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Kinetic and Mechanistic Investigations on Reductions of Aflatoxins by Lactic Acid

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Abstract—The kinetics of reduction of AFB_1 to AFB_2 and AFG_1 to AFG_2 by lactic acid has been investigated in dilute aqueous acidic solutions (pH 3.35–4.50) as a function of the concentrations of lactic acid, AFB_1, AFG_1 and hydrogen ion at 37 °C. The rate of the reaction was found to be first order with respect to the concentrations of lactic acid and aflatoxins and independent on hydrogen ion concentration. The experimental results are interpreted in terms of mechanisms involving an initial formation of transient oxonium intermediate, which tends to polarize the olefinic (C=C) carbon, which in turn causes the hydride abstraction from α-carbon atom of lactic acid in rate determining step. The proposed mechanisms involve an overall transfer of two protons and two electrons from lactic acid to AFB_1 and AFG_1 to give the corresponding reduced less toxic products AFB_2 and AFG_2 and the oxidised product pyruvic acid.

Aflatoxins (B_1, B_2, G_1 and G_2) are food-borne secondary toxic fungal metabolites produced by *Aspergillus flavus* and *Aspergillus parasiticus*. Consumption of aflatoxin in many parts of the world varies from 0 to 30,000 ng/kg/day.[1] Aflatoxins have been incriminated in hepatocellular carcinoma, acute hepatic failure and Reye's Syndrome. Exposure to aflatoxin may begin prenatally[2,3] persist during breast feeding[4,5] and continue in adult life. Visualizing the seriousness of aflatoxin problem in human and animal health, various methods were tested to control aflatoxin production. Uses of propionic acid, butyric acid[6] or lauric acid derivatives[7] have been shown to reduce fungal growth and aflatoxin production by *A. flavus*. Our earlier studies have also revealed that presence of lactic acid[8] or L-ascorbic acid[9] in culture medium significantly reduces mycelial growth and aflatoxin production. Amongst different aflatoxins present in culture medium, the concentrations of AFB_2 and G_2 were comparatively higher than B_1 and G_1.

Biochemical oxidation of lactic acid to pyruvic acid by lactate dehydrogenase is a well known enzymatic reaction in metabolic pathways in which NAD is reduced to $NADH_2$ involving an overall transfer of two protons and two electrons from lactic acid. A lactate dehydrogenase analogue of ascorbate dehydrogenase[10] system also seems to be efficiently operating in reduction of aflatoxins B_1 and G_1, to corresponding less toxic products aflatoxins B_2 and G_2, respectively. In order to understand the chemical nature of interaction of lactic acid, which behaves as a source of donor of two protons and two electrons during its interaction with aflatoxin, present investigation was undertaken to study the kinetic and mechanistic aspects of the redox reaction in aqueous solution.

A. parasiticus (NRRL 3240) obtained from Indian Agricultural Research Institute, New Delhi, was grown on sucrose-magnesium sulphate-potassium nitrate-yeast extract (SMKY) liquid medium at 28±2 °C for 10 days.[11] Culture filtrates were extracted with analytical grade chloroform (1:2; v/v) which was passed through anhydrous sodium sulphate. The chloroform extract was evaporated to dryness. The residue was dissolved in fresh chloroform, transferred to vials and labelled. All the chemicals used were of A.R. grade and A.R. grade lactic acid was used without further purification. All the reactions were run in freshly prepared double distilled water.

100 μL aflatoxin extract along with a pure aflatoxin standard (a gift from the International Agency for Research on Cancer, Lyon, France) were spotted on silica gel G coated activated TLC plates. Thereafter, the

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plates were developed in a solvent consisting of toluene/isoamyl alcohol/methanol (90:32:2; v/v).[12] The plates were air-dried and observed under long wave UV light (360 nm) for aflatoxins (B_1, B_2, G_1 and G_2). The aflatoxins were chemically confirmed by spraying trifluoroacetic acid or 25% sulphuric acid. Each spot was eluted separately and dissolved in chilled methanol. Aflatoxins were quantified using Shimadzu 160 UV–vis spectrophotometer at 360 nm according to the method of Nabney and Nesbitt.[13] Analysis of standard as well as mixture of aflatoxins were also done by HPLC in the beginning while initiating the studies involved in this work.

Aqueous solution of aflatoxin (3.89×10^{-6} mol dm^{-3}) was mixed with 4.44×10^{-4} mol dm^{-3} of lactic acid and incubated at 37 °C. In kinetic experiments the required pH of aflatoxin and lactic acid reaction mixture was maintained by adding dilute solution of HCl/carbonate free NaOH.[10] pH measurements were done with a digital pH meter (±0.01 pH) equipped with combined glass and calomel electrodes. An appropriate quantity of aflatoxin (solution A) and pre-equilibrated (ca. 30 min) solutions containing required concentrations of lactic acid and HCl/carbonate free NaOH (solution B) were made in separate containers. These containers were kept for sufficient time in thermostatic bath (temperature 37 °C). Each of the kinetic runs were started by mixing thoroughly solution A and B and the resulting solutions were subjected for finding the rate of change of concentrations of different aflatoxins with time.

Aqueous solution of 3.89×10^{-6} mol dm^{-3} aflatoxin containing mixtures of aflatoxin B_1, B_2, G_1 and G_2 (3:2:3:2) were thoroughly mixed with 4.44×10^{-4} mol dm^{-3} of lactic acid and incubated at 37 °C and aflatoxins were quantified at regular suitable intervals. Changes in concentrations of AFB_1 to AFB_2 (Fig. 1) and AFG_1 to AFG_2 (Fig. 2) revealed a significant decrease in AFB_1 and AFG_1 followed with increase in concentrations of AFB_2 and AFG_2. For reduction of AFB_1 to AFB_2 the rates (mol dm^{-3} h^{-1}) of decrease in the concentration of AF B_1 ($-$d [AFB_1]/d$t = 7.34\times10^{-8}$) and increase in the concentration of AFB_2 (d [AFB_2]/d$t = 6.21\times10^{-8}$), determined in the beginning, during first 6 h, were found to be higher than that of in the later part (after 6 h). After 6 h, the rates ($-$d[AFB_1]/dt) and (d[AFB_2]/dt) were 0.66×10^{-8} and 0.67×10^{-8} mol dm^{-3} h^{-1}, respectively. The rates of formation of pyruvic acid (PA), (d[PA]/dt), determined in the beginning (6 h) and in the later part (after 6 h) were 6.18×10^{-8} and 0.64×10^{-8} mol dm^{-3} h^{-1}, respectively. Similar results were obtained for reduction of AFG_1 to AFG_2 also. In beginning, during first 6-h rates (mol dm^{-3} h^{-1}) corresponding to ($-$d[AFG_1]/dt), (d[AFG_2]/dt) and (d[PA]/dt): 5.67×10^{-8}, 5.68×10^{-8} and 5.65×10^{-8} were higher than the respective rates 0.53×10^{-8}, 0.55×10^{-8} and 0.53×10^{-8}, determined after 6 h. These findings indicated that the rates of the formation of the products are almost identical with the rates of the decrease in the concentration of the reactants. Hence, in the experimental conditions, all the experimental rates were found to be almost equal for a particular reaction indicating that ($-$d[AFB_1]/dt) = (d[AFB_2]/dt) = (d[PA]/dt) and ($-$d[AFG_1]/dt) = (d[AFG_2]/dt) = (d[PA]/dt). These rates in turn will be also equal to the rate of decrease in the concentration of lactic acid (LA), that is ($-$d[LA]/dt).

Pyruvic acid obtained as the oxidation product of lactic acid was measured spectrophotometrically at 456 nm by using Salicyaldehyde.[14] In order to understand the stoichiometry of the reaction, experiments were conducted by maintaining slight excess of aflatoxins in the reaction mixtures and after completion of the reactions the amounts of aflatoxins (B_1 and G_1), respectively reduced to aflatoxins (B_2 and G_2), were estimated. The data revealed that the total concentration of the reduced products of aflatoxins ($B_2 + G_2$) was almost identical with the initial concentrations of lactic acid allowed in the reaction mixtures. Experiments conducted by maintaining excess of lactic acid in the reaction mixture also indicated that the concentration of the consumed lactic acid during the reaction, was almost equal to, the total initial concentration of AFB_1 and AFG_1 which was identical with the total concentration of the reduced products AFB_2 and AFG_2.The amount of pyruvic acid spectrophotometrically measured, after the completion of the reaction, was also found to be identical with the total concentration of the reduced aflatoxins B_2 and G_2. These results indicated that one equivalent of lactic acid reduces one equivalent of AFB_1 and AFG_1. Thus, the overall reactions between lactic acid and aflatoxins B_1

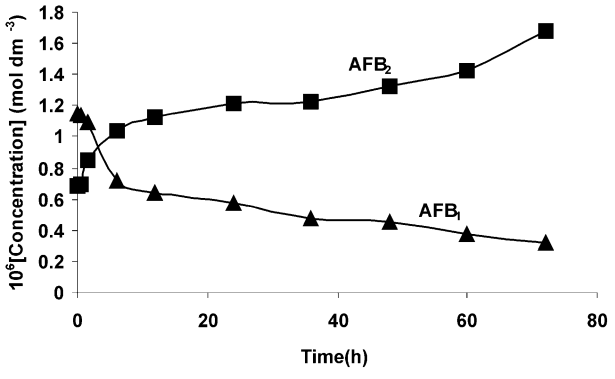


Figure 1. Time-dependent changes in the concentrations of AFB_1 and AFB_2 during interactions with lactic acid.

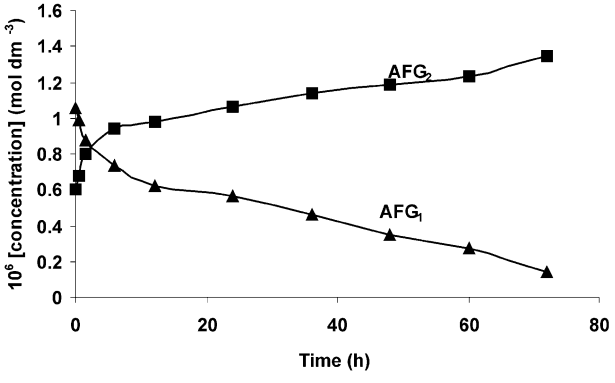


Figure 2. Time dependent changes in concentrations of AFG_1 and AFG_2 during interaction with lactic acid.

and G_1 are outlined as in eqs 1 and 2, where the oxidation product of lactic acid is pyruvic acid and the reduction product of aflatoxins B_1 and G_1 are aflatoxins B_2 and G_2, respectively.

$$CH_3CH\,OH\,COOH + AFB_1 \rightarrow CH_3CO\,COOH + AFB_2 \quad (1)$$

$$CH_3CH\,OH\,COOH + AFG_1 \rightarrow CH_3CO\,COOH + AFG_2 \quad (2)$$

Studies on reduction of AFB_1 to AFB_2 and AFG_1 to AFG_2 were performed in presence of lactic acid by performing a series of kinetic experiments at different initial concentrations of lactic acid and hydrogen ion at constant concentrations of AFB_1 and AFG_1 at 37 °C. An excess of lactic acid was used in all kinetic runs to ensure pseudo first order kinetic conditions and the corresponding rate constants (k_{obs}) in terms of concentration of aflatoxin were computed from the slopes of the plots of log ΔC versus time (multiplied by 2.303) where ΔC is the difference in concentrations at zero and experimental times. The reduction of aflatoxins studied at different concentrations of lactic acid (Table 1) indicated a linear increase in k_{obs} with increasing initial concentration of lactic acid. The plot of log k_{obs} versus log [lactic acid] was found to be linear with unit slopes, indicating that the reaction is first order with respect to concentration of lactic acid. First order kinetic plots of log ΔC versus time, in terms of aflatoxins concentrations, were linear. Also these first order rate constants k_{obs} were found to be constant on varying the initial concentrations of aflatoxins, which obviously indicates that the reaction is first order with respect to aflatoxin concentrations. No dependence on variation of the initial concentration of hydrogen ion on the rate of reduction of aflatoxins was observed in the said pH range (Table 1). Thus the rate of reduction of aflatoxins by lactic acid is first order in both [lactic acid] and [aflatoxins] following an overall second order kinetics.

Based on kinetic and experimental evidences the mechanism proposed for reduction of AFB_1 to AFB_2 and AFG_1 to AFG_2 by lactic acid are given in Schemes 1 and 2 respectively in which for brevity the whole structures of AFB_1 and AFG_1 are given in the first and last steps only. Formation of pyruvic acid as the oxidation product of lactic acid overruled the C–C bond scission of lactic acid in the activated state. Hence the cleavage of α C–H bond from the carbon bearing the functional group OH in lactic acid, seems to be reasonable to propose to be operative in the activation state for the two electron oxidation. Based on experimentally observed large kinetic isotope effect values, cleavage of α C–H bond during oxidation of lactic acid to pyruvic acid is already reported.[15]

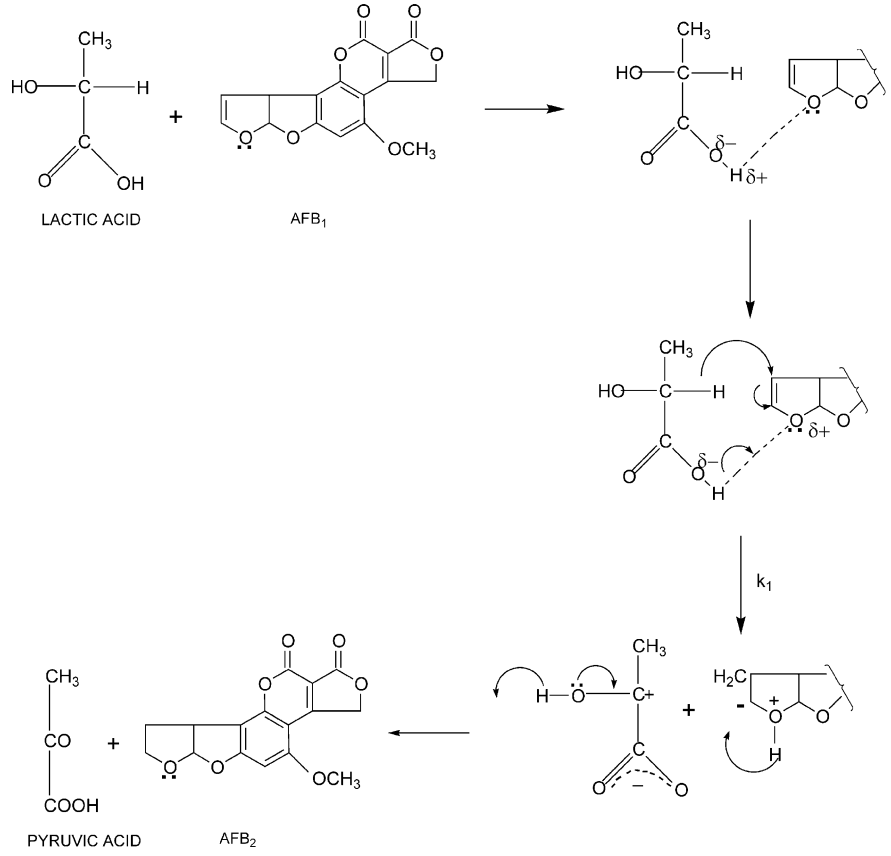
In order to ascertain the nature of cleavage of C–H bond, conducted experiments revealed that the oxidation of lactic acid was not affected by adding radical scavangers like allyl acetate. Also the oxidation of lactic acid, under Argon, failed to induce polymerization of acrylonitrile. These experiments suggest that a hydrogen abstraction mechanism is unlikely in view of the nil effect of radical scavangers on the reaction rate. Radical mechanism also could not be considered because radical formation could not be demonstrated by acrylonitrile polymerization during oxidation of lactic acid.

The above results are best explained by assuming an initial formation of transient oxonium ion, which is formed by the interaction of weakly dissociable proton of –COOH group in lactic acid[16] (pK = 3.862 at 25 °C) with the active oxygen of the alkenyl ether. The oxonium intermediate tends to polarize the olefinic (C=C) carbon, which in turn causes the hydride abstraction from α-carbon atom of lactic acid. The rate determining steps k_1 (Scheme 1) and k_2 (Scheme 2) are proposed to involve, hydride ion (H^-) abstraction from α-carbon atom of lactic acid by the polarized (C=C) carbon at the β-position. The next step then involves the rearrangements of the protons of, oxonium ion and alcoholic position of lactic acid to yield the reduced products AFB_2 (Scheme 1) and AFG_2 (Scheme 2) and the oxidised product pyruvic acid. Hence an overall transfer of

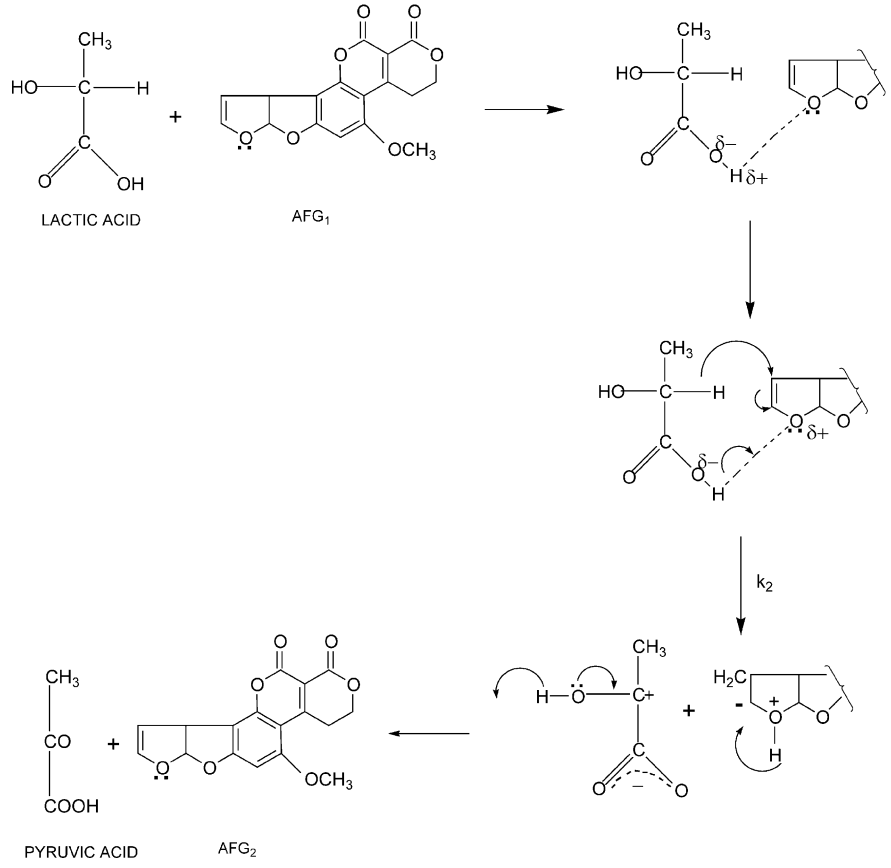
Table 1. Kinetic data for the reduction of AFB_1 and AFG_1 by lactic acid at 37 °C

[Lactic acid]×10⁴ mol dm⁻³	pH	$k_{obs}\times10^2$ h⁻¹ for AFB_1[a]	$k_1\times10^{-2}$ mol⁻¹ dm³ h⁻¹ for AFB_1[a]	$k_{obs}\times10^2$ h⁻¹ for AFG_1[b]	$k_2\times10^{-2}$ mol⁻¹ dm³ h⁻¹ for AFG_1[b]
0.25	3.35	0.30	1.20	0.29	1.16
0.50	3.35	0.59	1.18	0.57	1.14
1.00	3.35	1.17	1.17	1.13	1.13
2.20	3.35	2.56	1.16	2.48	1.11
4.44	3.35	5.15	1.16	4.97	1.12
4.44	3.80	5.18	1.17	4.98	1.12
4.44	4.05	5.28	1.19	5.00	1.13
4.44	4.50	5.34	1.20	5.02	1.13
4.44	3.35	5.18[c]	1.17[c]	4.97[d]	1.12[d]
4.44	3.35	5.17[e]	1.16[e]	5.00[f]	1.13[f]

[a][AFB_1] = 1.20×10^{-6} mol dm⁻³.
[b][AFG_1] = 1.14×10^{-6} mol dm⁻³.
[c][AFB_1] = 0.60×10^{-6} mol dm⁻³.
[d][AFG_1] = 0.57×10^{-6} mol dm⁻³.
[e][AFB_1] = 1.45×10^{-6} mol dm⁻³.
[f][AFG_1] = 1.37×10^{-6} mol dm⁻³.



Scheme 1.



Scheme 2.

two protons and two electrons take place during the reduction of AFB_1 to AFB_2 and AFG_1 to AFG_2 by lactic acid. Mechanism involving transfer of a hydride ion from lactic acid to the oxidant sodium *N*-chlorobenzene sulphonamide is also reported[17] in aqueous acidic medium.

The deduced rate equations describing the mechanisms of Schemes 1 and 2 are given from eqs 3–8.

$$\frac{-d[AFB_1]}{dt} = k_1 [AFB_1][CH_3CHOH\,COOH] \quad (3)$$

$$k_{obs} = (-d[AFB_1]/dt)/[AFB_1] = k_1[CH_3CHOH\,COOH] \quad (4)$$

$$k_1 = k_{obs}/[CH_3CHOH\,COOH] \quad (5)$$

where rate $= (-d[AFB_1]/dt) = (d[AFB_2]/dt) = (d[PA]/dt) = (-d[LA]/dt)$.

k_1 is the second order rate constant corresponding to the rate determining step in Scheme 2. Similarly, for rate constant k_2 (Scheme 2), the rate equations are given (eqs 6–8),

$$\frac{-d[AFG_1]}{dt} = k_2[AFG_1][CH_3CHOH\,COOH] \quad (6)$$

$$k_{obs} = (-d[AFG_1]/dt)/[AFG_1] = k_1[CH_3CHOH\,COOH] \quad (7)$$

$$k_2 = k_{obs}/[CH_3CHOH\,COOH] \quad (8)$$

where rate $= (-d[AFG_1]/dt) = (d[AFG_2]/dt) = (d[PA]/dt) = (-d[LA]/dt)$.

The rate constants k_1 and k_2 computed from eqs 5 and 8, respectively, were found to be constant verifying these rate equations and proposed mechanisms. The values of the second order rate constant k_1 and k_2 are 1.18×10^2 and 1.13×10^2 mol^{-1} dm^3 h^{-1}, respectively. These rate constants are appreciable but found to be less in comparison with the reductant L-ascorbic acid.[10] The reported[10] values of the rate constants k_1 and k_2 for reduction of AFB_1 to AFB_2 and AFG_1 to AFG, are 9.70×10^2 and 5.90×10^2 mol^{-1} dm^3 h^{-1}, respectively, in presence of the reductant ascorbic acid. The nature of transfer of electrons and protons from the reductants to aflatoxins seems to be the major factor responsible for making the ascorbic acid system relatively more efficient. The alteration of C–OH bond in ene-diol structure of ascorbic acid during reaction is easier than the cleavage of C–H bond of lactic acid which is involved in the mechanism.

The reaction of dilute solutions of lactic acid and aflatoxin leads the reduction of AFB_1 to AFB_2 and AFG_1 and AFG_2 involving an initial formation of transient oxonium intermediate, which tends to polarize the olefinic (C=C) carbon, which in turn causes the hydride abstraction from α-carbon of lactic acid. The overall reduction process involves the transfer of two protons and two electrons from lactic acid to the aflatoxins B_1 and G_1.

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