KINETICS OF THE AFLATOXIN B_1–SODIUM HYPOCHLORITE REACTION AND ITS APPLICATION TO THE STUDY OF AFLATOXIN B_1–BOVINE ALBUMIN BINDING

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The course of the aflatoxin B_1–sodium hypochlorite reaction was followed by fluorescence emission at 450 nm. The formation of intermediate compounds with a higher fluorescence quantum yield than that of aflatoxin B_1 was observed. This formation fitted a second order kinetics, and the measurement of the initial formation rate was used to calculate the concentration of the aflatoxin in solution. Bovine albumin exerted a protective effect on aflatoxin B_1 against the sodium hypochlorite action, which was related to the hydrophobic nature of the aflatoxin B_1–albumin binding. This effect was used to give a further insight of aflatoxin B_1–albumin interaction.

The aflatoxin B_1 (AFB_1) is a metabolite produced by the moulds *Aspergillus flavus* and *Aspergillus parasiticus*, and it is known as a toxic contaminant with carcinogenic properties, in human and animal foodstuffs[1].

The present report describes some kinetic characteristics of the reaction between AFB_1 and the hypochlorite anion. A protective effect of BSA on AFB_1 was found for this reaction, and it was useful to give additional data about the nature of the AFB_1–BSA binding process.

EXPERIMENTAL

Chemicals and Solutions

Crystalline AFB_1 was supplied by Serva Feinbiochemica GmbH & Co. (Federal Republic of Germany) and was dissolved in methanol (Merck) to give stock solutions which were kept in the dark. AFB_1 concentration was determined by spectrophotometry at 363 nm using $\varepsilon_{363} = 22\,000\ \text{mol}^{-1}\ \text{l}\ \text{cm}^{-1}$ (ref.[2]). Sodium hypochlorite solutions were prepared immediately before use and their concentrations were determined by titration as reported[3]. BSA (fraction V, powder) was provided by Sigma Chemical Co., (U.S.A.). All the reagents were of analytical quality.

Fluorescence Measurements

The fluorescence measurements were performed by using a 93200 Spectral Fluorescence Accessory attached to a Beckman DU-2 spectrophotometer, with a GEF 4T5 BL Mercury Phosphor excitation lamp and a Turner Primary filter which provided a maximal transmittance at 360 nm. No radiation was detected at wavelengths higher than 400 nm. The emission wavelengths were selected by the spectrophotometer monochromator.

The exposure time of the samples to exciting light was minimized to avoid photodecomposition of AFB_1. Correction for inner filter effect was usually unnecessary because of the low absorbance of the solutions at the selected wavelengths. The emission maximum of the AFB_1 was found at 450 nm in accordance with previous data[3]. All the studies were performed at 20°C. Phosphate buffer (50 mmol l^{-1}, pH 7·4) was used as medium for most of the experiments.

AFB_1–sodium hypochlorite and AFB_1–BSA–sodium hypochlorite mixtures were prepared from thermostatized solutions of the reactants.

Thin-layer Chromatography

TLC of AFB_1–sodium hypochlorite and AFB_1–BSA–sodium hypochlorite mixtures was accomplished on Silica Gel 60 G (Merck), 0·25 mm thick, activated at 110°C for 120 min, using a mixture of chloroform and acetone 90 : 10 v/v as the solvent system.

RESULTS AND DISCUSSION

AFB_1–Sodium Hypochlorite Reactions

The course of the reaction of AFB_1 with the hypochlorite anion was followed by the fluorescence intensity at 450 nm, both at constant AFB_1 concentration (Fig. 1) and at constant sodium hypochlorite concentration (Fig. 2). The shape of the curves obtained in the first experimental condition could indicate the formation of intermediate compounds with a high fluorescence quantum yield in relation with that of AFB_1.

The sodium hypochlorite concentration used in the second case (Fig. 2) was chosen so that the initial fluorescence increased almost three times, in a period of time (1 – 2 min) which made its measurements easily possible.

It is evident from Fig. 2 that the initial formation rate of fluorescent intermediate compounds was related to the initial AFB_1 concentration. Since the fluorescence increase was detectable without difficulty, it was interesting to test the possibility of using the initial velocity (or a related parameter) as a measure of the AFB_1 concentration in solution. Therefore, initial velocities (V_0) were calculated from the curves in Fig. 2, and the logarithms of V_0 values were plotted against the logarithms of the corresponding AFB_1 concentrations (Fig. 3). The plot yielded a good straight line with a slope 2, showing that the reaction was initially of pseudosecond order with respect to AFB_1. The resultant equation was:

$$\ln V_0 = \ln k + 2 \ln [AFB_1], \qquad (1)$$

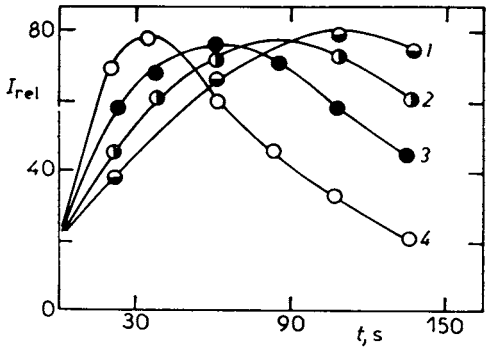

FIG. 1

Course of AFB_1–sodium hypochlorite reaction as followed by fluorescence at 450 nm, at different sodium hypochlorite concentrations. AFB_1 concentration 1·5 μmol l^{-1}, sodium hypochlorite concentrations: *1* 65, *2* 101, *3* 202, *4* 270 μmol l^{-1}. I_{rel} relative fluorescence, *t* time in sec

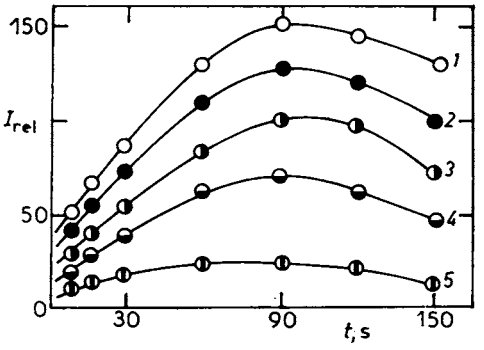

FIG. 2

Effect of AFB_1 concentration in the course of AFB_1–sodium hypochlorite reaction. Sodium hypochlorite concentration 65 μmol . l^{-1}. AFB_1 concentrations: *1* 2·52, *2* 2·10, *3* 1·68, *4* 1·05, *5* 0·32 μmol l^{-1}. I_{rel} relative fluorescence, *t* time in sec

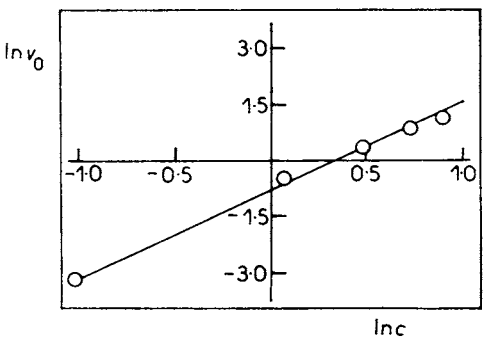

FIG. 3

Determination of the AFB_1 partial reaction order for the intermediate compound formation in the AFB_1–sodium hypochlorite reaction. V_0 initial velocity as measured from the data of Fig. 2, *c* AFB_1 concentration. Partial reaction order estimated: 2

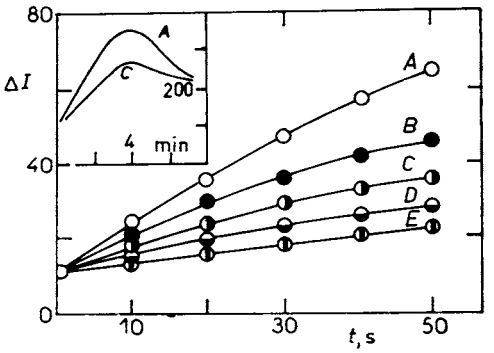

FIG. 4

Fluorescence enhancement ΔI during the course of the AFB_1–sodium hypochlorite reaction in the presence of different BSA concentrations. AFB_1 concentration: 1·51 μmol l^{-1}, sodium hypochlorite concentration 60 μmol l^{-1}, BSA concentration in μmol l^{-1}: *A* 0, *B* 0·25, *C* 0·50, *D* 0·67, *E* 1·0. Insert: Shape of the curves in the 0—6 min period, showing the similarity with curves in Fig. 2 (see text)

where k is an apparent rate constant, depending on temperature and sodium hypochlorite concentration.

This relationship enabled us to estimate AFB_1 concentrations from V_0 values when k had been previously determined. The V_0 estimations were carried out by the measurement of the fluorescence enhancement at a fixed time period (not longer than 0·5 min) in which the fluorescence *vs* time plots remained lineal.

This method, because of the important enhancement of the fluorescence quantum yield, provided higher sensibility than the direct measurement of AFB_1 fluorescence or absorbance.

BSA Effect on AFB_1–Sodium Hypochlorite Reaction

For the analysis of the effect of BSA on the interaction, the course of the reaction was followed as described above but in the presence of different BSA concentrations (Fig. 4). A clear decrease in the maximum fluorescence value reached was observed by increasing the BSA concentration. However, the shape of the curves remained the same either with or without BSA, a fact which strongly suggests that the reaction mechanism does not change by the action of the macromolecule. (Fig. 4, insert.)

The V_0 values also revealed a marked diminution at increasing BSA concentrations. The plot of V_0 against the reciprocal of BSA concentration (Fig. 5), showed that this parameter could be related to the concentration of AFB_1 not bound to

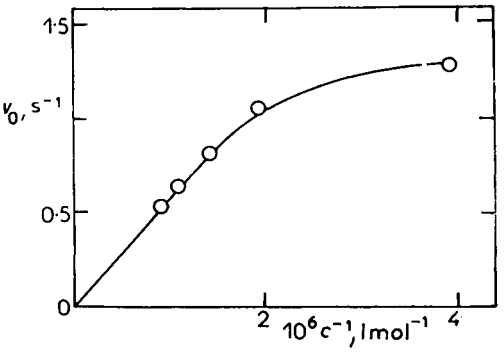

FIG. 5

Protective effect of BSA against the action of sodium hypochlorite on AFB_1 evidenced by the relation between reaction velocity (V_0) and the reciprocal of BSA concentration (c). AFB_1 concentration 1·51 μmol l^{-1}, sodium hypochlorite concentration 60 μmol . l^{-1}

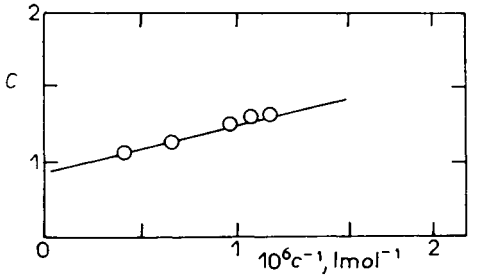

FIG. 6

Double reciprocal plot for the AFB_1–BSA interaction. The data were calculated from the protective effect of BSA against the action of sodium hypochlorite on AFB_1 as explained in the text. Sodium hypochlorite concentration 60 μmol l^{-1}, t 20°C, medium phosphate buffer pH 7·40, 50 μmol . l^{-1}; c free AFB_1, C bound AFB_1/BSA

BSA, inasmuch as it tended to be zero when AFB_1 was presumably totally bound in the presence of an excess of the macromolecule.

Further evidence to support the latter interpretation was brought forward submitting AFB_1–sodium hypochlorite mixtures, with and without BSA, to TLC as described in the Experimental part. After the run, a characteristic spot for AFB_1 (as compared with an AFB_1 standard) was observed for the AFB_1–sodium hypochlorite–BSA mixtures, whereas no AFB_1 spot was detected for the mixtures without BSA. This behavior was consistent with a protective effect exerted by BSA on AFB_1, hindering the oxidation of part of the toxin present in the mixture, very likely the fraction bound to the macromolecule. This fractions could be released from BSA during the run by the organic eluent.

The existence of such a protective effect can be employed, as in the case of the bilirubin–BSA system[5], for the study of the AFB_1–BSA interaction at equilibrium, by measuring V_0 for known AFB_1 and sodium hypochlorite concentration, in the presence of different BSA concentrations. Free AFB_1 concentrations were derived from the V_0 values by using equation (*1*), in the assumption that the reaction mechanism remained the same in the presence of BSA, as indicated by the shape of the curves in Fig. 4. Bound AFB_1 concentrations were calculated by the difference between total and free AFB_1 and used to obtain the bound AFB_1/BSA concentration ratios. The results obtained were displayed in a double reciprocal plot (Fig. 6), giving $2{\cdot}4.10^6$ mol^{-1} l as an estimation for the association constant at 20°C, and one binding site for AFB_1 by BSA molecule. Since the alternative hypothesis may be held that the V_0 diminution could be an effect of the BSA–sodium hypochlorite interaction, the study was repeated in the same conditions as above but in the presence of different sodium hypochlorite concentrations (20, 60, 100, and 160 $\mu mol\, l^{-1}$). The values obtained for the binding constant and for the number of binding sites were not significantly different ($p < 0{\cdot}05$) at the different sodium hypochlorite concentrations used.

Therefore the AFB_1–BSA interaction appears as another example of the protective action of albumin on small ligands bound to the macromolecule. Furthermore, the existence of such a protective effect against chemical reactions which take place in aqueous solution, provides an argument in favour of the hydrophobic nature postulated for the interaction[4,6], since it can be related to an AFB_1–BSA binding in which the ligand (or part of it) is buried into a hydrophobic region of the macromolecule.

The presence in the BSA molecule of one binding site for AFB_1 with a relatively high association constant, is interesting from a physiological approach, since it could provide the possibility of a storage action of BSA (or other albumin molecule with similar behavior) in the systemic circulation, besides of its transport or delivery role. The existence of more binding sites not able to protect the AFB_1 of the hypochlorite anion action cannot be detected by the method used.

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