against insulin as a source for this biologic effect. In this respect the activity closely resembles the "atypical" serum insulin described by Samaan *et al*(9,10); however, the present characterization of UILA from an obese human subject suggests that it is more closely related to albumin than to unmodified insulin.

Summary. The insulin-like activity found in urine protein from obese subjects during fasting was characterized by benzoic acid precipitate and Sephadex column chromatography. The major portion of the urine insulin-like activity was found in a single protein peak which was homogeneous by polyacrylamide gel electrophoresis, and its electrophoretic mobility was identical to human serum albumin. This active fraction was significantly neutralized by rabbit antiserum to human serum albumin. These results suggest that the insulin-like activity in urine of obese subjects is more closely associated with albumin than with unmodified insulin.

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Effect of Aflatoxin B_1 on the Deoxyribonucleic Acid Polymerase of *Escherichia coli*. (32274)

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Aflatoxin, a mixture of 4 toxic metabolites produced by *Aspergillus flavus*, has been characterized chemically(1-3). Aflatoxin B_1, the most toxic of the aflatoxins, is a potent carcinogen in rats(4,5) and trout(6). Legator (7) and Gabliks *et al*(8) reported that aflatoxin B_1 suppressed mitosis, inhibited DNA* synthesis, and produced giant cell formations in tissue culture in a manner similar to some alkylating agents. Spectroscopy and equilibrium dialysis studies have shown that aflatoxin B_1 binds to both native and denatured DNA *in vitro* (9). Microorganisms sensitive to aflatoxin as determined by growth inhibition include strains of gram-positive, spore-forming bacilli and one streptomycete (10).

Escherichia coli contains an enzyme, DNA polymerase, deoxynucleoside triphosphate: DNA deoxynucleotidyl transferase (EC 2.7.7.7). This enzyme polymerizes deoxynucleotides, as triphosphates, into DNA and the *in vitro* synthesis requires a DNA primer (11). In a study of the effect of lysogenic induction by mitomycin C on DNA polymerase, Pricer and Weissbach(12) described a change in the priming ability of the DNA of induced *E. coli* K 12 λ cells. Other investigators have also reported that mitomycin C acts at the DNA level in *E. coli* B(13).

* The abbreviations used are: DNA, deoxyribonucleic acid; TCA, trichloroacetic acid; EDTA, ethylene diaminetetraacetic acid; TTP, thymidine triphosphate; dCTP, deoxycytidine triphosphate; dATP, deoxyadenosine triphosphate; dGTP, deoxyguanosine triphosphate.

The purpose of this investigation was to examine biological changes and the alteration of DNA synthesis in *E. coli* cultured with aflatoxin B_1 and to compare these effects with those of mitomycin C a known alkylating agent.

Materials and methods. Growth and harvest of cells. For enzyme determinations, cultures of *E. coli* strain W1485† were grown on Koser Citrate Medium supplemented with 0.5% glucose and 0.5% yeast extract (pH 7.0) at 37°C with vigorous aeration. No other chemicals were added to the control cultures. Either crude aflatoxin preparation or crystalline aflatoxin B_1 was added to the culture medium to a final concentration of 1 μg/ml or 5 μg/ml crystalline aflatoxin B_1. Aflatoxin B_1 levels in the medium were determined by thin layer chromatography(14). A water solution of mitomycin C‡ was passed through a Millipore® filter and was added to the medium to a final concentration of 1 μg/ml. After 16 hours incubation, the cultures were harvested by centrifugation at 10,000 $\times$ *g* in the cold and were washed once with water. The cells were stored at −18°C until used.

For temperature studies, *E. coli* cultures were grown at 37° and 42°C with aflatoxin B_1, as above, except that standing cultures were used. Turbidity was measured as optical density of the cultures at 530 mμ on a Beckman B spectrophotometer. The bacteria were examined microscopically for abnormal morphological forms.

Preparation of native DNA primer. The supernatant fluids from aflatoxin B_1-treated, mitomycin C-treated or untreated frozen cells used as primers for *in vitro* DNA polymerase assays, were prepared from *E. coli* as described by Pricer and Weissbach(12). The concentration of each primer preparation was adjusted to contain approximately 22 μg DNA per 0.05 ml.

Preparation of DNA polymerase from E. coli. Packed frozen cells of *E. coli* (0.25 g), previously grown in media containing aflatoxin B_1, mitomycin C, or no added chemical, were suspended in 2 ml cold 0.05 M Tris-hydrochloride buffer (pH 7.4), kept in an ice bath, and disrupted by sonic oscillation with an ultrasonic disintegrator at maximum output for 20 minutes. Aliquots were taken for protein and DNA determinations. The sonicated mixture was centrifuged at 25,000 $\times$ *g* for 15 minutes in the cold, and the supernatant was used for measurement of DNA polymerase activity.

DNA polymerase assay. The DNA polymerase was measured by incorporation of tritiated thymidine 5′-triphosphate into an acid-insoluble DNA fraction. The procedure was a modification of that reported by Pricer and Weissbach(12). The assay mixtures were incubated in conical centrifuge tubes at 37°C. Each mixture had a final volume of 0.3 ml and contained 1 μmole $MgCl_2$, 0.3 μmole 2-mercaptoethanol, 0.25 μmole EDTA, 30 μmoles Tris-ethanolamine buffer at pH 8.75, 0.05 ml native DNA primer, and 3 mμmoles each of dGTP§, dATP¶, dCTP¶, and TTP¶. The radioactivity was added as 0.0587 mμmoles ^{3}H-TTP‖ containing 1.8 $\times$ 10^5 cpm. The enzyme preparation was added at levels of 0.005, 0.010, 0.015, or 0.020 ml and represented 100 to 300 μg protein per reaction vessel. This amount of protein produced a linear response of ^{3}H-TTP-incorporating activity between 10 and 30 minutes incubation. At the end of a 10 minute incubation period, the tubes were chilled at 0°C, and 0.2 ml of salmon sperm DNA (2.5 mg per ml), 1.0 ml water, and 4 μmoles of ATP were added with mixing. The tubes were heated at 100°C for 2 minutes and cooled to 0°C. Cold 10% TCA (3 ml) was added to each tube and contents were mixed. The precipitates were separated by centrifugation and were washed twice with cold 5% TCA. The precipitates were then dissolved in 0.5 ml concentrated formic acid and aliquots were transferred to vials containing 15 ml Bray's scintillation fluid(15). The counts per minute (cpm) were

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‡ Cancer Chemotherapy National Service Center, Nat. Cancer Inst.

§ Nutritional Biochemicals Corp., Cleveland, Ohio.

¶ California Corporation for Biochemical Research, Los Angeles, Calif.

‖ H-Thymidine 5′ triphosphate (4.8 curies/mM) was purchased from Schwarz Bioresearch Inc., Orangeburg, N. Y.

TABLE I. Effect of 5 μg/ml Aflatoxin B_1 and 1 μg/ml Mitomycin C on Total μg DNA per mg Protein of *E. coli* Cells Grown at 37°C.

Treatment	μg DNA/mg protein*	% of control
Control	111.6	100
Aflatoxin	66.73†	60
Mitomycin	70.25†	63

* Average of 5 independent experiments.
† Values significantly lower ($P < .025$) than corresponding control.

recorded by a Nuclear-Chicago spectrometer (Mark I) and were quench-corrected to disintegrations per minute (dpm). The counts obtained at 0 time were subtracted from those obtained after a 10 minute incubation.

Protein was assayed by the method of Lowry *et al*(16). DNA was determined according to the method of Webb and Levy(17). Thymidine kinase (ATP: thymidine 5'-transferase EC 2.7.1.21) was assayed as described by Childs and Legator(18). Data were statistically evaluated by ranking of unpaired replicates to determine the significance of differences between two treatments(19).

Results. Turbidimetric measurements of *E. coli* W1485 grown at 37°C indicated that this strain was relatively insensitive to aflatoxin B_1 at this temperature. However, aflatoxin had a definite inhibitory effect at 42°C. When the cultures were grown with 1 or 5 μg aflatoxin per ml of culture medium, the turbidity was reduced to 36 or 19% of the control values, respectively. These results were observed with either crude or crystalline aflatoxin B_1. Some elongated filaments were formed by the bacteria at 37° and 42°C, but at 42°C their reversion to normal forms by cytokinesis did not occur.

Table I shows the average DNA per mg protein of 5 independent experiments. The μg DNA per mg protein in bacteria exposed to 5 μg aflatoxin B_1 or 1μg mitomycin C was reduced to 60% or 63% of the control value, respectively. DNA synthesis was depressed significantly ($P < .025$) with aflatoxin B_1 and mitomycin C.

The results of a representative DNA polymerase assay are shown in Table II. Similar results were obtained in 4 additional experiments. The incorporating activity of DNA polymerase from *E. coli* cells grown with either aflatoxin B_1 or mitomycin C was significantly lower ($P < 0.005$) than the controls and the difference was significant regardless of the source of primer DNA. When the amount of control DNA primer or aflatoxin DNA primer in the assay was increased from 22 μg to 44 μg, the incorporating activity of the DNA polymerase of the control cells increased. No change in the incorporating activity of DNA polymerase from aflatoxin-treated cells was found with increased concentration of either DNA primer.

Thymidine kinase activity in extracts of *E. coli* did not change as a result of treatment with either aflatoxin or mitomycin.

Discussion. These data show that the DNA synthesis was significantly inhibited in *E. coli* grown in media containing 5 μg/ml of aflatoxin. The elongated filaments of *E. coli* observed at 37°C were also observed at 42°C. However, at the higher temperature, turbidity was reduced with increasing aflatoxin concentration. Appearance of abnormal morphological forms and decreased DNA synthesis indicates that the mechanism of cell division in *E. coli* was somewhat damaged by aflatoxin similar to the inhibition of DNA synthesis observed in tissue cultures(7,8). Aberrant morphological forms of *Flavobac-*

TABLE II. Incorporation of ^{3}H-Thymidine 5'-Triphosphate into DNA with Appropriate Primer DNA.*

Source of DNA polymerase	DPM × 10^3 per mg protein†		
	Control DNA primer	Aflatoxin DNA primer	Mitomycin DNA primer
Control cells	184.5	154.5	178.9
Aflatoxin-treated cells	83.6‡	56.5‡	79.9‡
Mitomycin-treated cells	94.2‡	52.1‡	68.8‡

* 22 μg primer DNA used per assay.
† Average of 9 replicate sample readings.
‡ Values significantly lower ($P < .005$) than the corresponding control.

terium aurantiacum have also been observed in the presence of aflatoxin B_1(20).

The inhibition of DNA synthesis by mitomycin C in *E. coli* is consistent with reported findings(21). Mitomycin C, like aflatoxin(2, 9), binds to DNA and is rendered toxic(22). Both alkylating agents, including mitomycin C(23), and ionizing radiation induced filament formation in *E. coli*(24). Adler and Hardigree(25) observed no cytokinesis in mitomycin C-treated filaments at 42°C; however, they observed cytokinesis in irradiated filaments at this temperature. Other investigators(26,27) have suggested that cell division repression with the production of long nonseptate filaments is controlled by a single gene in *E. coli*.

The inhibition of DNA synthesis in *E. coli* cultured with either aflatoxin B_1 or mitomycin C may result from decreased incorporating activity of DNA polymerase. The lack of effect on thymidine kinase indicates that aflatoxin and mitomycin do not influence a general enzyme inhibition. Many investigators(11,12,28-31) report that the state of the primer affects the action of DNA polymerase in bacterial and mammalian systems. However, our results show that the major effect of aflatoxin B_1 and mitomycin C in this investigation was to reduce DNA polymerase activity, regardless of the source of primer.

Summary. Aflatoxin B_1 and mitomycin C similarly affected *E. coli* cultures by (a) producing filamentous forms, (b) decreasing the incorporating activity of DNA polymerase, and (c) reducing the synthesis of DNA/mg protein.

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