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

James B. Stanley,* Louise S. Lee, Alva F. Cucullu, and Ines V. deGruy

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

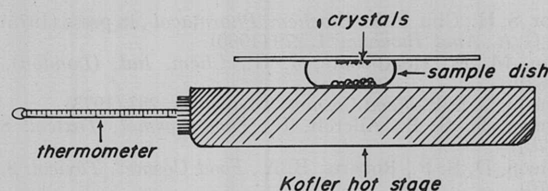

Figure 1. Assemblage for microsublimation.

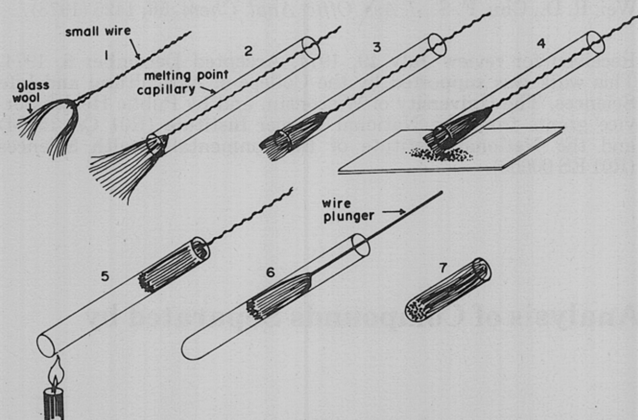

Figure 2. Procedure for transferring micro sample from microscope slide into melting point capillary for mass spectral analysis.

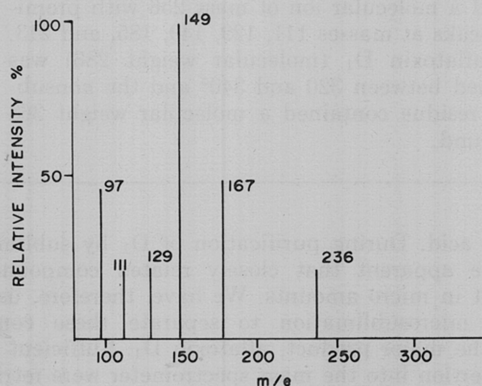

Figure 3. Mass spectrum of the mol wt 236 compound.

desired length for direct insertion into the mass spectrometer (step 7).

RESULTS

Mass spectral analysis of the crude aflatoxin D_1 and of the sublimate collected on a single microscope slide over the temperature range of 220–340° indicated predominance of aflatoxin D_1 (mol wt 286) and much smaller amounts of masses 236 and 256. Since the preparation before sublimation was nitrogen negative, the presence of masses 236 and 256 is indicative of molecular ions. The small amount of residue left in the subliming dish contained a mol wt 368 compound. As the temperature increased, the amounts and molecular weights of the sublimed compounds increased. Based on visual estimates of the amount of crystalline material on the microscope slide, approximately 100 ng of sublimate was collected from 220 to 240°. In Figure 3, the base peak is mass 149 with other prominent peaks at masses 97, 111, 129, and 236. Mass 236 is indicative of a molecular ion. Mass spectra of about equal weight collected from 214 to 260° indicate that the material was a mixture of compounds having molecular weights of 236 and 256. The 256 molecular weight compound predominated in the approximate 200-ng sublimate collected between 280 and 300°.

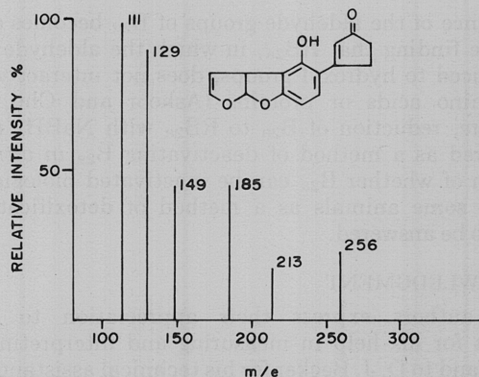

Figure 4. Mass spectrum of the mol wt 256 compound.

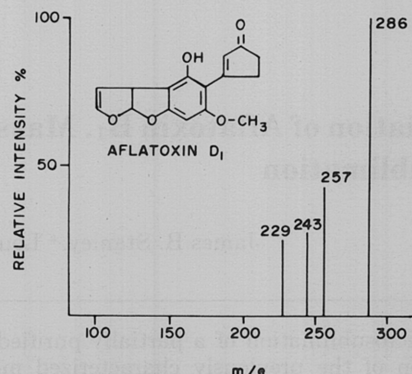

Figure 5. Mass spectrum of aflatoxin D_1.

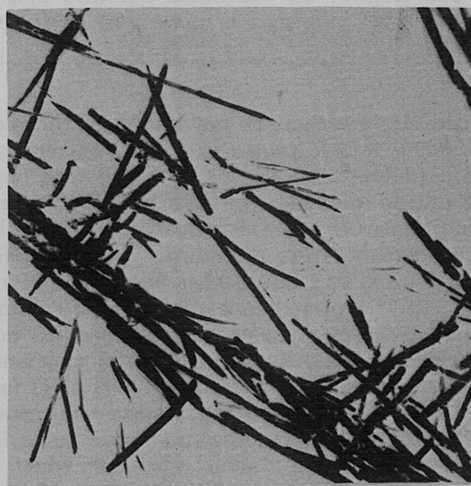

Figure 6. Photograph of crystalline sublimate obtained between 320 and 340° (aflatoxin D_1, mol wt 286).

In Figure 4, the base peak is mass 111 with other prominent peaks at masses 129, 149, 185, 213, and 256. Mass 256 is indicative of a molecular ion. This molecular weight is in agreement with the loss of the methoxyl group of aflatoxin D_1 as shown in Figure 5. Kiermeier and Ruffer (1974) report on the identification of such a compound after sodium hydroxide treatment of aflatoxin B_1 at 100°.

At least 2 mg of pure aflatoxin D_1 was collected between 320 and 340°, scraped from the slide, and weighed on a micro balance. The mass spectrum of this sublimate (Figure 5) indicates a molecular ion of 286 and other prominent peaks at masses 229, 243, and 257. Figure 6 is a photograph of a portion of this sublimate.

DISCUSSION

In view of the highly carcinogenic properties of aflatoxin B_1, information on breakdown products of detoxification is much needed. This information must necessarily be obtained on extremely small samples because of the scarcity of aflatoxin B_1 and the subsequent difficulty in obtaining enough of the ammoniation products for characterization. Fractional microsublimation effectively separated two new compounds from the major reaction product, aflatoxin D_1, and the micro-retrieval technique described was a major factor in obtaining enough of these compounds for molecular weight determinations.

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Effect of Chemical Treatments Causing Rapid Onset of Rigor on Tenderness of Poultry Breast Meat

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Postmortem treatment with either calcium chloride, dinitrofluorobenzene, or iodoacetate and aging at 35° as compared to 20° and antemortem treatment with epinephrine were used to investigate the effect of pH and adenosine triphosphate levels immediately after slaughter on isometric tension development and shear force of pectoralis major muscle of male chickens. These treatments either minimized postmortem glycolysis and synthesis of ATP, or accelerated glycolysis and ATP breakdown, and caused the muscle to go into rigor sooner than normal after death. Onset of rigor within 1 hr after slaughter and shorter than normal interval of time between death and maximum rigor development caused a higher isometric tension and toughness in meat. These damaging effects were minimal in muscle having high pH. The relation between rapid onset of rigor, pH, and tenderness is discussed.

Earlier studies on factors causing variation in tenderness in similar muscles from comparable animals have shown that pH values of 6.2 or lower and onset of rigor within 1 hr after slaughter caused toughness in poultry (Khan and Nakamura, 1970) and beef (Khan and Lentz, 1973). The work was extended to study whether this toughness is caused by rapid depletion of adenosine triphosphate (ATP) or by the acid condition produced immediately after slaughter. Since anaerobic glycolysis is involved in both lactic acid formation and ATP synthesis, it is difficult to separate the adverse effects on tenderness of these two chemical activities under normal conditions. However, this difficulty can be overcome by controlling the biochemical processes involved in postmortem glycolysis and in ATP synthesis and depletion. A study of these activities under controlled conditions would provide a more direct evidence on the involvement of accelerated glycolysis and onset of rigor in the development of changes in tenderness.

This paper describes tests made on pectoralis major muscles in which (a) the regeneration of ATP by phosphokinase was minimized by 2,4-dinitrofluorobenzene treatment (Dydynska and Wilkie, 1966), (b) the regeneration of ATP by adenylate kinase and anaerobic glycolysis was minimized by iodoacetate treatment (Padieu and Mommaert, 1960), (c) the rates of postmortem glycolysis and ATP depletion were accelerated by $CaCl_2$ treatment (Campions et al., 1971) or by raising the aging temperature, and (d) postmortem glycolysis was minimized by antemortem epinephrine treatment (DeFremery and Pool, 1963; Khan and Nakamura, 1970). Postmortem changes in pH, content of adenosine nucleotides, and isometric tension development were measured and shear force determined.

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EXPERIMENTAL SECTION

Tests were made on pectoralis major muscles excised from well-rested male chickens (Leghorn, pathogen free from a single flock, live weight 2–3 kg) immediately after slaughtering and bleeding. Thirty-six of these birds were administered sodium pentobarbital (25 mg/kg body weight) in the thigh muscles 15 min before slaughtering to minimize struggling at death and to obtain muscle tissue having a pH value and high-energy phosphate level comparable to that in live muscle. Six birds were administered epinephrine 12–16 hr before slaughtering as described earlier (Khan and Nakamura, 1970) to obtain muscle having high ultimate pH. Excised muscles were cut along the fibers into strips, 1 cm square in cross section and about 6 cm in length. Strips from adjacent locations were used for measuring changes in rigor tension, shear force, adenosine nucleotide content, and pH. All measurements were made on duplicate samples from the same muscle, and all experiments were repeated three times.