THE PURITY OF AFLATOXIN G_1 AND USE OF ANTIOXIDANT AND CHELATING AGENT ON THE PURIFICATION OF THE TOXIN BY THIN-LAYER CHROMATOGRAPHY

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Aflatoxin G_1 was originally identified as a compound emitting fluorescence under ultraviolet irradiation, and this emission has been widely used for identifying the toxin on thin-layer chromatographic (TLC) plates[1]. However, LIJINSKY AND BUTLER[2] recently reported that G_1 is a blue fluorescent compound and that the green fluorescence is due to a yellow impurity superimposed on the blue G_1 spot. Furthermore, they demonstrated that blue G_1 is toxic to ducklings. Moreover, ultraviolet adsorption data[3-5] available for G_1 toxin are inconsistent. The calculated ratios of absorbance (A) at 363 mμ to that at 265 mμ of G_1 from the reported data in methanol or in ethanol fluctuated from 1.61 to 1.87. Since the 363 mμ band is the excitation band[3,6] responsible for the fluorescence of the toxin, any substance absorbing in this ultraviolet region should change the fluorescence property. In view of these conflicting observations concerning the hue of fluorescence and purity of G_1, a careful re-examination of these points seems desirable.

During preparative separation of G_1 by TLC procedures, modifications of the toxin into a number of unknown derivatives occurred and thus hindered effective purification. An antioxidant and a chelating agent were used in an attempt to prevent such undesirable chemical changes. This paper describes the application of 4-methyl-2,6-di-*tert.*-butylphenol (BHT) and ethylenediaminetetra-acetic acid (EDTA) in the purification of G_1.

EXPERIMENTAL

Aflatoxin G_1

Aspergillus parasiticus 15957 (Commonwealth Mycological Institute Collection, Surrey, England) was used to produce aflatoxin on polished rice, following the procedures of SHOTWELL *et al.*[7], but without shaking during incubation. After eight days at room temperature, the moldy rice was extracted with chloroform and concentrated under reduced pressure at 35°. The concentrate was then filtered through anhydrous sodium sulfate and the yellowish filtrate applied to a number of preparative TLC plates. The plates were prepared with MN-Kieselgel G-HR (Brinkman) and developed by 3 % methanol in chloroform (v/v). Toxins were identified on TLC plates by comparison with standard toxin mixtures, obtained from the U.S.D.A., Southern Regional

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Research Laboratory, New Orleans, La. G_1, the predominant toxin in the extract, was scraped from the plates, extracted with chloroform by centrifugation, and concentrated. The concentrated G_1 preparation was further purified by repeating the procedure. After each purification, the G_1 band was extracted by chloroform and its ultraviolet absorption spectrum obtained (Beckman DK-2A spectrophotometer). The ratios of A 360 mμ/A 264 mμ were calculated from the spectra.

BHT and EDTA

Chloroform was refluxed in the presence of 2-thiobarbituric acid[8] and distilled to eliminate carbonyl and other impurities. The distilled solvent was collected in brown bottles containing BHT to give a final concentration of 0.02 %. Disodium salt of EDTA was dissolved in glass distilled water to 0.4 % level, adjusted to pH 7 and used in TLC plate preparations. Two parts of this water were mixed with one part of Kieselgel.

Chloroform extracts of G_1 from the plates were concentrated in a two-neck (℔ 14/20 and 10/18) pear-shaped flask (50 ml) under nitrogen and reduced pressure. One neck was connected to a nitrogen tank and the other to a water aspirator through a safety flask. Nitrogen was then bubbled through a capillary. A heating mantle was used to prevent frosting outside of the flask during evaporation of the solvent. This procedure not only decreased oxygen in the flask, but also reduced the time involved. After complete evaporation of chloroform, G_1 was dissolved in water, and its ultraviolet absorption spectrum was recorded.

Because of the sensitivity of the toxin toward light and in particular, ultraviolet light, efforts were made to avoid exposure during the entire procedure. In some of the latter experiments, the complete procedure was conducted in a dark room with a small red dark room safety light as a sole illuminant, and the toxin band was located on TLC plates at the edge of a low intensity ultraviolet lamp in the shortest time possible. All G_1 preparations before and after purification were examined on analytical TLC plates and compared for chroma and hue under visible and ultraviolet lights and for R_F values.

RESULTS AND DISCUSSION

A typical example of the relationship between repeated purification and ultraviolet absorption property of the toxin is shown in Fig. 1. The ratio of A 360 mμ/A 264 mμ of G_1 in chloroform obtained from the first purification was 2.15; the toxin was yellow and emitted a green fluorescence on TLC plates. During extraction of G_1 by chloroform, the yellow color was retained mainly on Kieselgel. The ratio gradually decreased very slightly until the fifth purification. In the sixth purification the ratio became 1.45 and then dropped to 0.43 in the seventh purification. This highly purified toxin was colorless and yielded a light blue fluorescence, but not a green fluorescence. However, the light blue fluorescence of G_1 was quite different from the purple blue fluorescence of B toxins, and they could be clearly distinguished. In another experiment (not shown), partially purified G_1 was further purified in the same manner except the spectra were obtained in ethanol after evaporation of chloroform. The ratio decreased from 1.45 to 0.49, and the resulting G_1 was colorless with light blue fluorescence. The light blue G_1 had the same R_F value as green G_1 and as G_1 in the

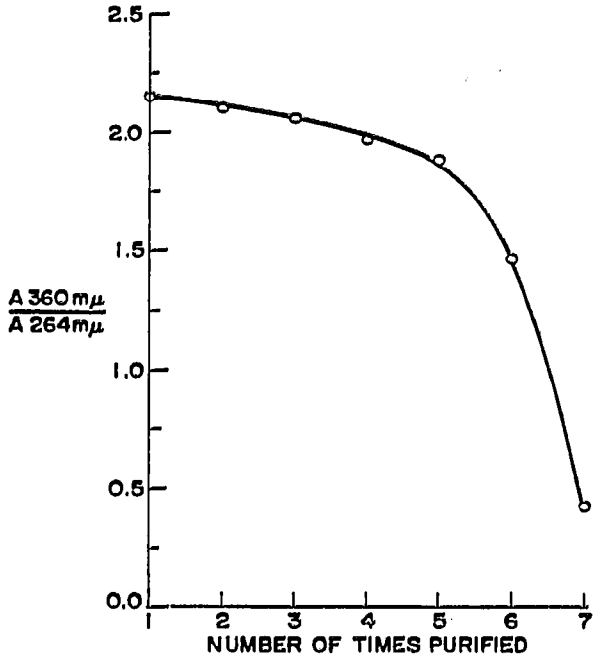

Fig. 1. Relationship between the ratio of A 360 mμ/A 264 mμ and number of times aflatoxin G_1 was purified by thin-layer chromatography.

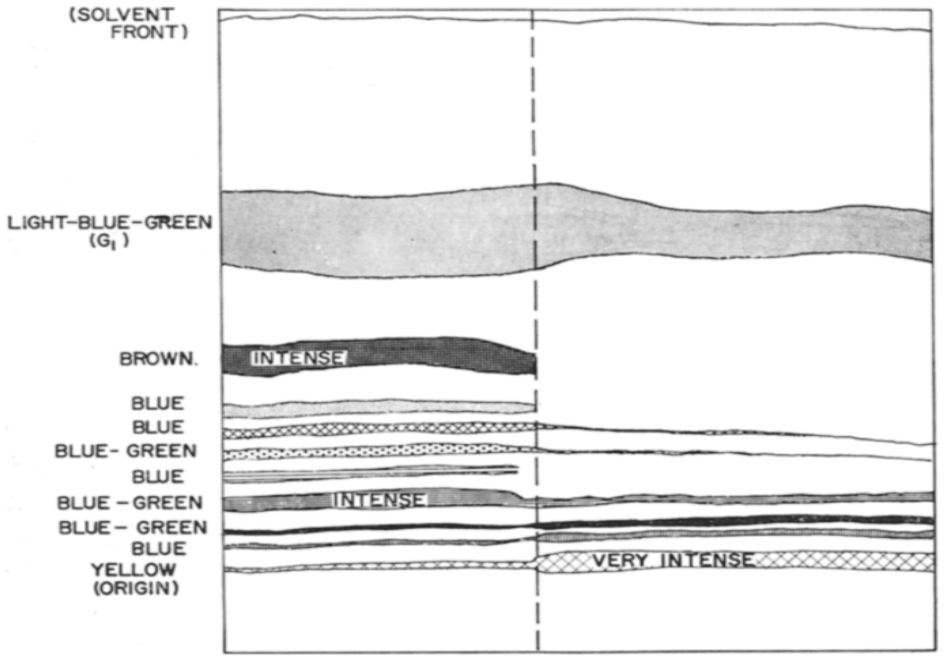

Fig. 2. Schematic thin-layer chromatogram of aflatoxin G_1 in chloroform (left) and in water (right).

standard toxin mixture. This observation confirms the report of LIJINSKY AND BUTLER[2] that the yellow substance having the same chromatographic mobility as G_1 is gradually eliminated during repeated purification. Apparently, the yellow substance behaves similarly in both solvent systems: 3 % methanol in chloroform and chloroform–diethyl ether–acetic acid (2:2:1)[2].

During purification, there were always about ten other fluorescent bands with lower R_F values than G_1, in addition to a yellow band at the origin. Also, a fluorescent band with higher mobility than that of G_1 was often observed. When aqueous G_1 solution was applied on TLC plates, there were usually fewer bands (Fig. 2). Some of these bands were yellow in visible light, and hues of the fluorescent bands were blue, brown, yellow or green. Under similar conditions, G_2 produced only one or two modified toxin bands confirming the report of ANDRELLOS *et al.*[9]. Apparently the numbers, kinds and intensities of such bands were very sensitive to several factors, such as the degree of unsaturation of toxin molecules, solvents, lights, oxygen, time of storage and other factors. These fluorescent compounds undoubtedly were derived from G_1 during preparation, storage and purification. Because of loss of toxin by such modification during purification, the amount of G_1 from four preparative TLC plates was recovered only as a single spot on analytical TLC plate after the seventh purification. For this reason, further purification was halted. Neither evaporation of solvent during concentration of the toxin under a stream of nitrogen, nor operations in a cold room improved this situation significantly.

BHT has been used successfully to protect lipids from autoxidation during chromatography, manipulation, and storage[10]. When chloroform stabilized with BHT was used as eluting and developing solvents, recovery of G_1 improved significantly; numbers of the non-G_1 fluorescent bands and intensities of these bands were much less than those of control plates without BHT (Fig. 3). However, G_1 bands were still

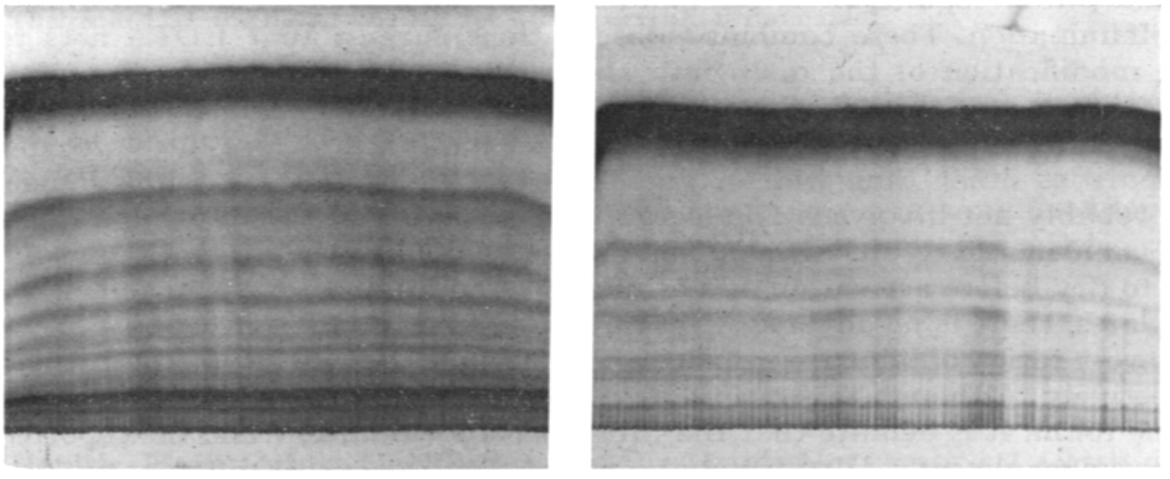

Fig. 3. Thin-layer chromatograms of aflatoxin G_1 developed with BHT containing solvent (right) and without BHT (left). The uppermost wide band is G_1, and others are modified derivatives of the toxin. The picture was taken under ultraviolet irradiation with aid of a yellow filter in a dark room.

yellow and emitted a green fluorescence. Increasing the antioxidant concentration to 0.05 % was no more effective than 0.02 %. Doing the purification in a dark room eliminated almost all the modified toxin bands on TLC plates. This observation suggested that BHT is effective only in protecting G_1 from auto-oxidative changes, but not from photosensitive modification. The auto-oxidized G_1, obtained by exposing aqueous G_1 solution in air at room temperature for several days, gave a typical pink chromogen[11] with 2-thiobarbituric acid. Photochemical changes of aflatoxins have been discussed recently[2,9]. BHT is practically insoluble in water at room temperature

and thus does not affect ultraviolet absorption spectra of G_1 in water. Furthermore, BHT did not affect toxin separation on TLC plates, and the excess BHT in the toxin concentrates appeared as a dark region under ultraviolet light near the solvent front.

An attempt was made to eliminate the yellow color from the toxin by paper partition chromatography (PPC) using Whatman No. 1 filter paper and water-saturated *n*-butanol. After 24 h at room temperature, G_1 appeared as a bluish fluorescent spot with an R_F value of 0.76. However, the expected yellow spot was not detected. The yellow subtance is apparently not produced during PPC. The toxin spot was eluted with water, and its ultraviolet absorption spectrum was examined. The ratio (A 366 mμ/A 264 mμ) was 0.78.

Since Kieselgel contains 0.001% iron, modification of G_1 into the yellow substance could be non-photochemical, as well as photochemical in nature, and catalyzed by iron or other metallic impurities in the sorbent during the purification using TLC plates. To test this possibility, EDTA was incorporated into the sorbent (0.8 % in Kieselgel) in the preparation of TLC plates by using EDTA added water. Surprisingly, G_1 became colorless and emitted a bluish fluorescence, and the ratio (A 366 mμ/A 264 mμ) of the toxin in water was 0.79. This ratio is in good agreement with that obtained from PPC and is much lower than those reported[3-5] (1.61–1.87). When the same toxin was separated on TLC plates without EDTA, the G_1 was slightly yellow and became increasingly yellow upon standing. Thus, the yellow substance is also formed from G_1 after separation of the band during standing in air under ordinary laboratory illumination. These combined observations suggest that EDTA acts to prevent the modification of the toxin into the yellow substance during chromatography and after separation on TLC plates, but has no effect on the substance already formed before TLC application and that formed at the origin before development. Metallic impurities other than iron in Kieselgel may be involved in this change. Calcium is probably not involved: Kieselgels, with and without calcium sulfate as a binder, gave an identical result. Although addition of EDTA to the sorbent requires a longer time to dry before activation of TLC plates (40 min) and results in somewhat fluffy TLC plates, there is no adverse effect on toxin separation.

The lowest ratios obtained were 0.43 and 0.42 (not shown) in chloroform and water, respectively. Although this ratio may not be accurate due to the low concentration of the toxin, it is definite that the ratio is smaller than 1, while the reported value[3-5] approaches almost 2. Ultraviolet absorption spectra of G_1 at different purified stages illustrate this situation (Fig. 4). A similar observation was made with G_2. The possibility that such spectral changes are due to the differences in concentration of the toxins was eliminated by dilution studies.

It is believed that the extra absorption in 360 mμ region due to the yellow impurity not only disturbs the color but also the intensity of fluorescence. Thus, quantitative determination of the toxin by use of the reported molar absorptivities[12,13] at 363 mμ is not more reliable than by measurement of fluorescence. Since the absorption band at 264 mμ is less disturbed by the yellow impurity, and since the molar absorptivity reported for this band is less variable (10,000–10,500) than that at 363 mμ (16,000–18,700)[3-5], the use of absorption at 264 mμ is probably more reliable than others in quantitative determination of the toxin. The position of absorption maximum for the 264 mμ band does not change by changing the solvent from chloroform to

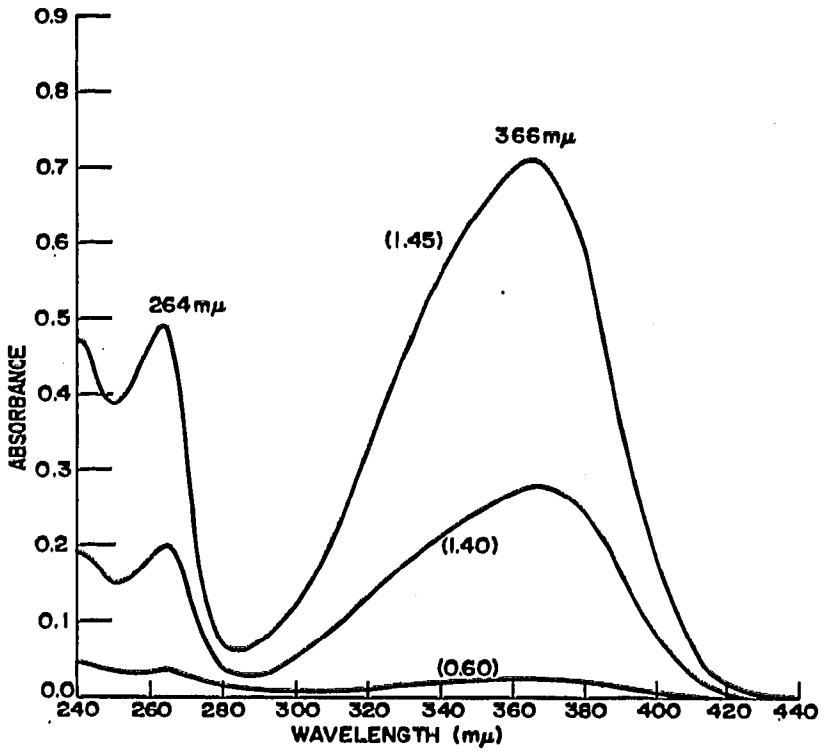

Fig. 4. Ultraviolet absorption spectra of aflatoxin G_1 in water at different stages of purification. The ratio of A 366 mμ/A 264 mμ for each spectrum is indicated.

ethanol or to water, while the latter band changes from 360 mμ in chloroform to 363 mμ in ethanol, or to 366 mμ in water.

The use of both BHT and EDTA in a dark room is highly beneficial to prevent undesirable changes; however, the highly purified toxin could not be obtained by single step purification on TLC plates. The nature of those impurities remains to be solved.

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SUMMARY

During purification of aflatoxin G_1 by TLC procedures, about ten additional modified toxins are separated as fluorescent bands. G_2 produces only one or two modified toxin bands under similar conditions. Use of BHT in developing and eluting solvents and EDTA in preparation of TLC plates and operation of the entire procedure in a dark room effectively prevent such undesirable changes of the toxin. Crude G_1 is usually yellow in visible light and emits a green fluorescence by ultraviolet irradiation. Upon further purifications it becomes colorless in visible light and a light blue fluor-

escent compound under ultraviolet light. During purification, the ratio of absorbance at 360 mμ or 366 mμ to that at 264 mμ changes from about 2 to 0.5. The high absorbance in the region of 360 mμ for the crude toxin is apparently due to an unknown yellow impurity. A similar change of the ratio is also observed during purification of G_2.

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