(3:3:1, v/v/v), n-butanol/acetic acid/water (5:1:2, v/v/v) and n-propanol/ethyl acetate/water (65:10:25, v/v/v) and tested for sugars by spray reagents. Ascending chromatography was performed on hydrolyzed and unhydrolyzed antioxidant solutions. Only the hydrolyzed sample contained a free sugar when sprayed with aniline oxalate. The sugar from the antioxidant appeared in the same area as a galactose standard and produced a brown color with aniline oxalate, suggesting that the unknown was a hexose. Reaction toward p-anisidine and p-anisidine HCl, light brown, indicated the sugar to be an aldohexose. Separation did not appear when the unknown was cochromatographed with the galactose standard.

Spectral analysis was employed for further identification of the antioxidant. The methanolic spectrum showed a peak at 290 nm. Spectral data resembled that of dihydroquercetin (taxifolin) (Table I). Spectrophotometric and chromatographic analysis demonstrated that the antioxidant was dihydroquercetin (taxifolin). Quantitative comparison of spectral curves for the antioxidant with an authentic sample demonstrated that the peanuts contained 3.4×10^{-4} mol/kg of dihydroquercetin.

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Lipid-Lipase Interactions. I. Fat Splitting with Lipase from *Candida rugosa*

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ABSTRACT

Commercial dry lipase from *Candida rugosa* (formerly *C. cylindracea*) was used to catalyze hydrolysis of tallow, coconut oil and olive oil at 26-40 C. A methodology was developed to yield results reproducible within ±10% and to achieve essentially complete hydrolysis. From the hydrolysis data, an empirical relationship was developed that shows that the percentage of free fatty acid formed is almost a linear function of the logarithm of reaction time and the logarithm of enzyme concentration. A 95-98% hydrolysis of the 3 substrates was achieved experimentally in 72 hr, requiring 15 units lipase per milliequivalent (U/meq) of coconut oil or tallow and 6 U/meq of olive oil. The kinetics of lipolysis were determined for all 3 substrates and were found to approximate first order. Lipolysis rate was higher for olive oil than for tallow and coconut oil; no significant differences were observed between the latter 2 substrates. No statistically significant change in overall reaction rate was found when the hydrolysis was run at 26 C, 36 C or 46 C. Although the literature cites calcium or sodium ions and albumin as beneficial adjuvants to enzymatic lipolysis, these additives appeared to have no significant beneficial effect on the reaction. On the other hand, hydrocarbon solvents and nonionic surfactants showed an adverse effect.

INTRODUCTION

The present industrial process for fat and oil hydrolysis involves pressures of ca. 700 psi and temperatures of 480 F or higher (1) for a period of ca. 2 hr to achieve 96-99% hydrolysis. The resulting products are extremely dark fatty acids and discolored dilute (10%) aqueous solutions of glycerol. The fatty acids are unusable as obtained and need to be redistilled to remove color and by-products. The glycerol layer, after concentration, usually is distilled to remove color and impurities. These processes are energy intensive and give rise to a variety of undesirable side reactions. For example, highly unsaturated fatty acids can polymerize, and, if the temperature rises above 450 F, anhydrides form that, if heating continues, decompose to yield ketones and hydrocarbons (1).

In the interest of conserving energy and to minimize thermal degradation, we set out to study enzymatic hydrolysis of triglycerides. This approach would lead to little or no additional color development, cause no chemical degradation and a more concentrated glycerol solution might also be achieved. A variety of lipases of different origins have been studied in the biochemical literature. Pancreatic lipase was the first fat-splitting enzyme to be thoroughly investigated. However, it is not useful for rapid total fat splitting because it is specific only for the α-positions of fatty triglycerides (2,3).

A 1965 patent (4) discloses the purification of a highly active lipase obtained from a newly found species of the yeast *Candida cylindracea,* now named *Candida rugosa* (5). Benzonana and Esposito (6) later showed that this lipase catalyzed complete hydrolysis of several natural oils to free fatty acids and glycerol in 8-20 hr. Another patent (7) describes the use of this same lipase in the hydrolysis of olive, soybean and linseed oils.

[1] Presented in part at the 73rd National Meeting of the American Oil Chemists' Society, May 1982, Toronto, Ontario, Canada.

The Meito Sangyo Company, a producer of this lipase, has obtained patents covering the hydrolysis of oils and fats using other lipases. The latest of these patents (8) cites the hydrolysis of beef tallow with a lipase from Meito Pl 679 bacteria in the presence of excess calcium hydroxide. Castor-bean lipase has also been used to catalyze complete hydrolysis of animal fats (9).

We set out to determine the kinetics of lipolysis of olive oil, coconut oil and tallow and the effects of temperature, concentration of lipase and pH. The minimum amount of lipase required for 95-98% hydrolysis was determined. Commercially available lipase from *C. rugosa* was used in this study. Because Ca (10,11) and Na (12) ions, along with albumin (13), have been cited as greatly enhancing enzyme activity, the effects of these on olive-oil lipolysis at room temperature were determined in the present study. Also examined were the consequences of adding a nonionic surfactant (12) to olive oil and hydrocarbon solvent to tallow (13,14) to hydrolyze the latter at room temperature. None of these additives proved beneficial, and some of them reduced lipolysis rates.

EXPERIMENTAL

Materials

Enzeco Lipase Concentrate (30,000 U/g according to supplier) from *C. rugosa* E.C. 3.1.1.3. was supplied by Enzyme Development Corporation, New York, NY. The enzyme was kept in a sealed container and stored in a refrigerator. The lipase assay was not checked, and the lipase was used as supplied. However, in order to check enzyme stability, samples of purified olive oil were periodically subjected to 16 hr lipolysis at 6 U/meq oil and consistently gave 75-80% lipolysis. Under these test conditions, the lipase activity remained unchanged for 2 years. Refined and bleached edible beef tallow (free fatty acid, 2.5%) was obtained from the Inolex Corporation, Philadelphia, PA; 76 F edible coconut oil (free fatty acid, 1.2%) from the Industrial Products Group, Stokely-Van Camp Corporation, Harrison, NJ; olive oil (free fatty acid, 1.7%), imported from Filippo Berio, Lucca, Italy, was purchased locally. Fatty acid complexed albumin (BSA) was purchased from Sigma Chemical Company, St. Louis, MO; Neodol 91-6 was supplied by the Shell Chemical Company, Houston, TX; Filtrol Grade 4 was furnished by the Filtrol Corporation, Los Angeles, CA. All other materials were purchased from Thomas Scientific, Philadelphia, PA.

Equipment

All pH measurements were made with a Corning 125 pH meter.

Substrate Purification

Edible tallow and coconut oil were used without further purification because they had already been bleached by the supplier. Olive oil was purified as follows. The oil, 5000 g, was placed in a 3-neck flask with a mechanical stirrer and heating mantle. Filtrol Grade 4 (275 g) was added to the stirred oil, and the mixture was heated under a 25-30 mm vacuum to 90-100 C. The temperature was held there for 1.5 hr while stirring and vacuum were continued. The vacuum was then released by bleeding nitrogen into the system, which was cooled to 70 C and filtered; the yield of filtered oil was 4425 g.

Lipolysis

The substrate, 3.01 g olive oil or tallow or 2.52 g coconut oil (ca. 3.3 millimoles or 10 meq) was placed in a 25 mL Pyrex glass-stoppered Erlenmeyer flask with a 1 in. Teflon-coated magnetic stir bar. A stirring speed of 60-90 rpm was used. The aqueous part of the reaction mixture consisted of 3.5 mL .1 M sodium phosphate buffer solution (pH 5.4) prepared according to Gomori's procedure (15) by adding the proper amount of enzyme and other additives as needed and stirring the solution until all of the enzyme had dissolved. This was added to the reaction flask. If buffer was not used, the enzyme was dissolved in distilled water. The reaction flask was set on a Bell-Stir stirring plate and placed in an incubator that kept the temperature within a 3 C range for a prescribed stirring period. At the end of the reaction period, the flask was placed on a steam bath or in a boiling water bath for 15 min to inactivate the enzyme. The reaction mixture was transferred to a 250 mL Erlenmeyer flask, and the reaction flask was rinsed with 50 mL neutralized 95% ethanol containing phenolphthalein into the reaction mixture. If the test sample is dissolved in the alcohol immediately after the prescribed reaction time, the heat-inactivation step above can be eliminated because the alcohol will also inactivate the enzyme. The alcoholic solution was titrated with 0.1 N sodium hydroxide solution to determine the total fatty acid generated.

Blanks were obtained by titrating the mixture of reactants after the enzyme had been inactivated. All data are averages of at least duplicate or triplicate runs, and most data are averages of 5 replicate runs.

RESULTS AND DISCUSSION

Methodology

The lipase generated by *C. rugosa* was selected for this study because of its nonspecific mode of action, which makes it eminently suitable for the complete hydrolysis of a variety of fatty substrates. Initially, a large body of data was generated with olive oil as the substrate as much of the published literature deals with it exclusively, and it has been the substrate of choice for assaying of lipase activity. However, the results obtained with olive oil were quite erratic, varying from batch to batch. Some lots of oil appeared to contain impurities that totally inhibited lipase action. Treatment with a bleaching clay, as described in the experimental section, removed the lipase inhibitors as well as color bodies. Tallow and coconut oil were both edible grades that had been refined and bleached by the manufacturers and were used without purification. We did not investigate the chemical nature of the inhibitors in olive oil. Surprisingly, in view of the common use of olive oil as a substrate for lipase standardization, this purification has not been documented in the literature.

The major reaction parameters of enzyme concentration, pH, temperature, effect of hydrocarbon solvent on tallow hydrolyses and effects of other additives on olive-oil hydrolysis were investigated in the enzyme-catalyzed hydrolyses of tallow, coconut oil and olive oil. Although the experimental procedure described above is quite simple, a careful technique must be used. Use of an oversized reaction flask is not advisable and excessive stirring speed could cause a separation of the enzyme from the main body of stirred liquid. Accurate timing for the 1 hr and 2 hr time intervals is critical, because the initial hydrolysis rate is rapid, and the enzyme action must be stopped promptly. If these precautions are observed, the data obtained are reproducible within ±10%.

Logarithmic Data Plots

The data obtained give the free fatty acid content of the reaction mixture, and the free fatty acid content equals the

degree of hydrolysis. The industrial practitioner of enzymatic lipolysis will find plotting the percentage of hydrolysis as a function of the logarithm of reaction time to be convenient, and Figure 1 shows results for tallow hydrolysis at 40 C at levels of 3, 6, 15, and 30 units of lipase per milliequivalent substrate (U/meq). Almost a linear relationship between these 2 parameters exists, regardless of enzyme concentration. The same data also can be plotted showing the percentage of hydrolysis as a function of the logarithm of enzyme concentration, which will also show an almost linear relationship. Similar linear relationships were observed for coconut oil and olive oil. Although these straight-line relationships are entirely empirical, extrapolation or interpolation of such plots provides the simplest and most practical way of estimating the time required for complete hydrolysis or activity of an enzyme solution of unknown activity.

Duplicate experiments were carried out to determine the minimum amount of lipase required to hydrolyze the 3 substrates completely after a 72 hr reaction time. For tallow and coconut oil, an average lipase level of 15 U/meq was required. An average of 6 U/meq was required for olive oil at 26 C. These data agree quite well with values extrapolated from semilogarithmic plots.

Analysis of Kinetic Data

To assess the reproducibility of the measurements of extent of hydrolysis, multiple independent analyses were obtained for each oil at each reaction time. As shown in Table I, the precision of the experimental determination of the extent of hydrolysis is within ±10%. We observed that 1 hr and 2 hr rates of reaction showed a high degree of variability. Consequently, in this study rate contants of the hydrolysis reactions were calculated from data for 4 hr, 8 hr and 16 hr periods.

A typical plot of experimental data fitted to a first order model is presented in Figure 2. The solid line is the linear regression fit to the indicated experimental data. All correlation coefficients for individual experiments fell within the range of .92-.99. The first-order rate constant for the overall hydrolysis reaction, k, is obtained from the slope of the regression line. Statistical comparisons of rate constants from different experiments were performed via a *t* test on the 2 slopes of the regression lines using pooled estimated standard deviations.

Figure 3 shows the effect of pH on lipolysis. Between pH 4.8 and 7.2, no appreciable change occurs in the extent of lipolysis within the ±10% accuracy of the methodology.

All experiments, except those recorded in Figure 3, were conducted at pH 5.4 in .1 M sodium phosphate buffer, and the pH did not change over the course of the reaction. At this pH, the enzyme is stable and possesses a high activity. According to the literature, buffering has an effect on lipolysis rate. Unbuffered lipolysis during the early stages of hydrolysis sometimes gives erratic results, and the main virtue of the use of buffer appears to lie in the generation of more reproducible data. Experiments were conducted to determine the effect of phosphate buffers on *C. rugosa* lipase activity. Tallow at 40 C was used as the substrate and

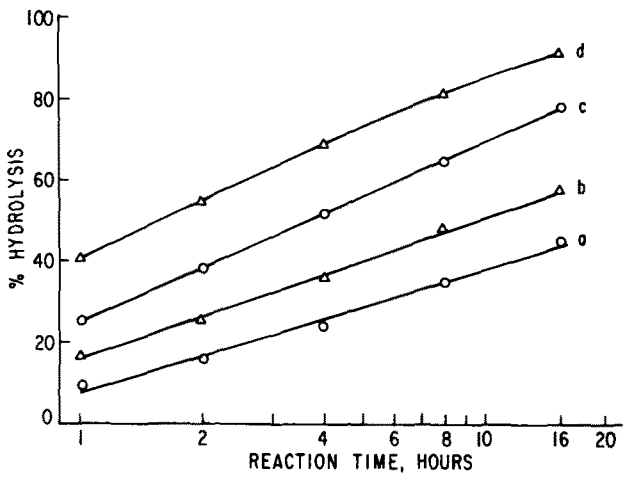

FIG. 1. Variation of tallow hydrolysis with reaction times at various lipase levels at 40 C in the presence of pH 5.4 phosphate buffer: (a) 3 U/meq, (b) 6 U/meq, (c) 15 U/meq and (d) 30 U/meq lipase level.

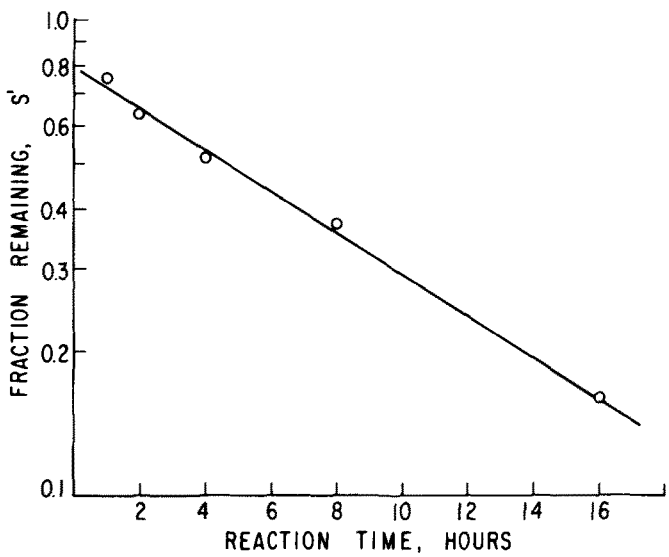

FIG. 2. Typical plot showing fit of experimental data to first-order reaction model. ○ = experimental data; — = linear regression line; coconut oil, enzyme concentration = 30 U/meq, pH = 5.4, .1 M sodium phosphate buffer; T = 26 C.

TABLE I

Precision of Hydrolysis Data[a]

Time (hr)	Olive oil			Coconut oil			Tallow	
	Average fraction hydrolyzed	s	n	Average fraction hydrolyzed	s	n	Average fraction hydrolyzed	s
1	.289	.0273	3	.164	.0158	3	.252	.0035
2	.459	.0319	3	.255	.0032	3	.398	.0079
4	.659	.0133	5	.376	.0049	5	.493	.0416
8	.808	.0460	3	.515	.0211	3	.685	.0287
16	.932	.0175	3	.682	.0147	3	.793	.0203

[a]pH 5.4, enzyme concentration: 15 U/meq in .1 M phosphate buffer; s = standard deviation; olive oil and coconut oil hydrolyzed at 26 C, tallow at 46 C.

2 enzyme concentrations (3 and 30 U/meq) were investigated. Statistical analysis of the results found no significant difference in reaction rate because of the presence of the phosphate buffer (P = .01).

The effect of enzyme concentration on the overall rate of hydrolysis can be seen in Table II. The data indicate decreasing enzyme use as the enzyme concentration is increased. This reduction is pronounced for tallow and coconut oil but is slight for olive oil up to an enzyme concentration of 15 U/meq. The slight specificity of the enzyme for oleic acid (11) might account for this. Because the enzyme reaction occurs at the interface between the phases, the potential exists for the reaction rate at elevated enzyme concentrations to be limited by surface area or diffusion of water into the oil phase.

The reproducibility of rate-constant determinations varies for the substrates studied. Overall rate constants determined in triplicate, Table II, reveal that the data for olive oil exhibit less precision than those for coconut oil and tallow. This indicates a generally higher level of error in the analytical measurements for the olive-oil experiments.

On a kinetic basis, olive oil is the best substrate of those evaluated for hydrolysis by the *C. rugosa* lipase under the conditions investigated (Table II). The experiments were carried out at different temperatures, tallow at 40 C, coconut oil and olive oil at 26 C. When the substrates are compared at 40 C, olive oil again has the highest overall apparent rate constant. Statistical analysis of the above data also show that no significant differences are found in the rates of tallow and coconut oil hydrolysis at equal enzyme levels (P = .05).

The differences in hydrolysis rate may possibly be related to the chemical structure of various oils. The degree of unsaturation is high for olive oil (ca. 85% oleic acid), moderate for tallow (ca. 40% oleic acid) and low for coconut oil. Although the *C. rugosa* lipase is considered to be nonspecific (6), it might slightly favor the hydrolysis of unsaturated lipids.

According to the supplier, the *C. rugosa* enzyme is stable up to temperatures of ca. 50 C. Experiments were conducted with olive oil to determine if an increase in hydrolysis rate could be achieved at higher temperatures. No statistically significant increase in overall hydrolysis rate was found in the temperature range 26-46 C (P = .05). At 50 C and above the enzyme becomes denatured.

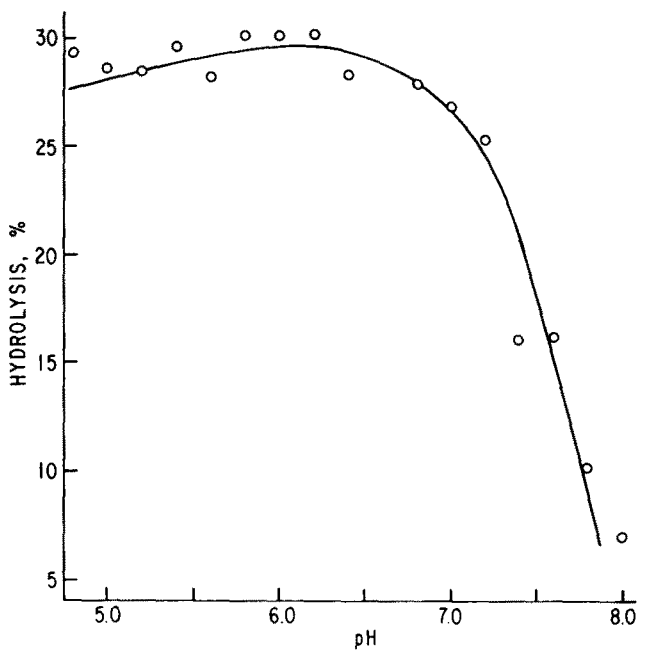

FIG. 3. Effect of pH on overall hydrolysis of olive oil, 2 hr reaction time, 26 C, 6 U/meq lipase.

Effect of Additives

If a low-boiling hydrocarbon solvent could be used in tallow hydrolysis, the reaction could be carried out at room temperature instead of at 46 C. In order to liquefy tallow completely at room temperature, an equal volume of hexane is required. However, as Table III shows, adding hexane results in a substantial reduction in lipolysis and does not represent a feasible approach. Temperature, as shown above, has no effect, and lipolysis at 26 C and 40 C proceeds to about the same extent.

Various studies on enzymatic hydrolysis (10-13) indicate that the addition of sodium or calcium ion, bovine serum albumin and nonionic emulsifiers drastically enhances the initial rates of enzyme-catalyzed lipolysis. These studies were usually limited to 15-20 min reaction times. Our studies indicate that this enhancement disappears when hydrolysis is carried out over longer periods of time.

TABLE III

Effect of Hexane on Tallow Hydrolysis[a]

Temperature (C)	Volume of hexane (mL)	Percentage of hydrolysis after 1 hr	2 hr	4 hr	8 hr	16 hr
26	3.5	1.0	2.1	3.4	6.0	16.2
40	3.5	1.6	2.3	3.3	7.6	14.0
40	0.0	16.5	24.7	35.4	48.0	56.9

[a]pH 5.4 .1M phosphate buffer, enzyme concentration = 6 U/meq (based on aqueous volume).

TABLE II

Effect of Enzyme Concentration on Overall Rate of Hydrolysis[a]

Enzyme concentration (U/meq)	Temperature (C)	Overall rate constant, k X 10^2, hr^{-1} Olive oil	Coconut oil	Tallow[b]
3	26	3.71	2.36	2.92
6	26	7.38	3.67	4.67
15	26	15.7[c] (s = 2.2)	6.39[c] (s = .20)	8.70[c] (s = .61)
30	26	18.2	10.0	
	40	26.3	8.49	10.5

[a]pH 5.4, .1 M phosphate buffer.

[b]T = 40 C in all experiments.

[c]Mean of 3 independent determinations; s = standard deviation.

The effects of such additives on enzymatic hydrolysis were examined as shown in Table IV. Although sodium ion has no effect, calcium ion appears to act as an inhibitor. The addition of .003% albumin gives essentially no improvement after 8 hr and 16 hr, as does the addition of 0.01% of a nonionic ether alcohol-type of surfactant (Neodol 91-6). However, as the concentration of the latter is increased to 0.1%, strong inhibition is evident during the first 8 hr of reaction time. Understanding the mechanisms that causes the reaction rate to increase dramatically after 8 hr is difficult.

TABLE IV

Effect of Various Additives on Olive-Oil Hydrolysis[a]

Additive	Percentage of hydrolysis after				
	1 hr	2 hr	4 hr	8 hr	16 hr
None	15.4	24.8	39.1	54.7	72.3
.1 M NaCl	14.5	23.8	45.1	60.5	74.0
.1 M $CaCl_2$	5.0	18.0	31.9	45.1	58.0
.003% Albumin	17.8	25.3	41.0	58.1	79.7
.01% Neodol	12.6	16.2	43.5	65.3	81.1
.1% Neodol	0.5	6.7	7.1	20.5	72.6

[a]pH 5.4, .1 M phosphate buffer, 26 C, enzyme concentration = 6 U/meq.

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Distribution of Ammonia-Related Aflatoxin Reaction Products in Cottonseed Meal

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ABSTRACT

The fate of aflatoxin during ammoniation of contaminated cottonseed meal was studied under conditions approximating those approved for commercial ammoniation of nonaflatoxin-contaminated meal. Uniformly ring-labeled ^{14}C-aflatoxin B_1 was added to 27.7 kg meal (14% moisture) that contained ca. 4000 μg naturally incurred aflatoxin B_1/kg. Distribution of the radiolabeled compound was used to trace the modification of aflatoxin B_1 after treatment with 4% ammonia at 40 psi, 100 C for 30 min. This treatment reduced the chemically detected aflatoxin B_1 to less than 4 μg/kg. In control nonammoniated meals, 90% of the radiolabeled material was accounted for in the methylene chloride extract. Duplicate 2-kg samples of the ammoniated meal were fractionated and the distribution of radioactivity was determined. Ca. 86% of the radioactivity was detected in the meal after initial air-drying. Ca. 25% of the added radioactivity was extracted from the air-dried meal with methylene chloride and another ca. 5% was extracted from this residue with methanol. Weak acid released 3% of the added radioactivity from the residue after methanol extraction, bicarbonate released 1% and Pronase digestion, including methylene chloride extraction of the residue, accounted for nearly 19% of the total added radioactivity. Only 37% of the added radioactivity remained in the meal matrix following solvent extractions and chemical and enzymic treatments.

INTRODUCTION

In 1969, based on the Food and Drug Administration's analytical capability at the time and with little knowledge of the risk from aflatoxin ingestion, an action level of 20 parts per billion (ppb) total aflatoxins (B_1, B_2, G_1 and G_2) was established. This action level remains in effect today and applies to commodities (raw or processed) that are susceptible to contamination, including feeds. A level of 300 ppb is allowed in whole cottonseed that will be used only for beef, swine and poultry rations.

Treatment with ammonia gas under pressure is an effective procedure for chemical inactivation of aflatoxins in cottonseed meal (1). Whole cottonseed has been decontaminated at ambient temperatures (2). Pressure inactivation reduced the aflatoxin content to less than 1 μg/kg but did not account for products formed from the toxin by the ammoniation treatment. Lee et al. (3) and Cucullu et al. (4) studied the products formed in a model reaction of aflatoxin B_1 and ammonium hydroxide with heat and pressure. They identified trace amounts (<0.1%) of one of the major products of this model reaction in ammoniated peanut