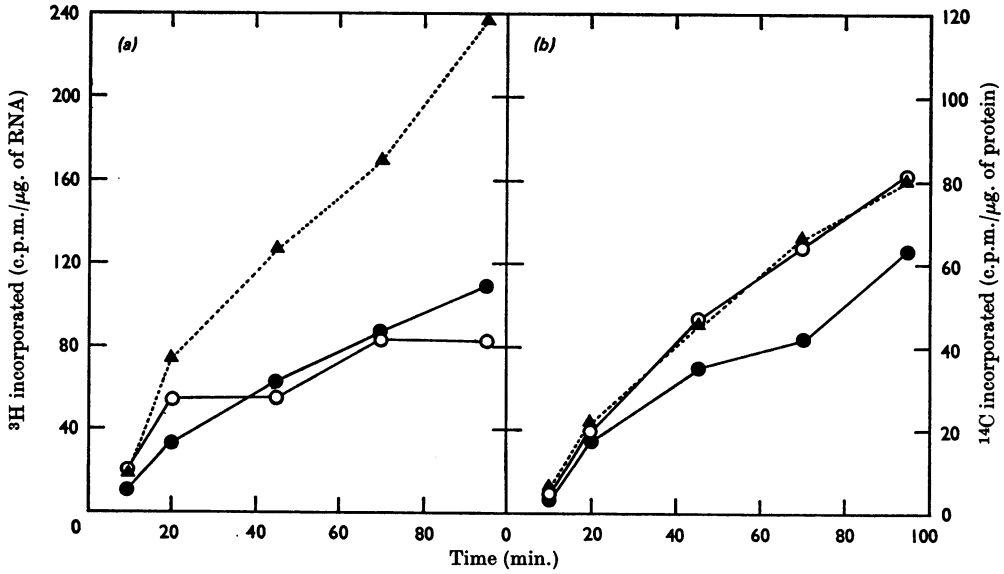


Fig. 2. Effects of aflatoxin B_1 (40μg./ml.) and actinomycin D (0·2μg./ml.) on the incorporation of [^{3}H]uridine into RNA (*a*) and of [^{14}C]leucine into protein (*b*) by HeLa cells. ▲, Untreated; ○, actinomycin D-treated; ●, aflatoxin B_1-treated.

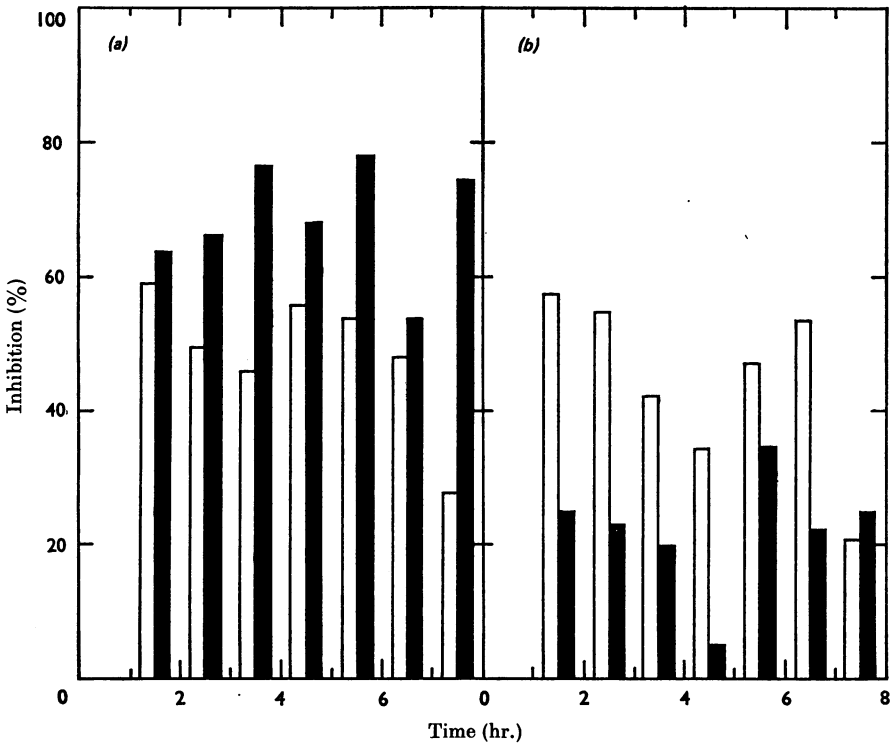


Fig. 3. Inhibitory effects of aflatoxin B_1 (40 μg./ml.) (*a*) and actinomycin D (0·2 μg./ml.) (*b*) on protein and RNA synthesis measured by incorporation of [^{14}C]leucine and [^{3}H]uridine respectively: HeLa cells were exposed to aflatoxin B_1 for 2 hr. or to actinomycin D for 30 min. At zero time the media were replaced with growth medium and incorporations were measured at regular times. The results are expressed as a percentage related to untreated controls at each time. White left-hand columns refer to RNA and black right-hand columns refer to protein.

was determined by labelling cells with [^{3}H]uridine in the presence and absence of the drugs. These concentrations were used because preliminary experiments had shown them to produce approx. 50% inhibition of [^{3}H]uridine incorporation into RNA. After an appropriate interval, RNA was extracted from these cells and fractionated by rate-zonal centrifugation in sucrose density gradients. The profiles obtained are illustrated in Fig. 4. All the ribosomal-RNA-precursor peaks present in untreated cells are evident in aflatoxin B_1-treated cells, although they are decreased in height and those representing the 32s and 18s components are diminished to a greater extent than that of the 45s component. In contrast, discrete ribosomal-RNA-precursor peaks are no longer recognizable in profiles from cells treated with actinomycin D, an effect that is in agreement with that reported in L cells by Perry (1964).

The disproportionate decrease in the height of the 32s and 18s RNA peaks compared with that for the 45s RNA component after treatment of the cells with aflatoxin B_1 suggested that the toxin may affect maturation of the ribosomal-RNA precursor. This maturation is known to involve methylation of the 45s component (Zimmerman & Holler, 1967); the effect of aflatoxin B_1 on ribosomal-RNA precursors may therefore be due to an effect on methylation. This was investigated in experiments in which [^{3}H]uridine and [^{14}C]-methionine were simultaneously incorporated into the RNA of untreated cells and cells treated with 40 μg. of aflatoxin B_1/ml. added 10 min. before the label. RNA was extracted from separated nucleoli so that the ratio of ^{14}C uptake to ^{3}H uptake in 45s RNA uncontaminated by heterodisperse RNA could be determined. The toxin affected incorporation of both ^{14}C and ^{3}H to the same extent and the experiment therefore lends no support to the possibility of a specific effect by aflatoxin B_1 on methylation of the 45s RNA.

To investigate further the conversion of fully methylated 45s RNA into 32s, 28s and 18s components, HeLa-cell monolayers were labelled with [^{14}C]methionine. The use of this radioactive label had the advantage of not labelling non-ribosomal

heterodisperse RNA to any great extent (Brown & Attardi, 1965). After cells had been exposed to [^{14}C]methionine for 15min., the medium was replaced with unlabelled medium containing 30μg. of non-radioactive methionine/ml., and 40μg. of aflatoxin B_1/ml. was added to half the cells. After 75min. the 'chased' RNA was extracted from untreated and aflatoxin B_1-treated monolayers and fractionated by density-gradient centrifugation. The radioactivity profiles obtained (Fig. 5) show a distinct difference: those from aflatoxin B_1-treated cells show persistence of the 45s ribosomal-RNA-precursor peak and a markedly diminished 18s component, indicating some delay in the maturation of the 45s RNA into its products, the 32s and 18s RNA.

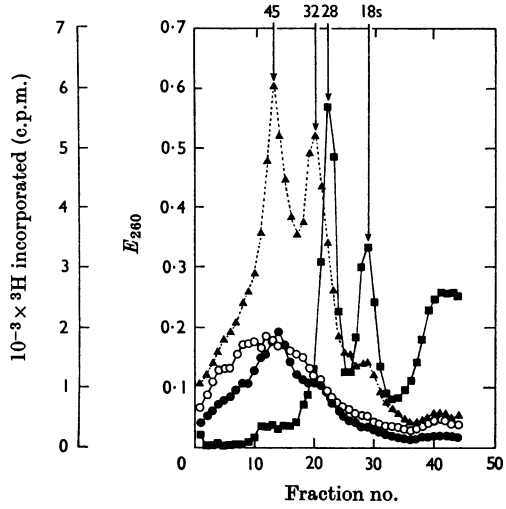


Fig. 4. RNA profiles obtained from HeLa cells untreated and treated with actinomycin D and aflatoxin B_1: cells were exposed to the toxins for 30min., then labelled with [^{3}H]uridine for 60min. in the presence of the toxin. ▲, Radioactivity in untreated cells; ○, radioactivity in actinomycin D-treated cells; ●, radioactivity in aflatoxin B_1-treated cells; ■, E_{260}.

Although both actinomycin D and aflatoxin B_1 inhibit RNA synthesis, their different effects on the synthesis of ribosomal-RNA precursors made the investigation of the effect of aflatoxin B_1 on the synthesis of other RNA species of interest. Experiments in which cell monolayers were labelled with [^{3}H]uridine for 60min. and then submitted to subcellular fractionation and extraction of RNA were then performed. The results illustrated in Fig. 6 show that aflatoxin B_1 (40μg./ml.) inhibits RNA synthesis to the same degree in both the nucleoli and nucleoplasm, indicating that synthesis of both ribosomal and non-ribosomal RNA is inhibited by aflatoxin B_1.

Effect of aflatoxin B_1 and actinomycin D on protein synthesis. In the experiments described in Fig. 4, where the doses of aflatoxin B_1 and actinomycin D

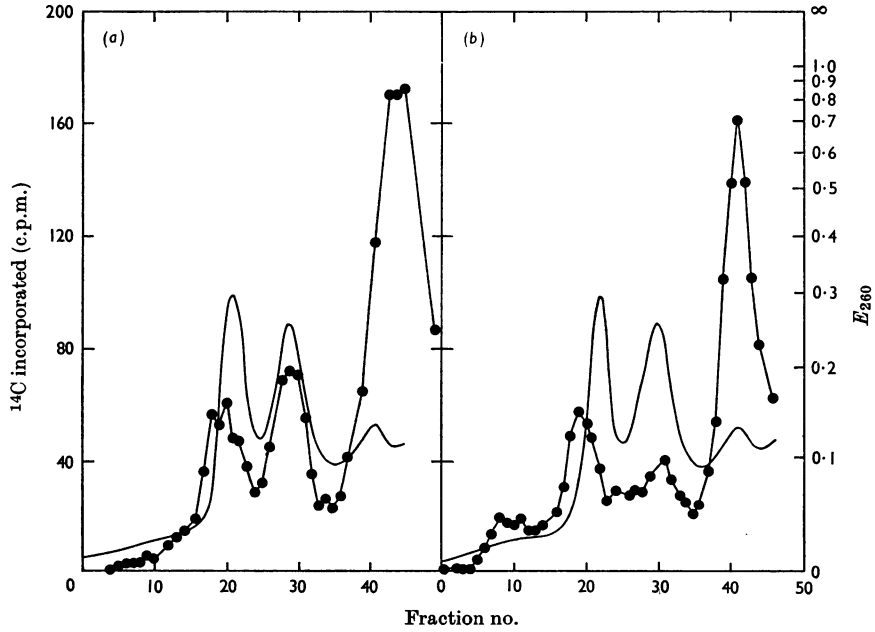


Fig. 5. Effect of aflatoxin B_1 on the maturation of the 45s ribosomal-RNA precursor labelled with [^{14}C]methionine: (*a*) untreated HeLa cells; (*b*) aflatoxin B_1-treated HeLa cells. ●, Radioactivity incorporated: ——, E_{260}.

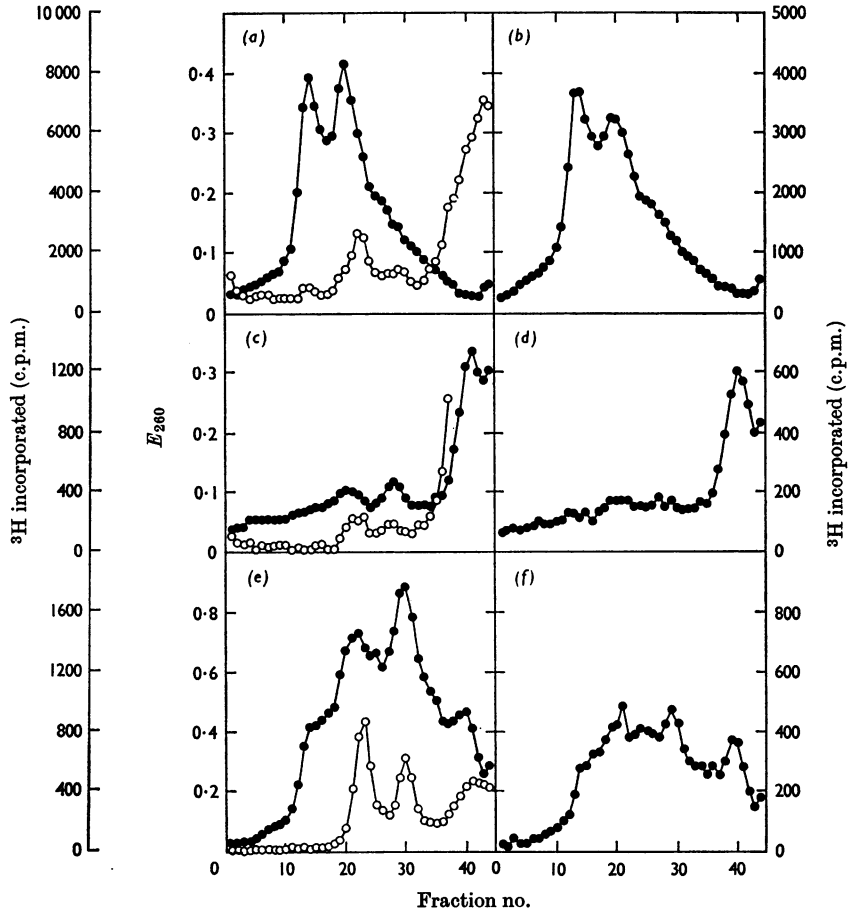


Fig. 6. Effect of aflatoxin B_1 (40μg./ml.) on RNA synthesis in nucleoli and nucleoplasm of HeLa cells. RNA profiles are from nucleoli (*a* and *b*), nucleoplasm (*c* and *d*) and cytoplasm (*e* and *f*): (*a*), (*c*) and (*e*), untreated HeLa cells; (*b*), (*d*) and (*f*), aflatoxin B_1-treated HeLa cells. ●, Radioactivity incorporated; ○, E_{260}.

were chosen to produce a similar degree of inhibition of RNA synthesis, aflatoxin B_1 for 1½hr. after its addition inhibited protein synthesis to a greater degree than did actinomycin D. A dose of actinomycin D that inhibits all forms of RNA synthesis leads ultimately to a secondary disaggregation of polyribosomes and thus to inhibition of protein synthesis (Penman *et al.* 1963). On the basis of this they inferred that the disaggregation of polyribosomes by actinomycin D resulted from the decay of messenger RNA. To determine whether the same interrelationship applied to the inhibitory effects of aflatoxin B_1, the relationship between inhibition of protein synthesis by aflatoxin B_1 and disaggregation of polyribosomes was investigated in experiments in which HeLa monolayers were treated with 40μg. of aflatoxin B_1/ml. for various times and labelled with ^{14}C-labelled protein hydrolysate for the last 1min. of each experiment. Cytoplasmic extracts from each experiment were fractionated by density-gradient centrifugation, and Fig. 7 shows the results of an experiment in which HeLa cells were exposed for 3hr. to 40μg. of aflatoxin B_1/ml., or to 2·5μg. of actinomycin D/ml. for 3 and 6hr. Fig. 7(*b*) shows that treatment with aflatoxin B_1 for 3hr. decreased the height of the E_{254} profile in the polyribosome region (fractions 14–24) and increased it in the 74s monoribosome region (fractions 26–29). This was accompanied by an equivalent decrease in incorporation of radioactive amino acids in the polyribosome region. Inhibition of protein synthesis by aflatoxin B_1 is thus clearly associated with the disaggregation of polyribosomes. If disaggregation of polyribosomes by aflatoxin B_1 is also due to decay of messenger RNA, the rate of disaggregation produced by both

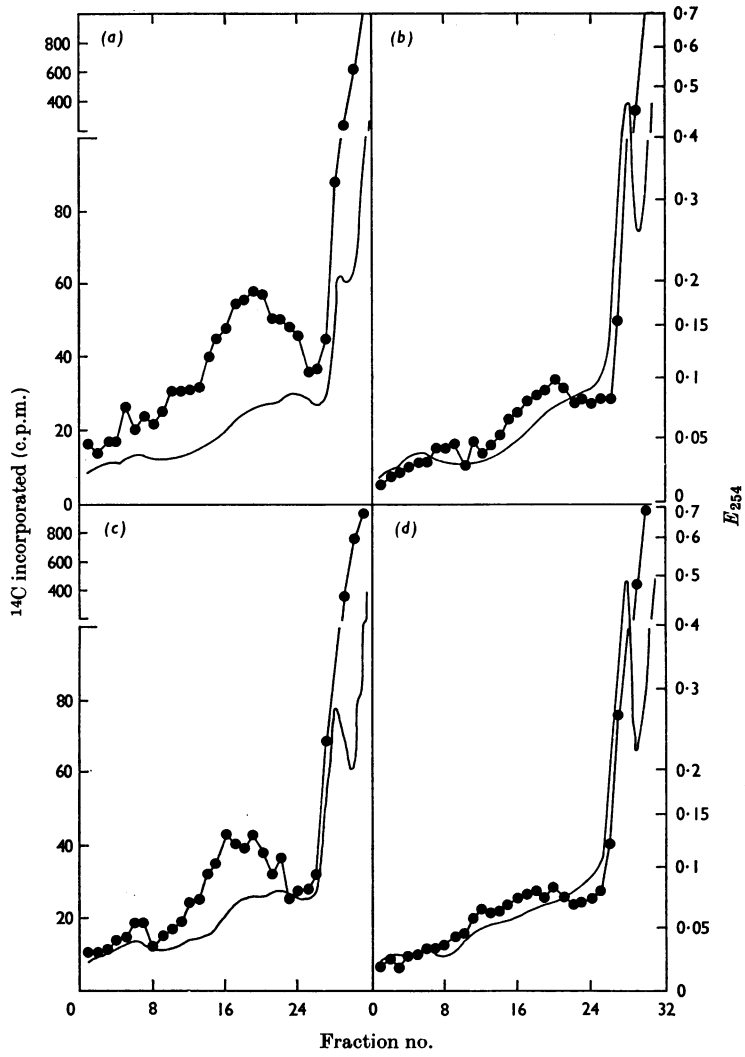


Fig. 7. Polyribosome profiles of cells treated with: (*b*) aflatoxin B_1 (40 μg./ml.) for 3 hr.; (*c*) actinomycin D (2·5 μg./ml.) for 3 hr.; (*d*) actinomycin D (2·5 μg./ml.) for 6 hr.; the control (*a*) is a profile from untreated cells. All cells were labelled with U-^{14}C-labelled chlorella protein hydrolysate for 1 min. before the polyribosomes were fractionated. ●, Radioactivity incorporated; ——, E_{254}.

drugs would be the same. But Figs. 7(*c*) and(*d*) show that cells treated with 2·5 μg. of actinomycin D/ml. for 3 hr. undergo less change in their E_{254} and radioactivity profiles than those treated with aflatoxin B_1 for the same period; only after 6 hr. treatment with actinomycin D do the profiles resemble those from cells treated with aflatoxin B_1 for the shorter period. The rate of disaggregation of polyribosomes by aflatoxin B_1 is therefore faster than can be accounted for by decay of messenger RNA. Further, no direct effect of aflatoxin B_1 on polyribosomes was demonstrable after centrifugation of isolated cytoplasmic preparations of HeLa cells treated with aflatoxin B_1 in concentrations up to 400 μg./ml. *in vitro* for 1 hr. at 4°. Incubation of cytoplasmic preparations with aflatoxin B_1 at higher temperatures was precluded by the instability of polyribosomes *in vitro*.

DISCUSSION

The cytotoxic action of aflatoxin B_1 and its inhibitory effect on DNA, RNA and protein synthesis in HeLa cells are similar to the effects

it produces in rat liver and in other human cells in culture (Legator, Zuffante & Harp, 1965; Zuckerman, Tsiquaye & Fulton, 1967; Zuckerman, Rees, Inman & Robb, 1968). HeLa cells thus provide a suitable system in which to study the effect of aflatoxin B_1 on nuclear RNA synthesis, and in which to compare its action with that of actinomycin D. Although both drugs are cytotoxic in HeLa cells (Clark, Love, Studzinski & Ellem, 1966) and inhibit incorporation of uridine into RNA, our experiments have revealed that their mechanisms of action are quite different. Aflatoxin B_1, at a concentration of 40 μg./ml., which decreases RNA synthesis by about 50%, rapidly inhibits protein synthesis to a marked degree, whereas actinomycin D, in doses that produce a similar depression of RNA synthesis, does not immediately inhibit protein synthesis.

Perry (1964) and Roberts & Newman (1966) have shown that small doses of actinomycin D selectively inhibit the synthesis of ribosomal-RNA precursor, leaving the rapidly labelled heterodisperse RNA relatively unaffected (Penman *et al.* 1968). In our experiments actinomycin D, at a concentration that approximately halved the amount of [^{3}H]uridine incorporated into RNA, completely suppressed the synthesis of ribosomal-RNA precursors, whereas aflatoxin B_1, at a concentration that inhibited [^{3}H]uridine incorporation to the same extent, allowed the continued but diminished synthesis of ribosomal-RNA precursor. This is evident from the easily detectable radioactively labelled peaks in the ribosomal-RNA precursor regions after treatment with aflatoxin B_1 and their absence after treatment with actinomycin D (Fig. 4). The inhibition of RNA synthesis to the same extent in nucleoli and nucleoplasm by aflatoxin B_1 indicates that, in contrast with actinomycin D, aflatoxin B_1 is non-selective in its action on ribosomal and heterodisperse RNA. This suggests that aflatoxin B_1 binds with DNA irrespective of its cellular localization or base composition.

From experiments *in vitro* and *in vivo* much evidence has been adduced to support the view that actinomycin D binds with the DNA helix and inhibits RNA synthesis through its action on DNA-dependent RNA polymerase (Reich & Goldberg, 1964). The failure of aflatoxin B_1 to affect the incorporation of uridine into the nucleotide pool although preventing incorporation of uridine into RNA *in vivo* suggests that its effect on RNA synthesis *in vivo* is similar to that of actinomycin D. But the failure of aflatoxin B_1 to inhibit RNA synthesis *in vitro* by DNA-directed RNA polymerases or rat liver nucleoli (King & Nicholson, 1967; Moulé & Frayssinet, 1968; Clifford, Rees & Stevens, 1967) does not support this suggestion. Our observations that the maturation of the 45s ribosomal-RNA precursor was inhibited by aflatoxin B_1 raises the possibility that the rate of ribosomal-RNA synthesis is controlled by the maturation of the 45s RNA. The effect on maturation could be explained by specific inhibition of methylation. Although we have failed to demonstrate any specific effect of aflatoxin B_1 on methylation, our experiments as far as they go do not exclude this possibility, nor could they unless the effect on methylation leaves [^{3}H]uridine incorporation unaffected.

Several observations on cells in culture suggest that protein synthesis is a necessary prerequisite for normal RNA synthesis (Zimmerman & Greenberg, 1965; Noteboom & Mueller, 1966). Complete inhibition of protein synthesis in L cells by cycloheximide is associated with a relatively greater decrease in the formation of 18s RNA compared with other ribosomal-RNA precursors, and with inhibition of passage of RNA from nucleus to cytoplasm (Ennis, 1966). Although the inhibition of protein synthesis produced by aflatoxin B_1 was not complete nor simultaneous with that of RNA synthesis, it is conceivable that this inhibition of protein synthesis would eventually affect synthesis of RNA.

Our observations that aflatoxin B_1 inhibited DNA synthesis in HeLa cells are similar to that reported by De Recondo *et al.* (1966) for rat liver. Since aflatoxin B_1 interacts *in vitro* with DNA (Clifford & Rees, 1966, 1967) and inhibits DNA synthesis in an *Escherichia coli* DNA polymerase system *in vitro* (L. E. Crook & K. R. Rees, unpublished work) it is probable that the toxin inhibits DNA synthesis by combining with DNA and thereby preventing transcription by DNA polymerase.

Disaggregation of polyribosomes and inhibition of amino acid incorporation into nascent protein chains by aflatoxin B_1 is more rapid than that produced by actinomycin D. This suggests that the mechanisms of action of the two drugs are different. Disaggregation of polyribosomes by actinomycin D is believed to be secondary to its inhibition of messenger-RNA synthesis (Penman *et al.* 1963). The more rapid effect of aflatoxin B_1 indicates that polyribosome disaggregation in this case and its subsequent effect on protein synthesis are not simply the result of inhibition of messenger-RNA synthesis but probably a consequence of some direct interaction between polyribosomes and the toxin or one of its derivatives. Interaction of the toxin with the RNA component of the polyribosomes is possible because interaction between aflatoxin B_1 and RNA has been demonstrated *in vitro* (J. I. Clifford & K. R. Rees, unpublished work); despite our findings that the addition of

aflatoxin B_1 to isolated HeLa-cell polyribosomes, at 4°, did not cause disaggregation or release of nascent protein chains, it is concluded that *in vivo* aflatoxin B_1 does directly disaggregate polyribosomes.

The effects of aflatoxin B_1 on heterodisperse RNA, DNA and protein synthesis that our investigation has revealed are in marked contrast with the effects of actinomycin D. Although some of the effects of aflatoxin B_1 may be accounted for by its interaction with the DNA helix, other factors are clearly involved.

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