Reduction of Aflatoxin B_{2a} with Sodium Borohydride

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Limited reduction of aflatoxin B_{2a} (B_{2a}) with sodium borohydride ($NaBH_4$) at neutral or slightly alkaline pH resulted in a new derivative, reduced aflatoxin B_{2a} (RB_{2a}). The compound was fluorescent and had an R_f value of 0.21 compared to 0.77 for B_{2a} on tlc plates developed in methanol–chloroform (10% v/v). It melted at 250–251° dec and had a molecular weight of 334 by mass spectrometry. Infrared spectra showed absorptions at 3500, 1760, 1684, and 1600 cm^{-1}; uv spectra in methanol and acidic solutions showed λ_{max} at 228, 265, and 362 nm; however, in basic solution the compound exhibited a bathochromic shift with λ_{max} at 245, 290, and 400 nm. Prolonged incubation of RB_{2a} with $NaBH_4$ resulted in further reduction of the compound. The data support the proposed structural changes of B_{2a} in which a phenolate ion is formed and two aldehyde groups are generated in basic solutions. Reduced aflatoxin B_{2a} was not toxic to chicken embryos at levels 100 times the LD_{50} of B_1.

Aflatoxin B_{2a} (B_{2a}), the hemiacetal of aflatoxin B_1 (B_1), is formed chemically either by treatment of the parent toxin with diluted acid (Pohland *et al.*, 1968) or by exposing the toxin to uv light in the presence of water (Wei and Chu, 1973) and is also produced biologically by a culture of *Aspergillus flavus* CMI, 01019b (Dutton and Heathcoate, 1968). It has been reported that B_{2a} is a major metabolite of B_1 in several animals (Patterson and Allcroft, 1970; Patterson and Roberts, 1970).

Several reports (Patterson and Roberts, 1972; Ashoor and Chu, 1975) have shown that in contrast to B_1, B_{2a} reacts readily with the amino group in amino acids and proteins and also inhibits the enzyme deoxyribonuclease I (DNase I) noncompetitively (Schabort and Pitout, 1971). These findings led to speculation on the possible role of B_{2a} for acute toxicity of B_1 (Patterson, 1973). The interaction between B_{2a} and amino acids and proteins is based on the hypothesis that B_{2a} undergoes structural changes at neutral and alkaline pH to form a phenolate ion with two aldehyde groups generated (Pohland *et al.*, 1968). Nevertheless, the existence of such a structure has not been experimentally demonstrated. This report presents evidence on the existence of two free aldehyde groups in B_{2a} in basic solutions by limited reduction of B_{2a} with $NaBH_4$ under conditions which do not reduce the carbonyl group of the cyclopentanone ring in the aflatoxin. Details for the preparation as well as physical and chemical properties of RB_{2a} are described.

EXPERIMENTAL SECTION

Chemicals. Adsorbosil-5 was purchased from Applied Science Laboratories, Inc., State College, Pa. Sodium borohydride, $NaBH_4$, was purchased from Fisher Scientific Co., Fair Lawn, N.J., and $[^3H]NaBH_4$ was purchased from New England Nuclear Co., Boston, Mass. All organic solvents were of analytical grade.

Analyses. Absorption was measured in a Beckman Model DU spectrophotometer, and absorption spectra were obtained by scanning various solutions at a range of 200–500 nm in a Beckman Model Acta III recording spectrophotometer with a light path of 1 cm. Infrared spectra were measured in potassium bromide disks with a Beckman Model IR-4 spectrophotometer. Mass spectra were obtained by a MS-9 mass spectrometer (Associated Electrical Industries, Manchester, England). Melting points were determined with a Monoscope IV melting apparatus (Glas-Triebel, Mannheim, W. Germany). Thin-layer chromatography (tlc) was done on glass plates (20 × 20 cm) coated to a thickness of 250 μ with adsorbosil-5. The developing solvents were either 6 or 10% (v/v) methanol in chloroform. Radioactivity measurements were made by mixing either an appropriate amount of extracted solution (generally less than 0.5 ml) or a radioactive spot scraped from a tlc plate with 10 ml of Bray's solution (Bray, 1960). The solution was subjected to counting by a Packard Tri-Carb Model 5017 liquid scintillation spectrometer for 10 min.

Preparation of B_{2a} and RB_{2a}. Aflatoxin B_{2a} was prepared by the method of Pohland *et al.* (1968). Initially, RB_{2a} was prepared in small amounts. One-tenth milliliter of $NaBH_4$ solution (2.6×10^{-2} *M*) in 0.05 *M* sodium phosphate buffer of pH 7.2 was added dropwise with constant stirring to 5 ml of B_{2a} (6×10^{-4} *M*) in 50% methanol-0.05 *M* sodium phosphate buffer of pH 7.2 and was kept at 4° for 30 min. Excess $NaBH_4$ was destroyed by adding 2 drops of 0.1 *N* HCl to the mixture with stirring after reaction. The reaction mixture was then subjected to tlc analysis. Aflatoxin B_{2a} was also reduced with $[^3H]NaBH_4$ (9.8×10^6 cpm/0.1 ml) in a similar manner. For large-scale preparation, 50 mg of B_{2a} (1.52×10^{-1} m*M*) in 50 ml of 50% methanol–phosphate buffer was allowed to react with 5 mg (1.32×10^{-1} m*M*) of $NaBH_4$ dissolved in 1 ml of phosphate buffer as described above. The reaction mixture was then evaporated under vacuum to remove methanol and freeze-dried. The residue was suspended in a small amount of methanol with 1 g of adsorbosil-5 to make a slurry which was layered carefully on top of a 2 × 40 cm adsorbosil-5 column equilibrated with 2% methanol in chloroform. Anhydrous sodium sulfate was added to the column to a height of 1 cm above the aflatoxin layer. The column was then eluted with 2% methanol in chloroform, and fractions were monitored for B_{2a} and RB_{2a} by tlc. Aflatoxin B_{2a} was eluted from the column in the first ten fractions followed by RB_{2a}. The fractions containing RB_{2a} were pooled, evaporated to dryness under vacuum, and crystallized twice from methanol–chloroform. A total of 40 mg of RB_{2a} was obtained in this manner. The compound was light yellow and had a melting point of 250–251° dec. For convenience, the compound is referred to as RB_{2a} to indicate that the carbonyl group in the cyclopentanone ring was not reduced under the conditions described.

In order to confirm that limited reduction (30 min) of B_{2a} did not involve the carbonyl group of the cyclopentanone ring, RB_{2a} was further reduced with $NaBH_4$ in one experiment and with $[^3H]NaBH_4$ in another by prolonging reduction time to overnight at 4°.

Aflatoxins B_{2a} and RB_{2a} in pyridine were acetylated with an excess of acetic anhydride. The reaction mixtures

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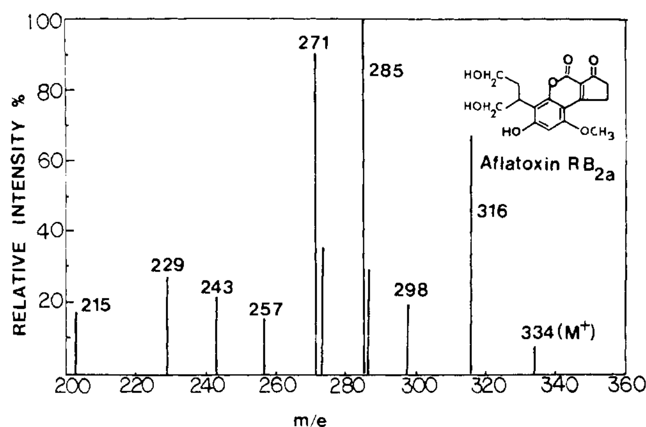

Figure 1. Mass spectrum of RB_{2a}. Peaks below *m/e* 200 are not shown.

Table I. Toxicity Assays of Aflatoxins B_1, B_{2a}, and RB_{2a} on 7-Day-Old Chicken Embryos

Aflatoxin	Added, μg/egg	Mortality: no. of deaths/no. of tested — 7 days after injection	14 days after injection
Control		0/8	0/8
B_1	0.8	4/8	5/8[a]
B_{2a}	20.0	0/8	0/8
	40.0	0/8	0/8
	80.0	0/8	1/8[a]
RB_{2a}	19.5	0/8	0/8
	38.0	0/8	0/8
	78.0	0/8	1/8[a]

[a] Includes 1 egg unhatched.

were left at room temperature overnight, evaporated under vacuum, redissolved in methanol, and purified by tlc.

Chicken Embryo Test. The toxicity of RB_{2a} and B_{2a} was tested on 7-day-old chick embryos (Leghorn-New Hampshire Cross, supplied by the University of Wisconsin Poultry Science Department) following the method described by Wei and Chu (1973).

RESULTS AND DISCUSSION

Tlc Analysis of Reduction Mixture of B_{2a}. Thin-layer chromatograms of the B_{2a} reduction mixture (30-min product) developed in 6% methanol in chloroform revealed two fluorescent spots with R_f values of 0.35 and 0.06 which were subsequently identified as B_{2a} and RB_{2a}, respectively. In contrast to B_{2a}, the reduced product did not migrate in methanol-chloroform systems with less than 5% methanol, indicating that the reduction product was more polar than B_{2a}. When the methanol concentration was increased to 10%, B_{2a} and RB_{2a} had R_f values of 0.77 and 0.21, respectively. The reduction yield was 86% as calculated by measuring unreacted B_{2a} using $\epsilon_{362\,nm}$ (MeOH) of 20,400. When $[^3H]NaBH_4$ was used, radioactivity was detected only in the RB_{2a} spot (2.2×10^5 cpm/μM). No back conversion of B_{2a} to B_1 was observed under these conditions.

Acetylation of RB_{2a} resulted in two spots with R_f values of 0.27 and 0.32 (solvent system, 6% methanol in chloroform). Control experiments of acetylation of B_{2a} also resolved two spots with R_f values of 0.84 and 0.98 in the same solvent system. Since it has been reported that B_{2a} gave two acetate epimers after acetylation (Pohland *et al.*, 1968), acetylation of RB_{2a} may have also produced two acetate epimers.

Mass Spectral Analysis of RB_{2a} and Acetyl RB_{2a}. The mass spectrum of B_{2a} is shown in Figure 1. The molecular weight of RB_{2a} by mass spectrometry was found to be 334. Two peaks at *m/e* 316 and 298 arising from the loss of two molecules of water indicated the presence of two hydroxyl groups. The mass spectrum of the acetate derivative of RB_{2a} revealed a mol wt of 460, indicating three acetylation sites in RB_{2a} compared to only one site in B_{2a} (Pohland *et al.*, 1968). The two additional acetate groups account for two additional hydroxyl groups in RB_{2a}. The acetate derivative of RB_{2a} had other peaks at *m/e* 418, 400, 358, 340, 305, 298, 285, 271, 243, 229, and 215.

Ir and Uv Spectrophotometric Analyses of RB_{2a}. The infrared spectrum (KBr) of RB_{2a} showed peaks at 1760 and 1684 cm^{-1} similar to B_1 (Asao *et al.*, 1965), indicating that the cyclopentanone ring was not reduced. The presence of hydroxyl groups in the new compound was evident from the existence of a broad peak at 3500 cm^{-1} in the ir spectrum. Other peaks were at 1600 and 1565 cm^{-1}.

The ultraviolet spectrum of RB_{2a} in methanol had absorption peaks at 228, 265, and 362 nm with ϵ of 14,900, 10,150, and 20,700, respectively. In acidic solutions, the compound had a similar absorption spectrum to that in methanol. However, the compound exhibited a bathochromic shift in basic solutions λ_{max} 245, 290, and 400 nm. Compared to the bathochromic shift in B_{2a}, RB_{2a} exhibited a more pronounced change at 400 nm, *i.e.*, the absorption ratio 400:362 nm at pH 10.0 was 2.1 for RB_{2a} compared to 1.7 for B_{2a}. Spectrophotometric titrations of B_{2a} and RB_{2a} at 400 nm showed that the absorbance of both compounds increased with the increase of pH, and gave typical titration curves with dissociation constants of 7.1 and 6.1, respectively. These results indicated that dissociation of the phenolic group in both compounds had occurred.

Further Reduction of RB_{2a}. Three spots resulted from further reduction of RB_{2a} with $NaBH_4$ and $[^3H]NaBH_4$ with R_f values of 0.06 (major), 0.27, and 0.32 compared to 0.21 for RB_{2a} in 10% methanol in chloroform. The three spots showed radioactivity when $[^3H]NaBH_4$ was used with a distribution of 85% for the major spot with an R_f value of 0.06 and 5.8 and 7.2% for the other two spots with R_f values of 0.27 and 0.32, respectively. The new products had a maximum uv absorption at 300 nm in methanol and in acidic solutions and exhibited a bathochromic shift to 375 nm in basic solutions. Since our primary purpose was to prove the presence of two free aldehyde groups in the B_{2a} molecule in basic solutions, no attempts were made to further characterize the new products. These results indicated the presence of additional sites of reduction in RB_{2a}, *i.e.*, the carbonyl group of the cyclopentanone ring of the molecule, and confirmed that limited reduction of B_{2a} involved only the two aldehyde groups of B_{2a}. Accordingly, care should be taken in preparation of RB_{2a} in order to limit the reduction to the aldehyde groups only.

Biological Activity of RB_{2a}. The results of the chicken embryo assays are given in Table I. At levels of 100 times the LD_{50} of B_1 (0.8 μg), neither B_{2a} nor RB_{2a} was toxic to 7-day-old chicken embryos.

CONCLUSIONS

The results obtained from this study provide evidence for the structural changes of B_{2a} in basic solutions leading to the formation of a phenolate ion with the generation of two aldehyde function groups (Pohland *et al.*, 1968). Aflatoxin B_{2a} is a major metabolite of B_1, and reacts readily with amino groups of amino acids and proteins (Patterson and Roberts, 1972; Ashoor and Chu, 1975). The biological

importance of the aldehyde groups of B_{2a} becomes evident from the finding that RB_{2a}, in which the aldehyde groups are reduced to hydroxyl groups, does not interact with either amino acids or proteins (Ashoor and Chu, 1975). Therefore, reduction of B_{2a} to RB_{2a} with $NaBH_4$ may be considered as a method of deactivating B_{2a} *in vitro*. The question of whether B_{2a} can be inactivated biologically *in vivo* by some animals as a method of detoxification remains to be answered.

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Ammoniation of Aflatoxin B_1. Mass Spectral Analysis of Compounds Separated by Microsublimation

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Microsublimation of a partially purified preparation of the previously characterized major reaction product of ammoniation of aflatoxin B_1, termed "aflatoxin D_1", effectively separated sufficient micro amounts of two additional compounds for mass spectral analysis. The mass spectrum of the sublimate obtained between 220 and 240° indicated a molecular ion of mass 236 with prominent peaks at masses 111, 129, and 149. Between 280 and 300° the mass spectrum indicated a molecular ion of mass 256 with prominent peaks at masses 111, 129, 149, 185, and 213. Pure aflatoxin D_1 (molecular weight 286) was sublimed between 320 and 340° and the nonsublimed residue contained a molecular weight 368 compound.

Aflatoxin B_1, a secondary metabolite of the molds *Aspergillus flavus* and *A. parasiticus*, is an extremely potent carcinogen (deIongh et al., 1964). Since this metabolite can be found in oilseeds that have been stored under adverse conditions, considerable research effort has been expanded on detoxification procedures (Dollear, 1969). One of the most promising approaches is ammoniation. Gardner et al. (1971) reported that aflatoxins in contaminated peanut and cottonseed meals were reduced to "nondetectable" levels (1 μg/kg) by the reaction of moistened meal with ammonia gas under pressure (40–50 psig) for ca. 30 min at 90–125°. Because of the apparent success of this approach to detoxification, the products formed from the reaction were studied in a model system in which pure aflatoxin B_1 was ammoniated. The major reaction product formed from the reaction of aflatoxin B_1 with ammonium hydroxide at elevated temperature and pressure has been isolated and characterized (Lee et al., 1974). This product, $C_{16}H_{14}O_5$, is nonfluorescent, exhibits phenolic properties, and lacks the lactone group characteristic of aflatoxin B_1. It was postulated that this new product, termed "aflatoxin D_1", molecular weight 286 (Lee et al., 1974), arises from the opening of the lactone ring of aflatoxin B_1 during ammoniation, formation of the ammonium salt of the resultant hydroxy acid, and decarboxylation of this β-keto acid. During purification of D_1 by sublimation it became apparent that closely related compounds were present in micro amounts. We have, therefore, used fractional microsublimation to separate these compounds from the major product, aflatoxin D_1. Sufficient samples for insertion into the mass spectrometer were retrieved by a novel glass wool broom technique.

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MATERIALS AND METHODS

Microsublimation was performed on the hot stage assemblage of a Kofler melting point apparatus. Heat was controlled by a variable voltage transformer and the temperature was programmed to increase 3°/min. Sublimates were collected on a series of microscope slides which covered the subliming dish (10 mm diameter × 2 mm deep) illustrated in Figure 1. Approximately 3 mg of material was sublimed over a range of 200–340° and the sublimates were collected over each 20° range. Micro amounts of each sublimate were swept from the microscope slide and inserted into the mass spectrometer (CEC 21-110B) according to the procedure outlined in Figure 2.

Steps 1 and 2 show the preparation of the glass wool broom for retrieving the sample from the microscope slide. In step 3 the glass wool is partially pulled up into a melting point capillary with a fine wire. The sample is picked up on the glass wool by sweeping the slide with the broom (step 4). In step 5, the glass wool containing the sample is pulled into the capillary; the thin wire is removed and the capillary is sealed. In step 6 the glass wool containing the sample is pushed to the sealed end of the capillary with a wire plunger. The capillary is cut to the