Final Report

Aflatoxins in Mammalian Cell Cultures

Contract N00014-67-C-0252
~~Grant No. NR 305-779~~

Department of the Navy

Office of Naval Research

Biochemistry 442

Principal Investigator: Ronald A. Chung
Carver Research Foundation
Tuskegee Institute, Alabama 36088

ABSTRACT

KB and HeLa mammalian tissue culture cells were cultured in the presence of 0.4, 1, and 4 ppm of aflatoxin. The incorporation of labeled uridine into the different RNA components separated by sucrose gradient ultracentrifugation and by methylated albumin column chromatography was studied.

All levels of aflatoxin increased the proportion of incorporation into RNA in the 4s region after 4 hours and 12 hours of exposure. The longer the time of exposure the more severe was the effect. It is not known whether this is the result of depolymerization or of stimulation of t-RNA synthesis or both.

Introduction

The biological effects of aflatoxins include lethality in animals receiving large dose histological changes in animals receiving small doses for extended periods and tumor induction (Wogan, 1966). Early field observations showed the duckling to be the most susceptible to acute aflatoxin poisoning. But some other animal species have recently been studied. Moule and Frayssinet (1968) administered 500 ug/kg of aflatoxin B_1 to partially hepatectomized rats, and reported that the inhibition of transcription primarily concerned nuclear RNA, preferentially nucleolar RNA as shown by a 60% inhibition of ^{3}H-thymidine incorporation into DNA. Inhibition of incorporation of ^{14}C-orotic acid into RNA was 80%. Therefore, the dissappearance, as early as 15 minutes after aflatoxin treatment, of newly synthesized 45s RNA was thought to be accompanied by a segregation of the morphological components at the ultrastructural level.

Clifford and Rees (1966) and Sporn et al., (1966) studied the alteration, in vivo, of RNA metabolism in rat liver by aflatoxin. Sporn et al. reported marked blocking of cytidine incorporation into nuclear RNA within 70 minutes after treatment with 0.5 mg of aflatoxin B_1, and a lowering of the RNA:DNA ratio of the nucleus. Clifford and Rees (1966) who reported an inhibition of incorporation of ^{14}C-orotic acid into the ribosenucleic acid of the rat liver cell nucleus, observed no inhibition of incorporation of orotic acid into the nucleotide pool. However, they observed a marked inhibition of incorporation of nucleotide precursors into nuclear RNA. This would suggest an inhibition of RNA polymerase, the only enzyme which functions between the two stages.

To further investigate the effects of aflatoxins on protein synthesis in vivo Clifford and Rees (1966) studied the induction of tryptophan pyroll-

ase by substrate or cortisone in the livers of control and B_1-treated rats. While they observed no inhibition in the substrate induced enzyme, they reported a 51% inhibition in the cortisone-induced enzyme.

High incidence of hepatomas in various animals fed contaminated peanut meal and other diets has been reported. In rats liver and renal damage, and bile duct hyperplasia have been reported (Newberne et al., 1964). Ashley et al. (1965) and Sinnhuber et al. (1965), have reported hepatocarcinoma in trout and rainbow trout, respectively. In ducklings, Prickett and Salmon (1965) observed massive infiltration of the liver, pancreas and kidneys by a homogeneous non cellular "collagenous" or amyloid matrix among other findings.

Extensive investigations have been conducted to determine the effects of aflatoxins in various assay test system such as cells in culture and embryonated eggs.

Juhasz and Greczi (1964) showed methanol extracts of aflatoxins (from peanut meal) to be toxic to calf kidney cells. Legator and Winthrow (1964) reported the inhibition of mitosis in human embryonic lung cells by small aflatoxin concentrations. Gabliks et al. (1965) reported the destruction (T_D50) of human liver and HeLa cells as well as primary cell cultures of whole duck embryos when cultures were exposed to 1-4 ppm.

Various reports of in vitro studies of effects of aflatoxin on protein synthesis have been released. Wogan (1966) reported toxicity of aflatoxin to chick embryos. Clifford and Rees (1966) and Sporn et al. (1966) reported various effects of aflatoxin on protein metabolism. Sporn et al. (1966) suggested that the observed binding of aflatoxin to DNA suggested that this binding was a crucial aspect of carcinogenic properties. Clifford and Rees who used liver slices reported an immediate inhibition of incorporation of

orotic acid into RNA, a later inhibition of leucine incorporation into protein, but observed no inhibition of the entry of amino acids into the liver slice.

Recently, the effect of aflatoxin on embryonated eggs was studied. Townsley and Lee (1967) reported that aflatoxin B_1 inhibits cell cleavage in fertilized mollusk eggs without preventing fertilization or nuclear division. This reaction is very dramatic since fertilized eggs in the presence of aflatoxins were multinuclear, whereas the controls were multicellular larvae.

In studying the mode of action of the aflatoxins, it is important to understand the metabolic fate of these compounds in animals.

DeIongh et al. (1964) observed that lactating cattle fed subtoxic levels of aflatoxin containing peanut meals excreted in their milk a compound which was toxic to animals. Although no aflatoxin B_1 was detected, this violet fluorescent "milk-toxin" or M_t aflatoxin caused lesions similar to those caused by aflatoxin B_1. The metabolic fate of aflatoxins in rats has been studied in rats by Adye and Mateles (1964) and by Wogan (1966). The B_1 aflatoxin labeled in the methoxy carbon was obtained by adding methyl methionine to the culture medium. The C^{14} appeared in aflatoxin B_1 only in the methoxy function. By adding acetate-1^{14}-C as the only source of carbon, the label was detected only in ring carbons (Wogan, 1966).

In a study of the distribution and exretion of C^{14} during a 24 hour period after administration of labeled methoxy compounds to rats, Shank and Wogan (1965) observed a recovery of 25-30% of C^{14} in CO_2; 25% in urine, 25% in the feces and intestines and 6-9% in the liver. When labeled ring compounds were used, however, the radioactivity was largely excreted in the feces, the liver retaining only 6-9%.

Methods and Procedure

Cell Strain

The KB cell line used to study cellular responses to aflatoxin B_1 was maintained in Carver Research Foundation Laboratory of Tuskegee Institute. The original strain was obtained from Dr. Dixon at Southern Research Institute on February 6, 1958.

It was isolated from a human carcinoma of the mouth by Harry Eagle in 1954, in a medium consisting of basal medium (Eagle 90 percent, human serum 10 percent) (Eagle, 1955) and in the course of 350 subsequent transfers has been adapted to 5 percent calf serum.

The KB cell line has been used extensively in the studies of cell metabolism, cancer chemotherapy screening, tumorigenicity and viruses.

It has a generation time of 30 hours (Karon et al., 1965).

Isotopic Tracer

L-uridine-2-C^{14} (Specific activity 51.5 mc/mM) uniformly labeled was purchased from the New England Nuclear Corporation, Boston, Massachusetts. This tracer was diluted to a concentration of 2 uc/ml with sterile distilled water and kept at 5°C as a stock solution.

Aflatoxin

The aflatoxin B_1 used in this study was produced from isolate ATTC 15517 of Aspergillus flavus Linke x Fries according to a modified method of et al. (1966) and Davis et al. (1966).

The basal medium (YES) used for culturing A. flavus contained 2% yeast extract (Difco) and 20% sucrose.

Five liter flasks containing 1 liter of medium per flask were autoclaved for 15 minutes at 20 lb. p.s.i. The pH of the medium was adjusted to 5.9 afte:

which the medium was inoculated with spores from stock (1-3 weeks old) cultures of *A. flavus* and incubated at 28°-30°C. for 10-14 days as stationary cultures.

Aflatoxin was extracted from the filtrates by refluxing for 1-3 hours with chloroform in a 90°C water bath. The extracts (lower layer in the separatory funnel) were filtered through cheese cloth, pooled and concentrated in vacuo to 30-50 ml. They were dried over anhydrous sodium sulphate which was subsequently removed by filtration. The clarified filtrate was concentrated in vacuo, and the concentrate (5-10 ml) treated with 10 volumes of hexane at -20°C. Crude aflatoxin was precipitated and was dried in vacuo.

The silicic acid column used for chromatography was prepared by packing silicic acid as a slurry in 1% ethanol in chloroform (v/v) into a chromatographic tube (28 x 400 mm). The final dimensions of the column were 2.8 by 20 cm. The aflatoxin components were eluted with 1% ethanol in chloroform and 20 ml fractions collected with an automatic fraction collector.

Samples were anlayzed by thin layer chromatography using chromatoplates prepared with Macherey Nagel D516 MN kieselgel G-HR adjusted for aflatoxin separations and obtained from Brinkman Instruments, New York. The chromatograms were developed with chloroform-aceton 9:1 (v/v) (Eppley, 1966) and the chromatoplates viewed with a UVL-22 ultra violet lamp obtained from Ultra Violet Products Incorporated, San Gabriel, California. Aflatoxin components were recovered from chromatograms and purified by repeated thin layer chromatographic analysis until an intense blue band (R_F 0.55) (Asao *et al.*, 1965) was obtained.

The aflatoxin B_1 obtained from thin layer chromatography was dissolved in chloroform and concentrated in vacuo to 5 ml. The clarified solution

was treated with 2 volumes of hexane overnight at -20°C. Crystals were collected by evaporating the supernatant with a continuous stream of nitrogen.

Confirmation of the presence of aflaxtoxin was performed according to the method of Andrellos et al. (1964).

To a small portion of purified aflatoxin in a test tube was added 0.2 ml of glacial acetic acid and 1 drop of thionyl chloride. The tube was stoppered and its contents shaken till a homogenous solution was obtained.

After standing for 5 minutes at room temperature, the solution was evaporated to dryness in a hot water bath under a stream of nitrogen, the solution and residue being kept at the bottom of the tube. The residue was then redissolved in 20 ml of chloroform (redistilled) and about 0.15 ml applied on a thin layer plate. 0.1 ml of standard was similarly treated, when R_Fs of 0.50 and 0.43 were obtained for standard and for thionyl chloride-treated aflatoxin.

Experimental Design

Cell Culture Method

A stock solution of aflatoxin containing 4ppm was prepared by dissolving 1 mg (0.001g) of aflatoxin B_1 in 0.2 ml propylene glycol in a small vial (Legator, 1966). This solution was treated with 10 ml of Eagle's 90 Calf Serum 10 medium and filtered over Swinnex 25 millipore ($1 \pm 0.1\ \mu$) (25mm) filter purchased from the Millipore Corporation, Bedford, Massachusetts. The resulting solution was made up to 250 ml with more medium. 180 ml of this solution (containing 0.144 ml 1,2-propane-diol) was used for experimental purposes. The other two concentrations were prepared by diluting 45 ml of stock solution to 180 ml (1.0 ppm) and by diluting 18 ml of stock solution

to 180 ml (0.4 ppm). All four concentrations contained 0.144 ml propylene glycol/180 ml medium. The solutions were poured into Pyrex 9825 (16 ml) centrifuge tubes in 10 ml portions and frozen at -8°C.

KB cells were incubated in Eagles basal medium at 37°C. for 48 hours and were then exposed to 0, 0.4, 1.0 and 4.0 ppm of aflatoxin B_1, and uridine-12^{14}-C. Cultures exposed for 1 hour received 1.0 uc while those exposed for 4 and 12 hours were treated with 0.2 uc. Two sets of experiments were run. Experiments were evaluated individually.

RNA Extraction

At the end of the labeling period, cells were harvested and the RNA extracted according to the method of Scherrer and Darnell (1962).

The incorporation of radioactivity was stopped by washing the cell pellet with chilled 0.14M NaCl followed by Earle's saline solution. The pellet was dissolved in 2.0 ml of 10^{-2}M sodium acetate buffer (pH 5.1) containing 2 ug/ml polyvinyl sulphate (PVS). Ten percent (10%) sodium dodecyl sulphate (purified) was added to a final concentration of 0.5% (0.1 ml). After lysing for 5 minutes, the reaction mixture was treated with an equal volume of hot (60°C) 90% redistilled phenol (Karon et al., 1965). The reaction mixture was shaken for 3 minutes in a 60°C. water bath and quickly brought to 4°C. by chilling in a -20°C. ice bath.

The emulsion was broken by centrifuging at 20,000g (15-16000 rpm) for 1 minute on head 874 of the Model B-20 of the IEC refrigerated centrifuge. After carefully removing the aduqous layer, extraction was repeated twice on this layer and the resulting extract treated with 0.05 ml 10% sodium dodecylsulphate (SDS), a few drops of polyvinyl sulphate 0.2 ml 0.1M NaCl and two volumes of chilled 95 percent ethanol (Suzuki and Kolgore, 1967).

The reaction mixture was frozen for 6-12 hours at -20°C after which the precipitate was removed by centrifugation. The precipitate was washed several times with cold ethanol to remove traces of phenol.

RNA Separation

Methylated Bovine Albumin

Bovine albumin used in preparing the methylated bovine albumin-coated kieselguhr (MAK) column was prepared according to the method of Mandell and Hershey (1960).

Crystalline bovine albumin (5 g) purchased from Johns-Manville Company, New York, was weighed accurately and placed in a large (1 liter) erlenmeyer flask. To this was added 500 ml absolute methyl alcohol and 4.2 ml (12 N) concentrated hydrochloric acid.

The mixture was placed in a dark chamber and was stirred occasionally. After 5 days, the mixture was removed, shaken vigorously and poured into 50 ml centrifuge. After centrifugation, the precipitate was washed twice with methanol and twice with anhydrous diethyl ether in the centrifuge.

The ether was evaporated in air and finally by suction (in vacuo over) KOH).

The white powder obtained was stored over KOH in a dessicator.

The MAK-Column

The methylated albumin-coated kieselguhr (MAK) column used in separating the RNA components was prepared according to the modified method of Mandel and Hershey (1960) and Suzuki and Kilgore (1967).

A suspension of 4 gm. of kieselguhr (Merck) (purified and calcined) obtained from Brinkman Instruments Incorporated, New York, in 20 ml of 0.1M NaCl (containing 0.05M phosphate buffer pH 6.8) was boiled for 5-10 minutes

to expel carbon dioxide. It was rapidly cooled by placing in a regrigerated contrifuge at -20°C. To the suspension was added 1.0 ml of a 1% solution of esterified albumin and the mixture was stirred for 15 minutes. After adding 2.0 ml buffered saline (0.1M NaCl) solution and stirring for 5 minutes, the suspension was transferred to a 20 x 200 mm tube, and washed with several 10 ml portions of 0.1M NaCl. Elution was achieved with a pump delivering 3 lb psi and the final volume of the washings was 250 ml. Equilibration was accomplished by washing the column with 1 liter of 0.10M buffered saline under air pressure (gravitational flow), followed by 250 ml of 0.3M phosphate -buffered saline solution under air pressure of 3 lb psi with a small pump.

RNA Separation

A sample containing less than 1.0 mg of RNA in 2.0-2.5 ml of 0.01M sodium acetate buffer (pH 5.1) containing 10^{-4}M Mg^{++} and 0.05M NaCl was applied to the column and washed with 5 ml portions of 0.3M NaCl (total volume 20 ml). RNA was eluted with a linear gradient of 0.3M - 1.5M NaCl at an adjusted flow rate of 2.0 - 2.2 ml/min, and 5 ml fractions were collected with an automatic fraction collector. All saline solutions were buffered with 0.05M phosphate buffer and adjusted to a pH of 6.8 (Suzuki and Kilgore, 1967).

RNA components were separated by ultracentrifugation using a linear sucrose gradient from 5-20% w/w concentration and centrifuged for 6-10 hours at 40,000 rpm in a Beckman model L preparation ultracentrifuge.

Tissue culture cells were homogenized and then fractionated by centrifugation at 700xg for 20 min, at 13,000xg for 20 min, and at 144,880xg for 30 min to precipitate nuclear, mitochondrial, microsomal and supernatnant fractions.

RESULTS AND DISCUSSION

The patterns of change in RNA specific activity in nuclear, mitochondrial and microsomal fractions of HeLa cells exposed to the various levels of aflatoxin were not different (Figures 1-3). However, in Figure 4, specific activity of RNA in supernatant fraction of cultures exposed to all levels of aflatoxin was higher at 8 hour than that of the control culture (0 ppm). But by 12 hours all cultures were similar.

Figures 5-8 show RNA components separated by sucrose gradient ultracentrifugation and Figures 9-12 show RNA components separated by methylated albumin (MAK) column chromatography. After 1 hour there was very little effect on the radioactivity of any RNA component but by 4 hours the incorporation of labeled uridine into RNA components in the 4s region of all cultures exposed to aflatoxin was greater than that of the 0 ppm control cultures. This effect persisted up to 12 hours with a tendency to be greater than at 4 hours. The increase in RNA specific activity at 8 hours in the supernatant fraction (Figures 1-4) may be explained in part by the increased incorporation of labeled uridine into RNA components at the 4s region. Whether this is a reflection of degradation of higher molecular RNA or to a stimulation of soluble RNA synthesis is not clear from this study.

Several workers (Moule and Frayssinet, 1968; Clifford and Rees, 1966; Sporn et al., 1966) have reported that aflatoxin inhibit the incorporation of labeled precursors into nuclear RNA. In the present study, the primary

effect of aflatoxin was on RNA in the 4s region which include, to a large extent, t-RNA. However, in view of the findings of Pong and Wogan (1968) that polysomes were degraded into dimers and monomers as a result of aflatoxin treatment, it could be possible that the increased incorporation into 4s RNA reflects depolymerization of higher molecular weight RNAs.

Shoental (1966) has suggested that the action of aflatoxin on nuclear and cytoplasmic components appears to be reversible unless necrosis occurs. It is not known whether mammalian cells, in the present study, if allowed to grow under normal conditions after exposure to aflatoxin, would again maintain normal metabolic patterns.

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Publications resulting from this grant:

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Mgbodile, Marcel U. K. Effect of Aflatoxins on RNA Synthesis in KB Cells. M. S. Thesis. Tuskegee Institute, August, 1969.

Emeh, Chukwudi Obi. Aflatoxin Effect on the Kinetics of RNA Synthesis in KB Cells. M. S. Thesis. Tuskegee Institute, June, 1969.

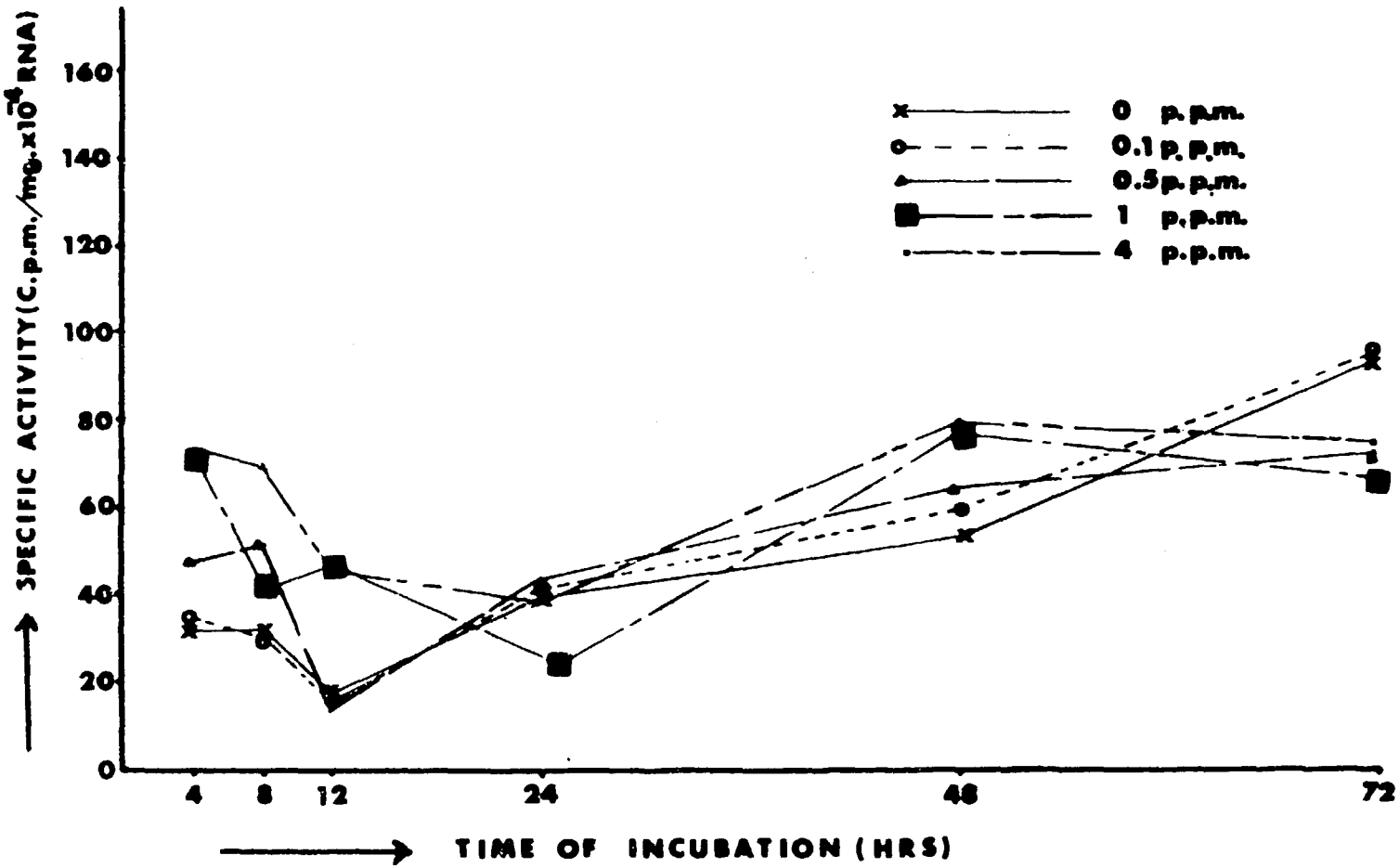

FIGURE 1. EFFECT OF AFLATOXIN B_1 ON SPECIFIC ACTIVITY OF RNA IN NUCLEAR FRACTION OF HELA S CELLS

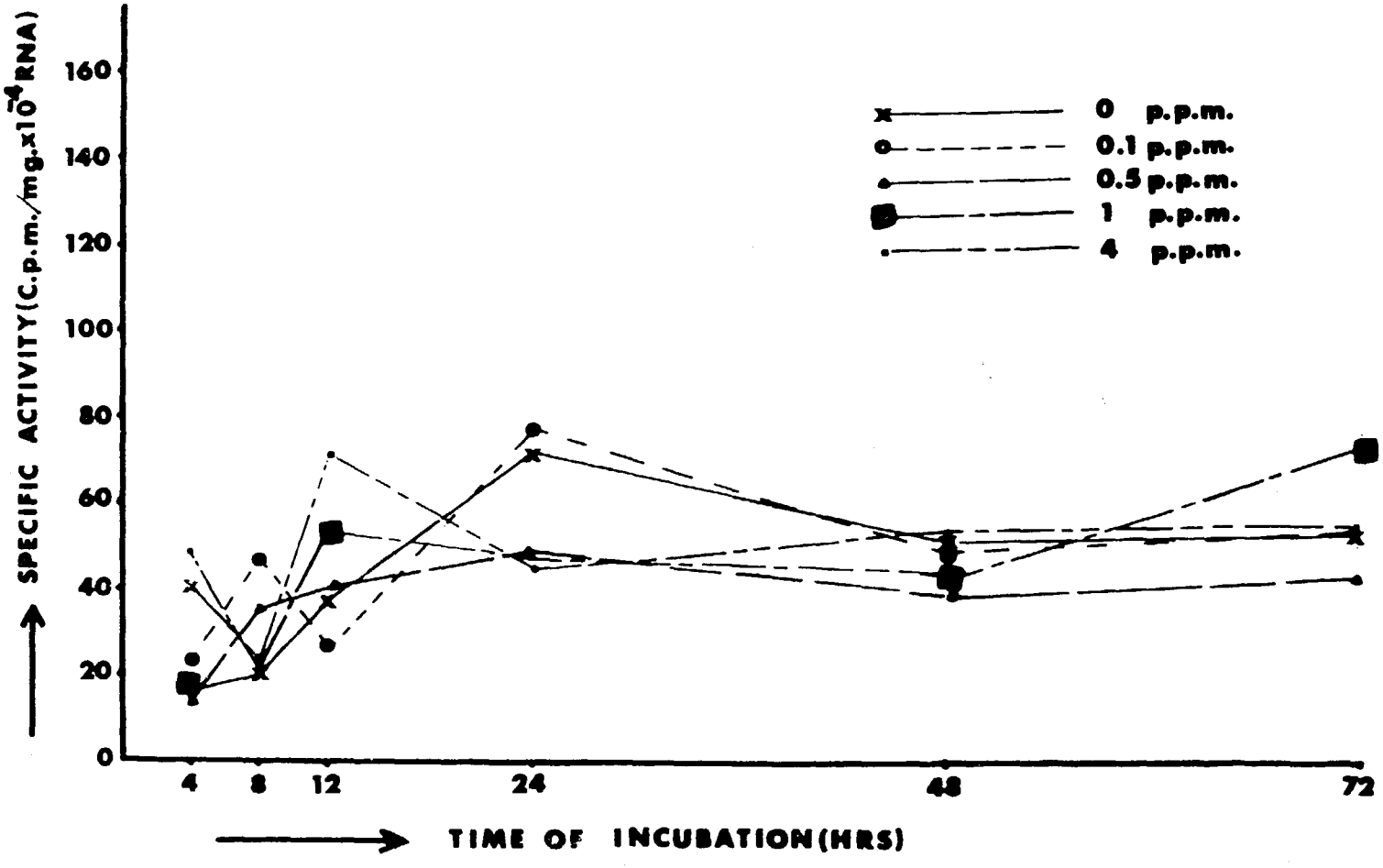

FIGURE 2. EFFECT OF AFIATOXIN B_1 ON SPECIFIC ACTIVITY OF RNA IN MITOCHONDRIAL FRACTION OF HELA S CELLS

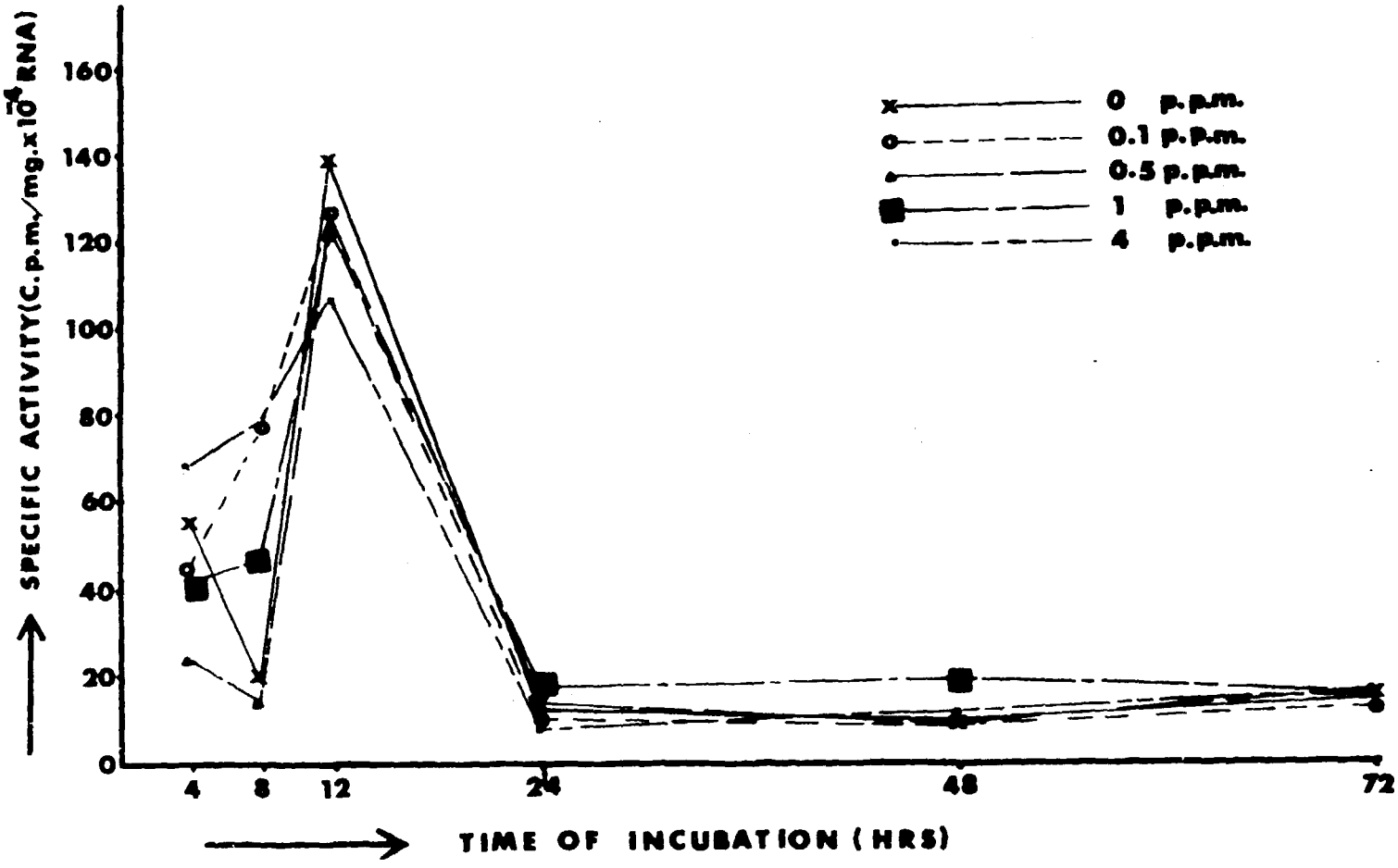

FIGURE 3. EFFECT OF AFLATOXIN B_1 ON SPECIFIC ACTIVITY OF RNA IN MICROSOMAL FRACTION OF HELA S CELLS

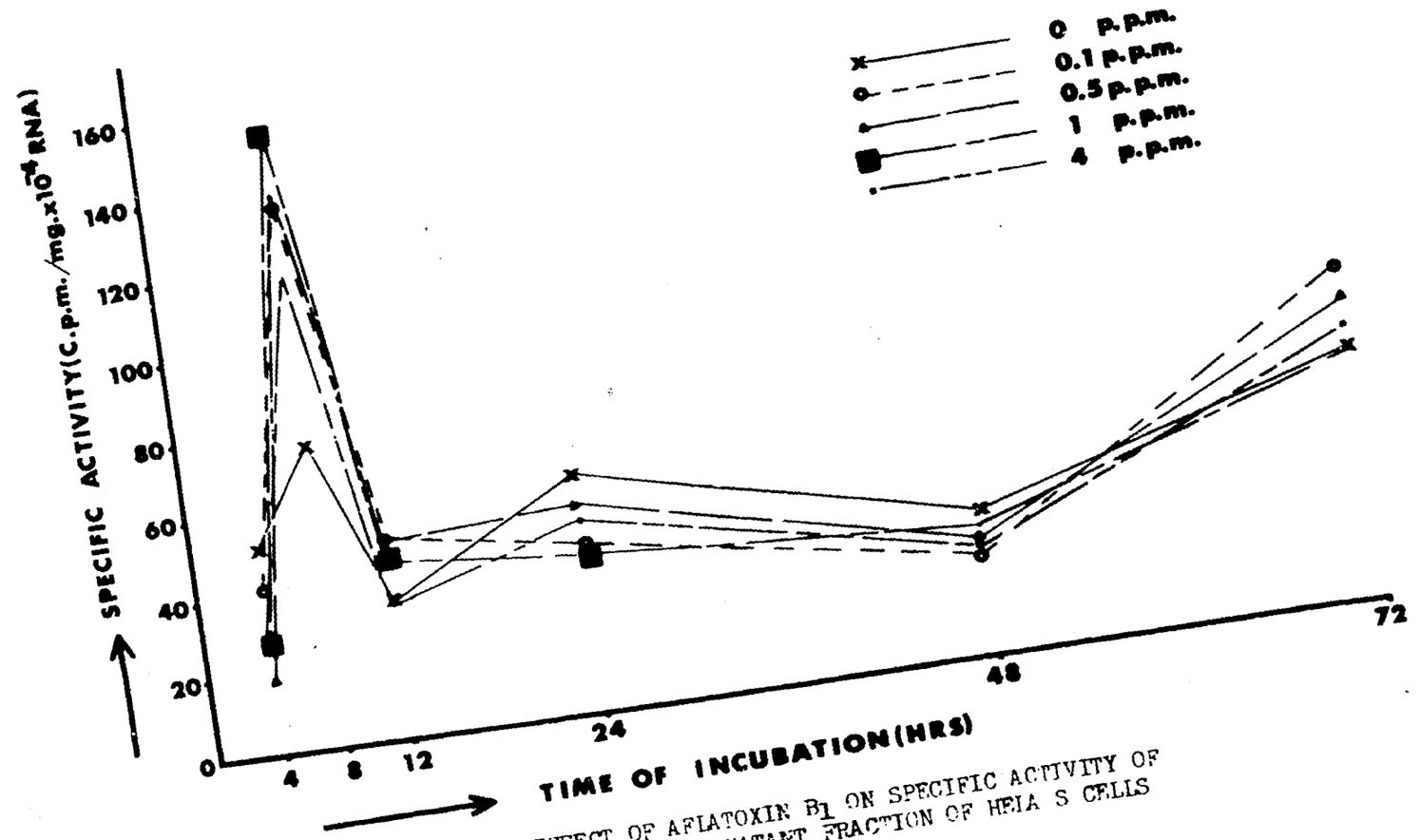

FIGURE 4. EFFECT OF AFLATOXIN B_1 ON SPECIFIC ACTIVITY OF RNA IN SUPERNATANT FRACTION OF HELA S CELLS

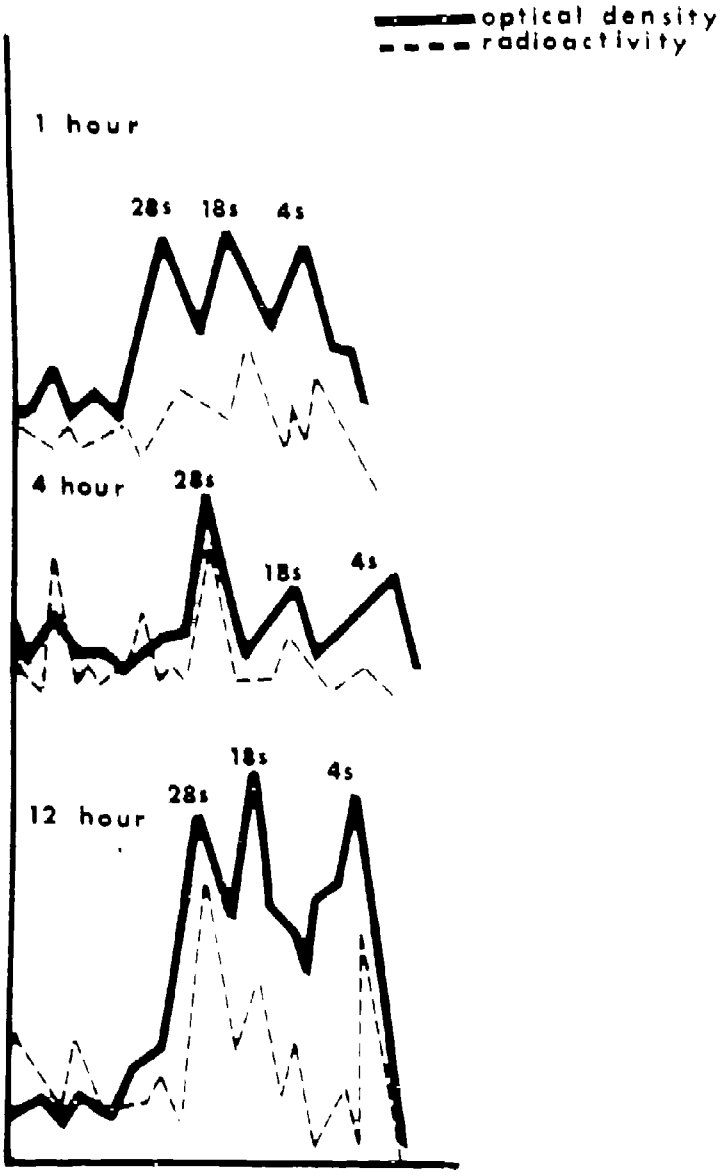

Figure 5. Sucrose gradient— o ppm aflatoxin

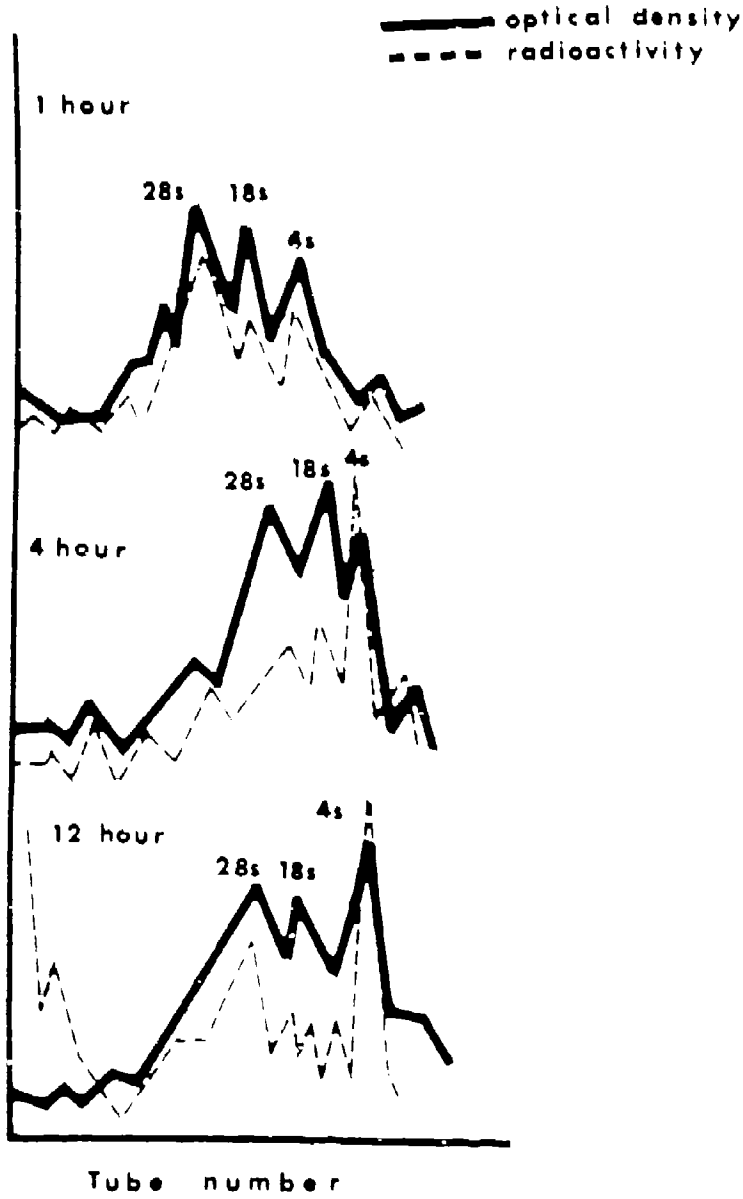

Figure 6. Sucrose gradient--4 ppm aflatoxin

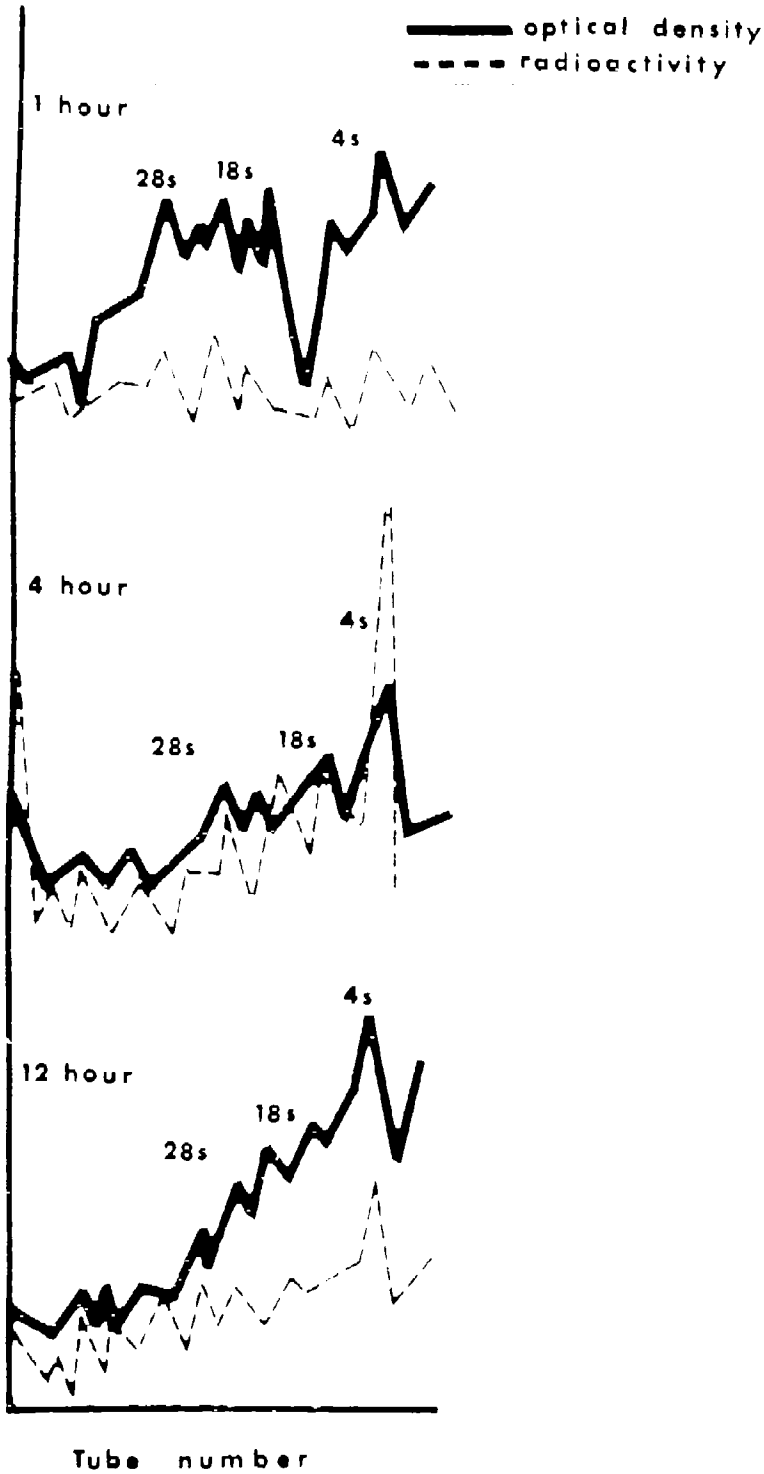

Figure 7. Sucrose gradient - 1.0 ppm aflatoxin

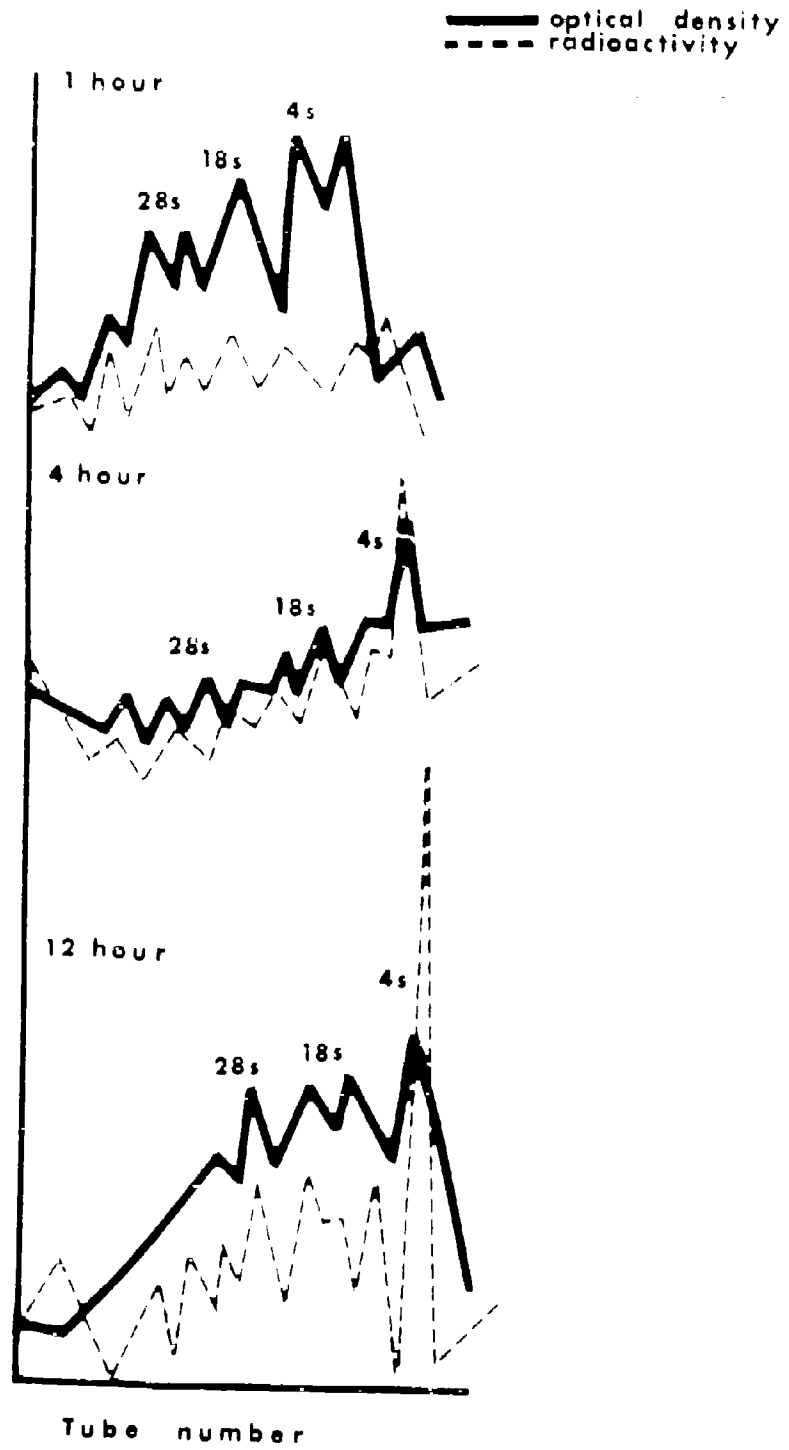

Figure 8. Sucrose gradient-4.0 ppm aflatoxin

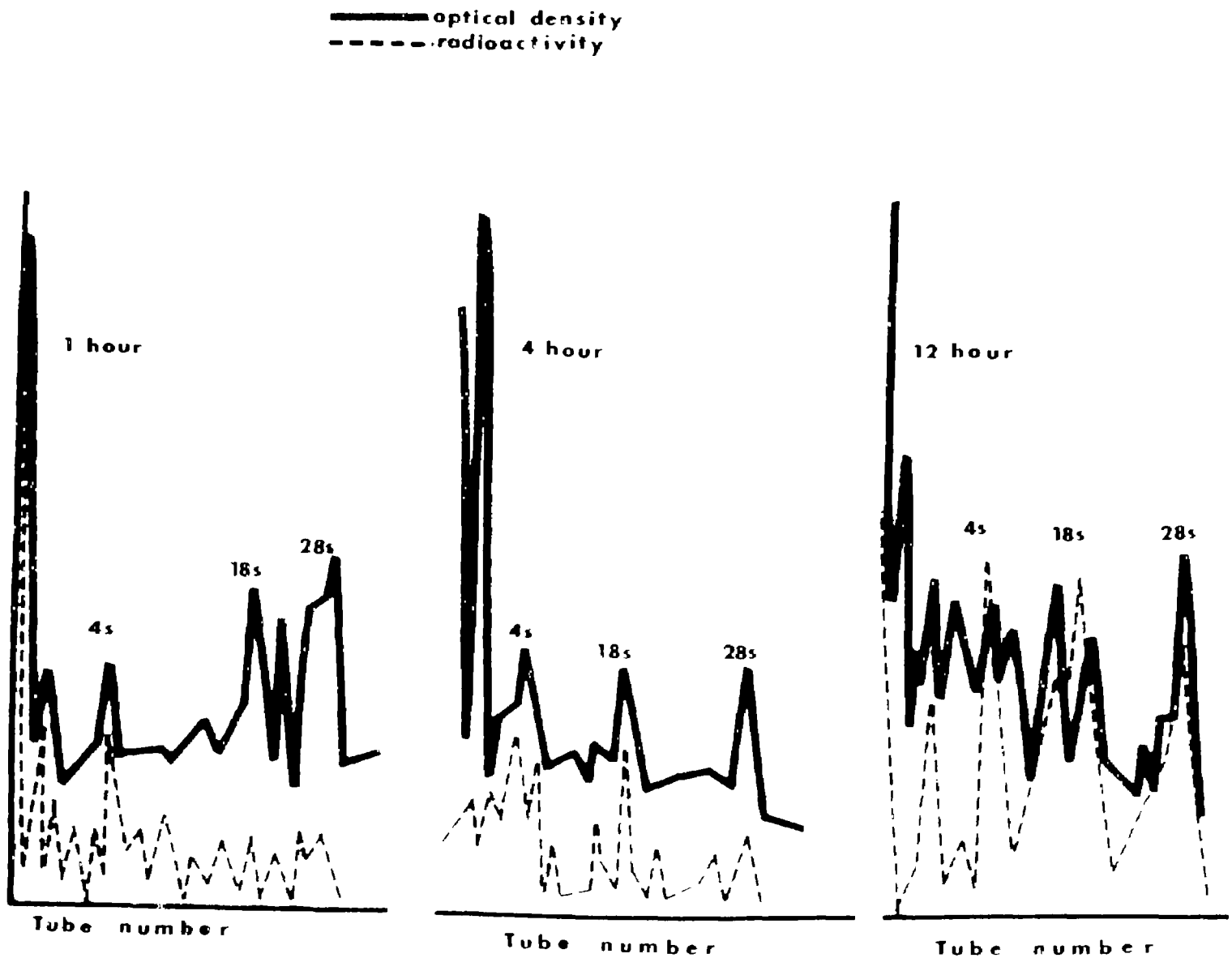

Figure 9. MAK column – O ppm aflatoxin

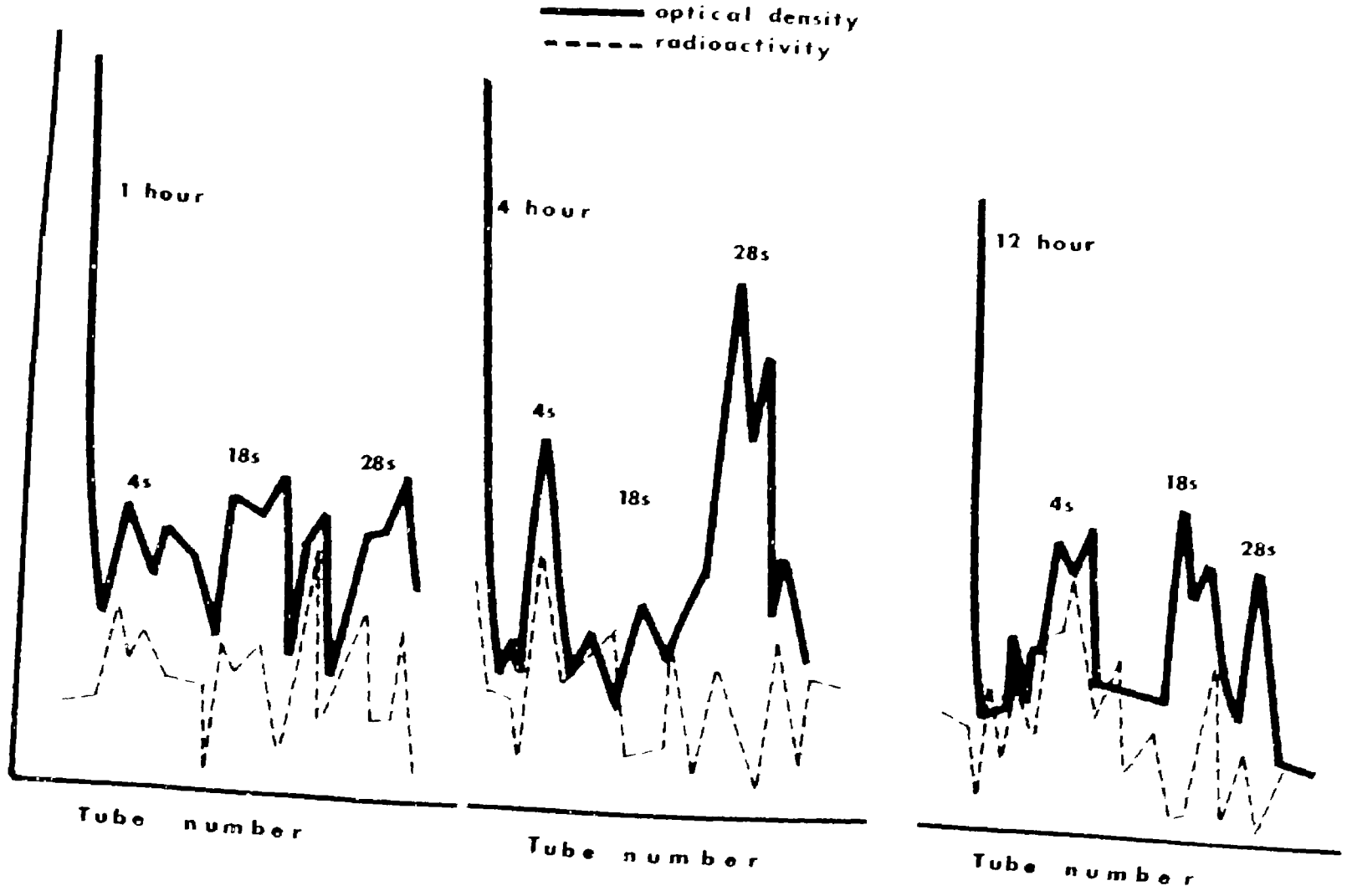

Figure 10. MAK column - 0.4 ppm aflatoxin

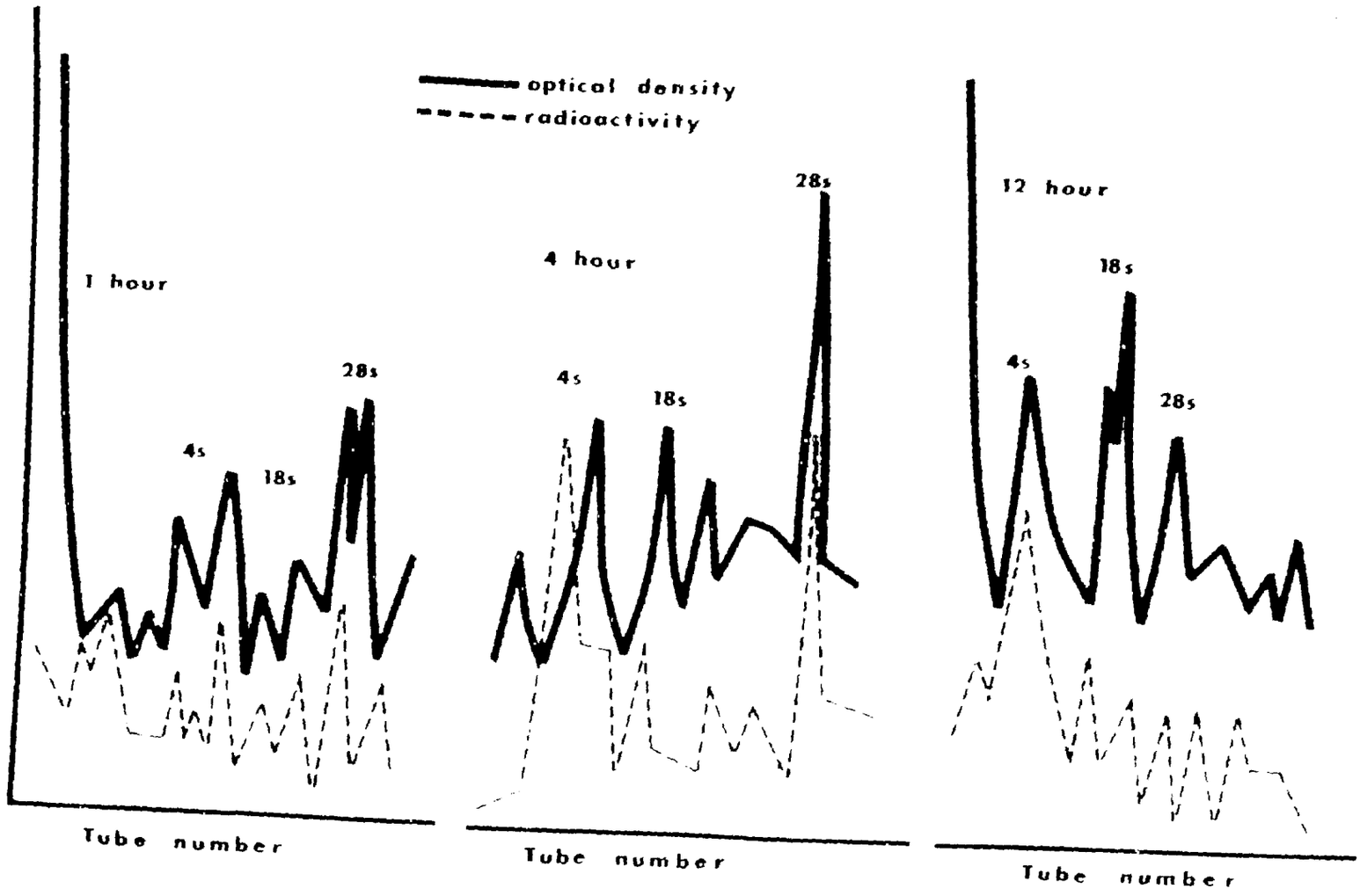

Figure 11. MAK column - 1.0 ppm aflatoxin

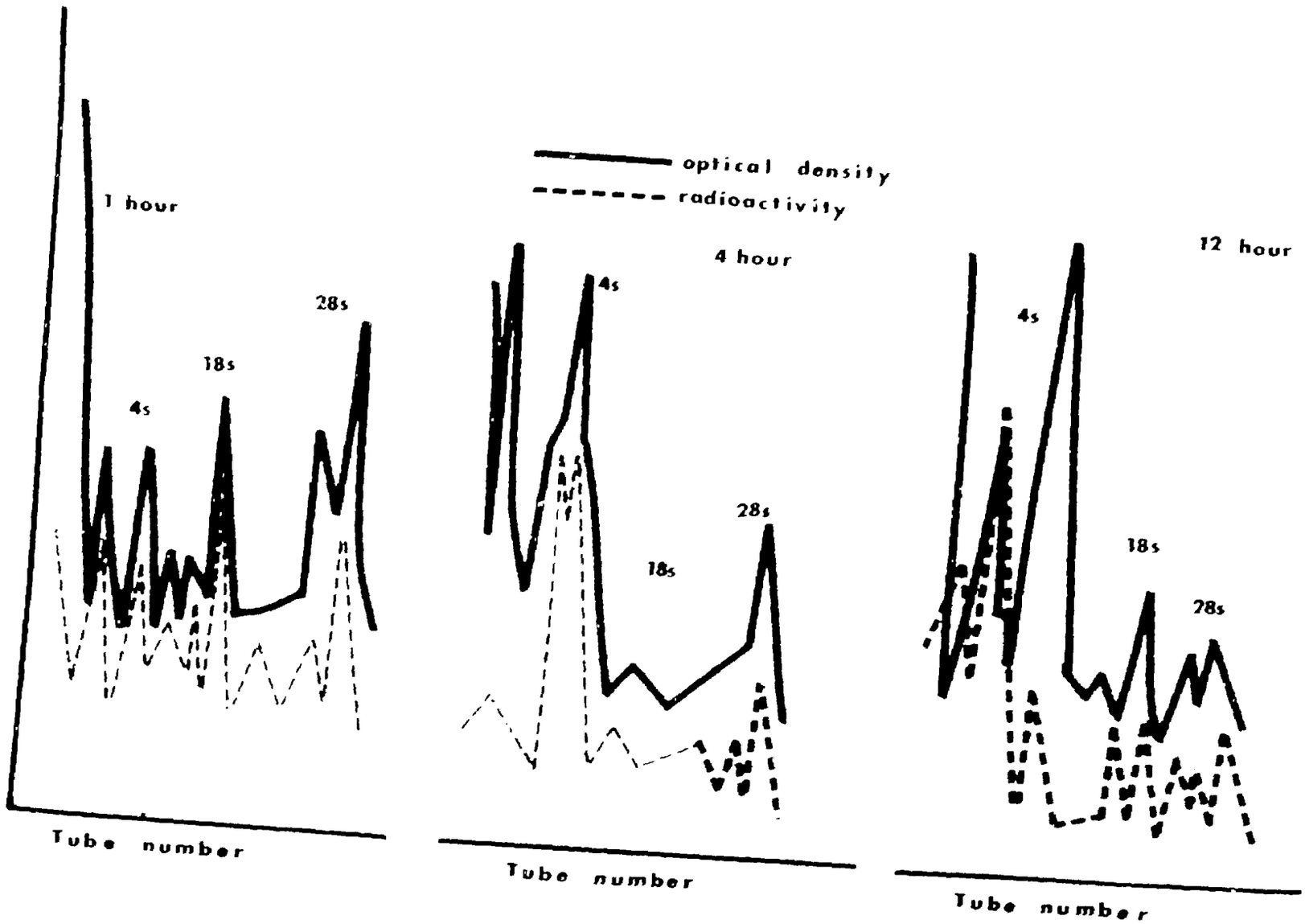

Figure 12. MAK column - 4.0 ppm aflatoxin