Kinetic Study of Acid-Catalyzed Conversion of Aflatoxins B_1 and G_1 to B_{2a} and G_{2a}

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ABSTRACT

Adjusting dilute aqueous solutions of aflatoxins B_1 and G_1 to pH 1, 2 and 3, and heating over a range of 40-100 C resulted in the conversion of B_1 to B_{2a} and G_1 to G_{2a} as major products. Both B_{2a} and G_{2a} were identified by co-thin layer chromatography with authentic B_{2a} and G_{2a} and M_1 on silica gel plates developed in two different solvents. The rate of disappearance of B_1 or G_1 at given temperature and at constant pH was found to be first order with respect to each aflatoxin. At given temperature the conversion is strongly pH dependent, a 10-fold increase in H^+ ion (1 pH unit) producing about a 9-fold increase in the reaction rate, indicating first order dependent of the rate on H^+ ion concentration.

INTRODUCTION

Oilseeds, among other agricultural products, are subject to sporadic invasion by toxigenic strains of the molds *Aspergillus flavus* and *Aspergillus parasiticus*, with the subsequent elaboration of toxic fluorescent mold metabolites known as aflatoxins (1). When such mold damaged oilseeds are processed, a portion of the aflatoxins are removed in the crude oil (2-4). Since these compounds are lactones, they are readily alkali soluble, are removed during conventional alkali refining and bleaching operations (2), and are presumably concentrated in the byproduct soapstock from the refining. Based on the observations that mild acidulation of alkaline solutions of aflatoxins results in relactonization and regeneration of the aflatoxin structure (2,5), these compounds may be regenerated during the acidulation of aflatoxin contaminated alkaline soapstocks for the recovery of the fatty acids. In fact, Cucullu et al. (5) have recently reported the presence of aflatoxins in several samples of alkaline and acidulated soapstocks obtained from the refining of aflatoxin contaminated crude oils.

Soapstocks are conventionally acidulated with sulfuric acid, heated, and the fatty acid recovered either by continuous centrifugal, or gravity settling operations. In the continuous system, a heated alkaline soapstock slurry, 190-200 F, is treated with sulfuric acid at a pH of 3-4, and held for minimal reaction time prior to centrifugal separation of the fatty acids (6,7). Batch systems usually employ acidulation with an excess of mineral acid, boiling of the acidulated mixture for 4-6 hr, followed by overnight settling to recover the fatty acids (7).

Several investigators have reported that strong acids catalyze the addition of water to the vinylene group of the dihydrofuran moiety of aflatoxin B_1 to yield a hydroxy analog of aflatoxin B_2 with the hydroxyl group substituted at the 2 position. This derivative has been termed aflatoxin B_{2a} (8), aflatoxin B_1 hemiacetal (9) and hydroxydihydro B_1 (10). An analogous derivative, G_{2a}, is produced from acidulation of aflatoxin G_1 (11). It has been reported that aflatoxins B_{2a} and G_{2a} are much less toxic than are B_1 or G_1, producing no detectable toxicity or liver lesions when fed to day-old ducklings at dosages of 66 times the LD_{50} value of B_1, and 19 times the LD_{50} of G_1, respectively (11).

The present study was undertaken to obtain information on the fate of aflatoxins B_1 and G_1 under simulated soapstock acidulation conditions. For this purpose, model systems were used in order to obtain kinetic data on the rate of disappearance of aflatoxins B_1 and G_1 as a function of pH, temperature and time.

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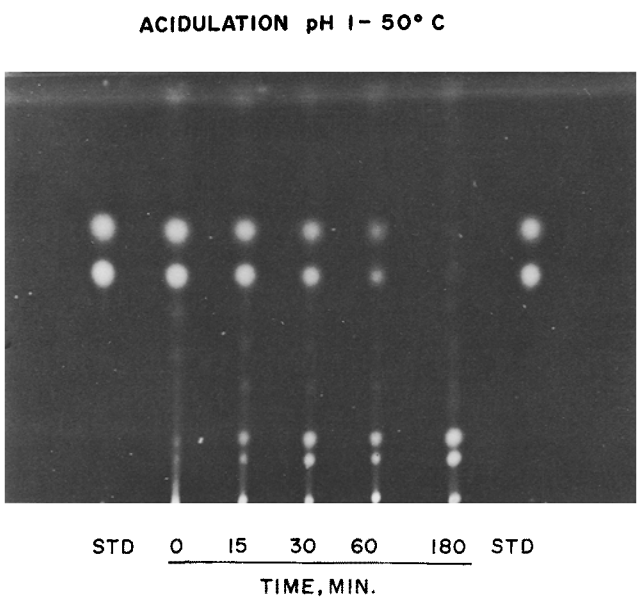


FIG. 1. Thin layer chromatograms from acidulation of aflatoxins B_1 and G_1 at pH 1 and 50 C, 0-180 min reaction. Plate developed in chloroform-acetone 9:1.

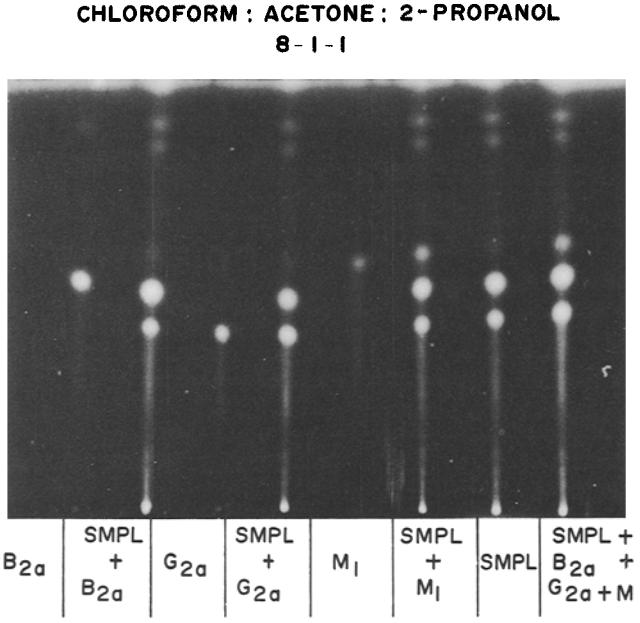
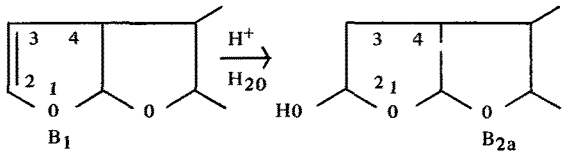


FIG. 2. Thin layer chromatography of B_1 and G_1 conversion products (pH 1, 50 C) with authentic B_{2a}, G_{2a} and M_1. Plate developed in chloroform-acetone-2-propanol 8:1:1.



EXPERIMENTAL PROCEDURE

A standard aflatoxin solution was prepared by dissolving accurately weighed 1 mg portions of crystalline aflatoxins B_1 and G_1 in 100 ml of chloroform. Aliquots of the standard solution representing 50 μg each of B_1 and G_1 were pipetted into a beaker, the solvent removed under a stream of nitrogen, and the aflatoxin dissolved in 500 ml of water previously adjusted to pH 12 with sodium hydroxide. While stirring, the solution was adjusted to the desired reaction pH (1,2 or 3) with 6N sulfuric acid, with a calibrated glass electrode as pH indicator. The reaction mixture was transferred to a preheated 1 liter round bottom flask fitted with a Variac controlled heating mantle, stirrer, thermometer and condenser, and quickly raised to the desired reaction temperature in the range of 40-100 C (± 0.5 C). The pH remained constant during all acidulation experiments.

As soon as the reaction temperature was attained, usually within 5-8 min, a 50 ml 0 time aliquot was withdrawn by pipette, transferred to a graduated 250 ml cylinder. The reaction was quenched by addition of 20 ml of 20% lead acetate solution and 60 ml of acetone, followed by rapid cooling to room temperature. A similar procedure was followed for 50 ml aliquots removed after reaction, at a given pH and temperature, for intervals ranging from 15-360 min.

The reaction aliquots were then diluted to 200 ml with water, 4-5 g of Celite analytical filter aid were added, and after mixing the contents were filtered through paper. A 100 ml aliquot of the filtrate was extracted twice with 50 ml portions of chloroform, in a separatory funnel, to quantitatively extract residual aflatoxin B_1 and G_1 in the aliquots. After evaporation of solvent, residues were dissolved in a known volume of benzene-acetonitrile 98:2 v/v, and duplicate 5 μl aliquots along with duplicate 5 μl aliquots of an aflatoxin standard (1.0 μg of B_1 and G_1/ml) dissolved in the same solvent were spotted on thin layer plates coated with Adsorbosil-1 silica gel (0.5 mm wet thickness). Plates were developed in chloroform-acetone 9:1 v/v for 12 cm solvent travel to separate residual aflatoxins B_1 and G_1 from fluorescent reaction products, and then scanned with a recording fluorodensitometer as outlined by Pons et al. (12) to determine the amounts of residual aflatoxins B_1 and G_1 in the reaction aliquot chromatograms. Thin layer chromatography (TLC) followed by fluorodensitometric measurement of aflatoxins on the thin layer plates allowed an estimate within ± 2-4% of the amount of B_1 and G_1 remaining after reaction, for a given time at given reaction temperature and pH.

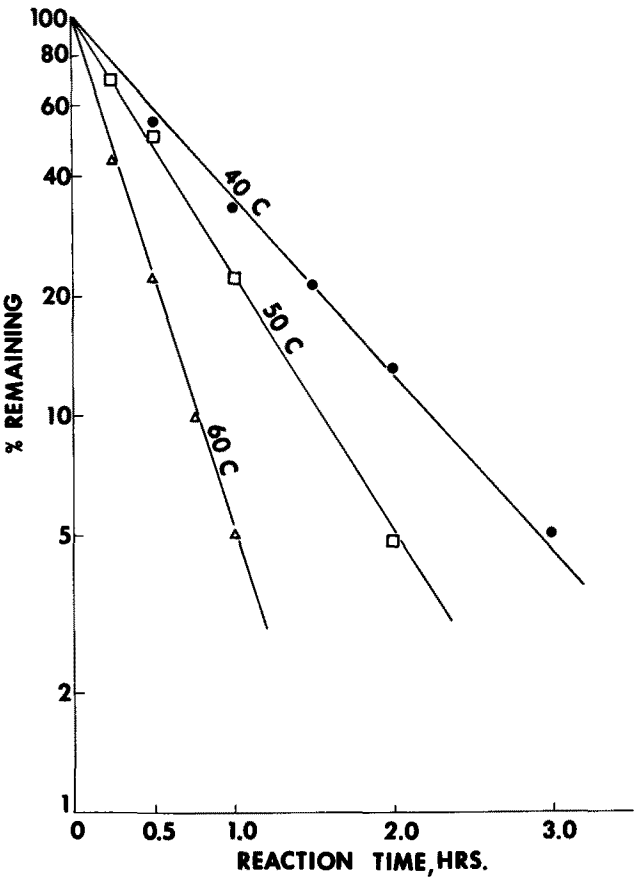


FIG. 3. First order reaction plots, acidulation of aflatoxins B_1, at pH 1, 40-60 C.

RESULTS

Conversion Products

TLC of aliquots of the reaction products on silica gel coated plates showed a gradual decrease in the amounts of B_1 and G_1 with time, and the appearance of two major fluorescent derivatives with low R_f. A typical chromatogram is shown in Figure 1 for the reaction of B_1 and G_1 at pH 1, and 50 C.

The low R_f reaction products of B_1 and G_1 at pH 1 and 50 C were subjected to co-TLC with aflatoxins B_{2a} prepared according to Pohland et al. (9); G_{2a} prepared

TABLE I

Specific Reaction Rate Constants and Catalytic Coefficients for Conversion of Aflatoxins B_1 and G_1 to B_{2a} and G_{2a}

Reaction temperature, C	Specific reaction rate constant, k min^{-1} x 10^3						k_H[b]	
	Aflatoxin B_1			Aflatoxin G_1			B_1	G_1
	pH 1	pH 2	pH 3	pH 1	pH 2	pH 3		
40	16.5[a]	1.39	0.233	12.8[a]	0.979	0.095	0.179	0.107
50	28.6[a]	2.90	0.450	23.5[a]	2.00	0.205	0.342	0.213
60	48.0[a]	5.80	0.838	41.7[a]	3.91	0.423	0.633	0.410
70	78.1	11.2[a]	1.50[a]	71.4	7.36[a]	0.835[a]	1.13	0.762
80	124	20.7[a]	2.61[a]	119	13.4[a]	1.59[a]	1.97	1.37
90	191	37.0[a]	4.39[a]	192	23.5[a]	2.91[a]	3.33	2.39
100	289	64.2[a]	7.19[a]	303	40.0[a]	5.16[a]	5.50	4.06

[a]Experimental data; all others estimated from plot of *ln k* vs. 1/T.
[b]$k_H = k/C_{H+}$.

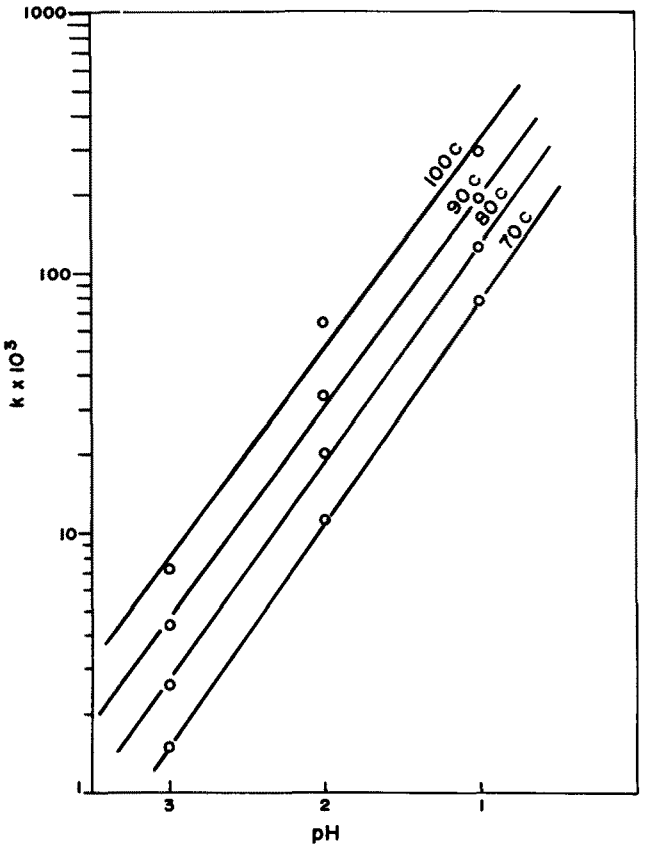


FIG. 4. Semilog plots of k against pH for acidulation of aflatoxin B_1 at 70-100 C.

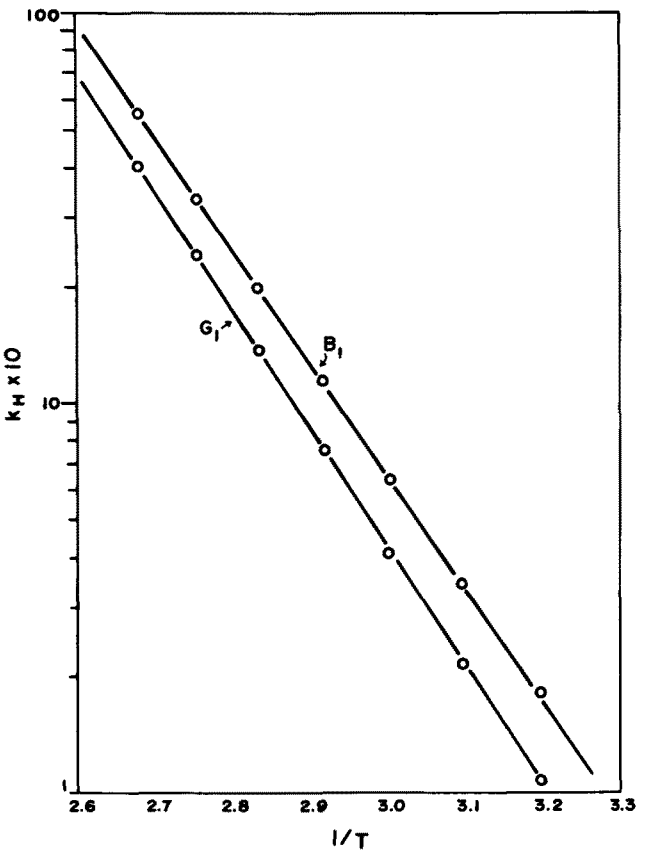


FIG. 5. Semilog plots of k_H against 1/T, aflatoxin B_1 and G_1.

in a similar manner, and with authentic aflatoxin M_1. Plates were developed in chloroform-methanol 9:1 or in chloroform-acetone-2-propanol 8:1:1 to increase the R_f of the fluorescent conversion products, and to effect separation of the three hydroxy aflatoxin derivatives. The chromatograms shown in Figure 2 representing the co-chromatography of the B_1 and G_1 reaction products at pH 1 and 50 C developed in chloroform-acetone-2-propanol 8:1:1 clearly identify the major conversion products as B_{2a} and G_{2a}, respectively. The hydroxy analog of B_1 with the OH group in the 4 position in the terminal furan ring, aflatoxin M_1, is not a major reaction product. Although the G_1 analog with OH in the 4 position GM_1 (13), was not available for chromatography, the chromatograms show no evidence of any major hydroxy derivatives other than B_{2a} or G_{2a}.

Both B_{2a} and G_{2a} were found to be unstable, decomposing to yellowish nonfluorescent derivatives during acidulation treatments and even during storage of reaction aliquots in solution. Attempts to actually measure the amounts of B_{2a} and G_{2a} produced during the course of acidulation reactions were unsuccessful. Early reaction time aliquots showed gradual increases in B_{2a} and G_{2a}, but those from longer reaction times showed an actual decrease, undoubtedly owing to decomposition of the hydroxy derivatives. Consequently all kinetic data developed in the study were based on the rate of disappearance of B_1 and G_1 as a function of pH, temperature and time.

Reaction Rates

Semilog plots of the percentage of B_1 or G_1 remaining against reaction time at constant temperature, and pH yielded typical first order reaction plots similar to those shown in Figure 3 for the acidulation of aflatoxin B_1 at pH 1, 40-60 C.

It should be emphasized that the reaction is first order with respect to B_1 or G_1 only at constant H^+ ion concentration.

The experimental reaction rate data were fit to a first order irreversible kinetic equation by minimizing the objective function,

$$\text{Sum of square} = \sum_{i=1}^{n} (Y^i - Y^c)^2$$

wherein $Y_c = Y_o e^{-kt_i}$, and n is the number of experimental data points, Y_o the initial percentage of aflatoxin remaining (100), Y_i the experimentally determined percentage remaining at time of reaction t_i, and Y_c the percentage of aflatoxin remaining as calculated from the time of reaction and the optimized rate coefficient k. The minimization was accomplished on a digital computer through successive optimizations of the rate coefficient by use of the Simplex procedure described by Nelder and Mead (14).

Arrhenius parameters were determined from a linear least square plot of $ln\ k$ against 1/T. For purposes of comparing results at different levels of acidity, ideality was assumed and the energy of activation (E_a) was computed as the negative slope of the line multiplied by the molar gas constant R (1.987 cal/deg/mole). Enthalpy of activation (ΔH^*, $k$cal/mole) was calculated from the relationship $\Delta H^* = E_a - RT$. The free energy change (ΔF^*, $k$cal/mole) was then calculated from the Eyring equation for absolute reaction rates (15), as R T times the natural logarithm of $2.0842 \times 10^{10}\ T/k$ (in sec^{-1}), and the change in entropy (ΔS^*, cal/deg/mole) from the thermodynamic relationship at constant temperature (16), $\Delta S^* = (\Delta H^* - \Delta F^*)/T$.

Calculated specific reaction rate constants, $k^{min\text{-}1} \times 10^3$, are tabulated in Table I. Constants listed at pH 1 (70-100 C), pH 2 (40-60 C) and pH 3 (40-60 C) were estimated from the slope and intercept of the regression equation of $ln\ k$ of experimental rates on the reciprocal of

TABLE II

Thermodynamic Constants Calculated From Catalytic Coefficients, k_H

Constant	Aflatoxin B_1		Aflatoxin G_1	
	40 C	100 C	40 C	100 C
ΔH^a	12.6	12.5	13.4	13.3
ΔF^b	22.0	23.8	22.3	24.0
ΔS^c	-30.0	-30.3	-28.4	-28.7

[a] *k*cal/mole.
[b] *k*cal/mole.
[c] cal/deg/mole.

TABLE III

Calculated Time Required to Convert 95% of Aflatoxin B_1 to B_{2a}

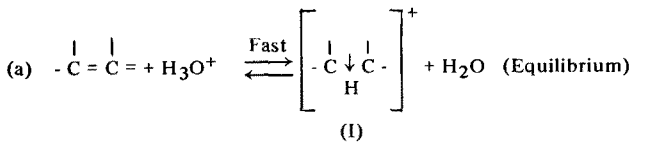
Temperature, C	$t_{0.05}$[a]		
	pH 1	pH 2	pH 3
40	3.0 hr	36.0 hr	214 hr
50	1.7 hr	17.2 hr	111 hr
60	1.0 hr	8.6 hr	59.6 hr
70	38 min	4.5 hr	33.2 hr
80	24 min	2.4 hr	19.1 hr
90	16 min	1.4 hr	11.4 hr
100	10 min	47 min	7.0 hr

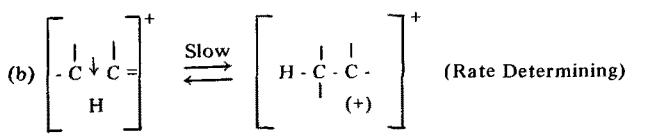
the absolute temperature.

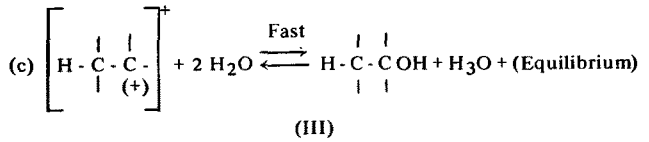
The conversion reaction is of course temperature dependent and the average relative increase in the rate per 10 C increase in temperature was 1.8 for both B_1 and G_1. The pH has a marked effect on the reaction rate as may be seen in Table I. The average relative increase in the reaction rate per 10-fold increase in H^+ ion concentration (1 pH unit) was 7.5 for B_1 and 9.5 for G_1 for an average of 8.5. This indicates that the conversion reaction is also approximately first order with respect to H^+ ion concentration. The semilog plots of k against pH for the acidulation of aflatoxin B_1 at 70-100 C, shown in Figure 4, illustrates the first order dependence of the rate on H^+ concentration.

Mechanism

The acid-catalyzed hydration of aflatoxins B_1 or G_1 to yield the saturated hydroxy derivatives B_{2a} or G_{2a} (8-11) should be analogous to the proposed mechanism for the hydration of olefins (17,18). Such reactions involve rapid proton attack at the C = C bond to produce an activated π complex (I), a carbonium ion (II), followed by addition of water to yield the carbinol (III) the rate determining step, being the slow conversion of the π complex (I) to the carbonium ion (II).







Thus the rate of hydration is actually second order, depending on the concentration of olefin and H^+ ion

$$\left[-\frac{dC}{dt}\text{ olefin} = k\ (C\ \text{olefin})\ (C_{H+})\right]$$

and is pseudo-first order at constant pH.

The experimental unimolecular rate constante, k, tabulated in Table I are actually the sum of two terms:

$$(a)\ k = k_O + k_H\ C_{H^+}$$

where k = the rate for the uncatalyzed reaction in pure water (pH 7) and k_H = the catalytic coefficient. Using the regression of $ln\ k$ on pH, estimated values for k_o for aflatoxin B_1 at pH 7 were found to be 0.417×10^{-7} (40 C) and 0.500×10^{-5} (100 C), insignificant in terms of the magnitude of the acid catalyzed k. Thus equation (a) reduces to $k = k_H\ C_{H+}$, and $k_H = k/C_{H+}$. Mean catalytic coefficients, k_H, calculated from the $k$constants at pH 1, 2 and 3 are also listed in Table I for aflatoxins B_1 and G_1. There is a definite trend toward slightly lower coefficients for aflatoxin G_1 as compared to B_1. Semilog plots of k_H vs. 1/T show good fit of the experimental data for both B_1 and G_1 as illustrated in Figure 5.

Since the k_H coefficients at given temperatures (Table I) represent an average of the experimental data obtained at pH 1, 2 and 3, they were used in the regression of $ln\ k_H$ on 1/T to calculated the free energy change (Δ F*), enthalpy of activation (Δ H*), and entropy of activation (Δ S*) as previously outlined. These data are shown in Table II, for 40 C and 100 C. The influence of H^+ ion catalysis on the enthalpy of activation is appreciable. For example, Δ H* for B_1 at 100 C is about 12.5 *k*cal while Δ H* calculated for the uncatalyzed reaction in pure water (pH 7) was about 18.0 *k*cal. Entropies of activation (Δ S*) were negative ranging from about -28 to -30 E.U. Consideration of the proposed mechanism for olefin hydration (18) suggests that these large negative entropies result from restriction in the freedom of motion. of the activated complex due to the bonding of water molecules in the transition state.

Taft (18) suggests that all hydrations whose rates parallel H^+ ion concentration, involve mechanisms by which water is bonded in the transition state and result in large negative entropies of activation owing to loss of translational motion. Where water is not involved in the transition state, hydration rates parallel the Hammett acidity function h_O, and the entropy of activation is actually positive (18). He reported Δ S* values ranging from + 2 E.U. for the hydration of isobutene to -23 E.U. for the hydration of crotonaldehyde and β,β-dimethylacrolein. Thus the hydration of aflatoxin B_1 proceeds by a mechanism different from that proposed for simple olefins (17,18).

The rate constants k, tabulated in Table I, can be used to calculate the time required to convert any fraction of the original B_1 or G_1 to B_{2a} or G_{2a}, respectively. The time required to convert any portion of aflatoxin B_1 or G_1 at given pH and temperature, is important for conversion of aflatoxins during the acidulation of contaminated soapstocks. The times required for 95% conversion ($t_{0.05}$) outlined in Table III, emphasize that the conversion reaction is quite slow at pH 3, fairly rapid at pH 2 and very rapid at pH 1. Low acidulation pH and prolonged holding times at high temperature, as usually employed in batchwise soapstock acidulations, would favor the inactivation of any aflatoxins present. Where short holding time, ca. 5-10 min, is practiced, as in continuous acidulation (6,7), high temperature (100 C) at pH 1 would seem to be optimum

conditions to assure rapid conversion of aflatoxin contamination in soapstocks to relatively innocuous derivatives.

ACKNOWLEDGMENTS

R.R. Benerito, A.G. Pierce, Jr., R.J. Berni, and L.E. Brown of the Southern Regional Research Laboratory gave consultation and technical help. A.C. Keyl, Western Regional Research Laboratory, ARS, USDA, supplied authentic aflatoxin M_1.

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[Received August 26, 1971]